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AKADÉMIAI DOKTORI ÉRTEKEZÉS

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**AZ ERBB FEHÉRJÉK KLASZTERIZÁCIÓJÁNAK BIOFIZIKAI KARAKTERIZÁLÁSA ÉS
BIOLÓGIAI JELENTŐSÉGE**



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Rövidítések jegyzéke

ADCC	antitest függő celluláris citotoxicitás (antibody-dependent cellular cytotoxicity)
APMA	aminofenil-higany-acetát (aminophenylmercuric-acetate)
AR	amfiregulin
BiFC	kétmolekuláris fluoreszcencia komplementáció (bimolecular fluorescence complementation)
BSA	borjú szérum albumin (bovine serum albumin)
BTC	betacellulin
CDC	komplement függő citotoxicitás (complement-dependent cytotoxicity)
CDK	ciklin dependens kináz
CFP	cián színű fluoreszcens protein (cyan fluorescent protein)
CTX-B	kolera toxin B alegység
EGF	epidermális növekedési faktor (epidermal growth factor)
eGFP	„javított” GFP (enhanced GFP)
EGFR	EGF receptor
ELISA	enzyme-linked immunosorbent assay
EPG	epigén
EPR	epiregulin
FITC	fluoreszcein-izotiocianát
FCS	fötális borjú szérum (fetal calf serum) vagy fluoreszcencia korrelációs spektroszkópia
FRAP	fluoreszcencia visszatérés fotoelhalványítás után (fluorescence recovery after photobleaching)
FRET	fluoreszcencia (vagy Förster típusú) rezonancia energia transzfer
FSAB	FRET szenzitizált akceptor fotoelhalványítás (FRET-sensitized acceptor bleaching)
GAMIG	egér ellenes kecske immunglobulin (goat anti-mouse immunoglobulin)
GFP	zöld fluoreszcens protein (green fluorescent protein)
GP	generalizált polarizáció
GPI	glikozilfoszfatidil-inozitol
HA	hialuronsav (hyaluronic acid)
HA10	tíz monoszacharid egységből álló hialuronsav oligoszacharid
HABC	hialuronsav kötő komplex (hyaluronic acid binding complex)

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HER	humán EGF receptor
HRG	heregulin
IGF1R	inzulinszerű növekedési faktor-1 receptor (insulin-like growth factor 1 receptor)
L _d	folyékony rendezetlen (liquid disordered)
L _o	folyékony rendezett (liquid ordered)
MAPK	mitogén aktiválta protein kináz
4-MU	4-metilumbelliferon
mYFP	monomer YFP
N&B	„number and brightness” analízis
NRG	neuregulin
OF	Oligofectamine
PALM	fotoaktivációs lokalizációs mikroszkópia (photoactivation localization microscopy)
PBS	foszfát pufferelt só (phosphate buffered saline)
PCR	polimeráz láncreakció (polymerase chain reaction)
PI3K	foszfatidilinozitol 3-kináz
PLA	proximitás ligációs vizsgálat (proximity ligation assay)
PLAP	placentáris alkalikus foszfatáz (placental alkaline phosphatase)
RCA	gördülő cirkuláris amplifikáció (rolling circle amplification)
ROI	érdeklődésre számot tartó terület (region of interest)
QDot	kvantum pont (quantum dot)
SPT	egyedi partikulum követés (single particle tracking)
shRNS	kis hajtű RNS (small hairpin RNA)
siRNS	kis interferáló RNS (small interfering RNA)
SD	standard deviáció
SEM	középérték közepes hibája (standard error of the mean)
STED	stimulált emissziós depléciós mikroszkópia (stimulated emission depletion microscopy)
STORM	sztochasztikus optikai rekonstrukciós mikroszkópia (stochastic optical reconstruction microscopy)
TGF α	transzformáló növekedési faktor α (transforming growth factor α)
TM	transzmembrán

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TMA-DPH	4'-(trimetilammonio)-difenilhexatrién
YFP	sárga fluoreszcens protein (yellow fluorescent protein)
Y2H	élesztő két-hibrid (yeast two-hybrid)

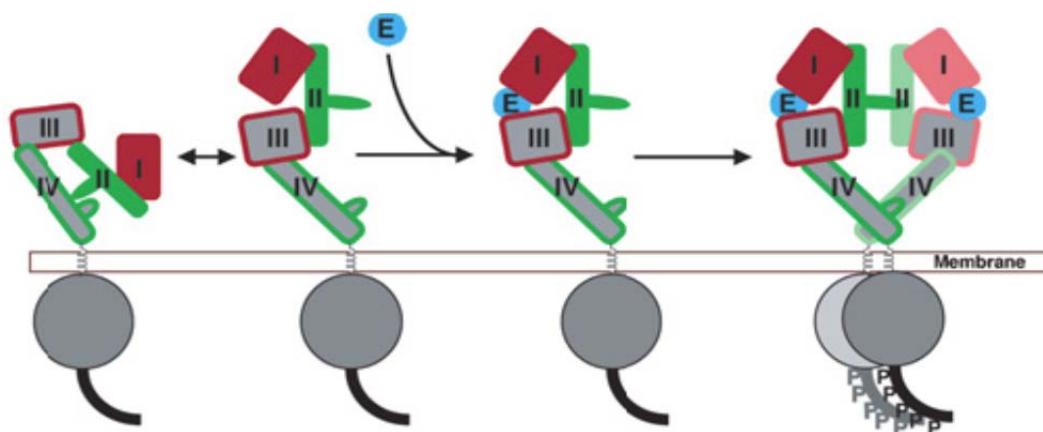
1. Irodalmi háttér

1.1. Az ErbB fehérjék: klaszterizáció mindenek felett

A transzmembrán jelátvitel vizsgálata központi jelentőségű a sejt- és molekuláris biológiai kutatásokban, hiszen sejtek fiziológiás és patológiás aktivációs folyamatainak első lépésének megismerése mind alapkutatási, mind orvosi szempontból rendkívül fontos. A receptor tirozin kinázok a jelátviteli folyamatok egyik legrészletesebben tanulmányozott résztvevői. Közülük az epidermális növekedési faktor (epidermal growth factor, EGF) receptorcsalád képezi ezen dolgozat legfontosabb tárgyát. Jelentőségét az adja, hogy kb. 40 éve ismert a receptort aktiváló növekedési faktor, az EGF (1), melynek receptora azóta a legrészletesebben tanulmányozott és ismert transzmembrán receptor tirozin kinázzá vált. A családnak négy tagja van, melyeket ErbB1-4 receptoroknak, ill. emberben HER1-4 (HER=human epidermal growth factor receptor) fehérjéknek neveznek. Az ErbB1 fehérje azonos a család névadó tagjával, az EGF receptorral (EGFR). Az 1970-es években a receptoraktiváció paradigmáját az EGFR-ra írták le, amikor a családnak még csak ez a tagja volt ismert. Eszerint a receptor inaktív állapotban monomerként van jelen a plazmamembránban, majd a ligand (EGF) megkötése után reverzibilisen dimerizálódik, amelyet a receptor intracelluláris kináz doménjének aktivációja és a transzmembrán jelátvitel aktivációja kísér (2). Azóta a kép sokkal árnyaltabbá és bonyolultabbá vált. Ennek megértéséhez tekintsük át először röviden az ErbB fehérjék szerkezetét.

Mind a négy fehérje hasonló felépítést mutat. Tartalmaznak egy 620 aminosavból álló extracelluláris domént, egy rövid α -helikális transzmembrán szegmenst és egy intracelluláris részt. Ez utóbbi további részekre osztható: a membránhoz legközelebb a 40 aminosavat tartalmazó juxtamembrán domén, majd a tirozinkináz domén és végül a C-terminális végen a foszfortirozint kötő effektor molekulák kötőhelyei találhatóak (3, 4). A 2000-es évek elején az összes ErbB fehérje extracelluláris részének röntgenkristallográfiai elemzése feltárta részletesebb szerkezetüket (5-11). Az L1 (I) és az L2 (III) szubdomének leucinban gazdagok és képesek a ligand megkötésére, míg a ciszteinben gazdag CR1 (II, S1) és CR2 (IV, S2) szubdomének a receptor dimerizációjában játszanak fontos szerepet. Ligandum hiányában az ErbB2 kivételével a többi receptor ún. zárt konformációt vesz fel, amelyben a II. és IV. domének között intramolekuláris híd képződik. Ez a kapcsolat stabilizálja ezt a térszerkezetet, és megakadályozza a dimerizációt, mivel a II. domén dimerizációs karjának intramolekuláris kölcsönhatása kizárja a II. domének közötti intermolekuláris kapcsolódást.

Zárt konformációban a ligandot kötő domének távol vannak egymástól, de képesek ligandot kötni (12). Egyik elmélet szerint a ligand kötődés indukálja a zárt konformáció nyíltba való átmenetét. Más elképzelések szerint a receptor konformációja fluktuál a zárt és nyílt konformációk között, és a ligand kötődése „csak” stabilizálja a nyílt konformációt (13). Ligandum kötését követően az I. és III. domének egymáshoz közel kerülnek, felszakad a II-IV domének közötti intramolekuláris híd, és exponálódik a II. domén dimerizációs karja, amely így intermolekuláris kapcsolatokat tud stabilizálni (1. ábra).



1. ábra. Az EGF receptor ligandfüggő aktivációja.

Forrás: *Ferguson, Biochemical Society Transactions, 2004, 32:742*

Az ErbB2 extracelluláris doménjének viselkedése több szempontból eltér a fentiekétől. Egyrészt a receptor konstitutívan nyílt konformációban van, amiben a dimerizációs kar exponált (8). Másrészt az I. és III. domének közötti ligandkötő zseb túl kicsi. A fenti strukturális információ összhangban van a már régóta ismert sejtbioológiai eredményekkel, melyek szerint az ErbB2 nem képes növekedési faktort kötni, és a többi ErbB fehérje preferált heterodimerizációs partnere (14, 15). Az ErbB2 fehérje konstitutívan nyitott konformációja ellenére nem hajlamos molekuláris szintű homodimerizációra, amiért valószínűleg extracelluláris doménjének negatív töltése és következményes elektrosztatikus taszítása felelős (8). Egyes újabb eredmények szerint az ErbB2 extracelluláris doménje még sincs konstitutívan aktív konformációban, mert rejtett, domének közötti gátló kölcsönhatásokat tartalmaz (16). Tovább bonyolítja a képet az, hogy magas expressziós szint mellett az ErbB2 mégis képez homoasszociátumokat, melyeket azonban valószínűleg nem a már leírt dimerizációs kar, hanem a molekula egyéb részei (pl. transzmembrán vagy intracelluláris domének) vagy a lipid környezet stabilizálnak (17, 18).

A fenti relatív egyszerű modell, mely szerint az extracelluláris domén ligand indukált konformáció-változása okozza a dimerizációt, számtalan észlelést nem tud megmagyarázni. Valószínűleg az extracelluláris domének önmagukban nem képesek az ErbB fehérjék közötti heterodimerizáció kiváltására (19), pedig a ligandumok ilyen klasztereket is létre hoznak (20, 21). A dimerek összetételét egyrészt az ErbB fehérjék expressziós szintje, másrészt a ligandum típusa határozza meg, amelyeket három csoportra oszthatunk (22, 23):

- a) EGF-szerű ligandok, amelyek leginkább az ErbB1-hez kötődnek: EGF, TGF α (transzformáló növekedési faktor α), AR (amfirogulin), EPG (epigén).
- b) Neuregulinok, más néven heregulinok, melyek a neuregulin receptorokhoz (ErbB3 és ErbB4) kötődnek: négy neuregulin gén ismert (NRG-1=HRG, NRG-2, NRG-3, NRG-4), ezen belül a HRG és a NRG-2 rendelkezik α és β izoformával is.
- c) HB-EGF (heparint kötő EGF-szerű növekedési faktor), BTC (betacellulin) és EPR (epiregulin), melyek egyaránt kötődnek az ErbB1-hez és az ErbB4-hez is.

Tehát a növekedési faktor kötődik a saját receptorához, majd annak homo- vagy heterodimerizációját váltja ki, ami a receptor aktivációjához vezet. A dimerek stabilizálásában a transzmembrán domén (24) és a kináz domén is részt vesznek (25). Az utóbbi szerkezetének röntgenkristallográfiás vizsgálata érdekes betekintést engedett az aktiváció és a dimerizáció közötti kapcsolatba (26). Ezek szerint a receptor monomer állapotában a kináz domén a Src vagy a ciklin dependens kinázok (CDK) öngátolt konformációjához hasonlít. A ligand kötés és az extracelluláris domének dimerizációja, melyet a transzmembrán domén konformációváltozása közvetít a membránon keresztül (27), a kináz doméneket térbeli közelségbe hozza, és így azok egy aszimmetrikus dimert hoznak létre. Ennek szerkezete hasonlít a ciklin-CDK komplex konformációjához, tehát az egyik EGFR kináz domén ciklin szerepét játssza és aktiválja a másik EGFR tirozin kinázát. Ezt követően az aktivált receptor keresztfoszforilálja a másik receptor C-terminális doménjében levő tirozin oldalláncokat, amely a következő fejezetben leírtaknak megfelelően a jelátviteli folyamatokat aktiválja. A fentiek tükrében értelmet nyer az, hogy egyáltalán miért van szükség a receptor tirozin kinázok aktivációjához a dimerizációra. A több transzmembrán szegmenssel rendelkező receptorok (pl. G fehérjéhez kapcsolt receptorok) esetében a ligand megkötődése az extracelluláris domén konformáció-változásán keresztül a transzmembrán szegmensek átrendeződéséhez vezet, amely elégséges ahhoz, hogy kiváltsa az intracelluláris domén konformációjának megváltozását. Bár az ErbB fehérjék esetében is kimutatható

konformáció-változás a transzmembrán doménben (27), egyetlen transzmembrán szegmens konformációjának átalakulása valószínűleg nem elég ahhoz, hogy önmagában indukálja az intracelluláris domén konformáció-változását. Ehelyett a receptorok az extracelluláris, transzmembrán és intracelluláris domének együttműködése révén dimerizálódnak, ami a kináz domének egymáshoz közelkerülése után kiváltja a keresztfoszforilációt.

Bár a fenti monomer→dimer átmenet ligand indukálta bekövetkeztét már egyedi molekula szinten is kimutatták (28), ugyanezen közlemény megmutatta ezen elképzelés korlátait. Kimutatták, hogy a monomer és dimer állapoton kívül létezik egy „összezárt” (co-confined) pszeudo-dimer is, amelyben a receptor monomerek molekuláris közelségbe kerülnek egymáshoz, de távolságuk mégis nagyobb annál, hogy közvetlen monomer-monomer kontaktus tartsa össze őket. Elképzelhető, hogy közös lipid doménben történő megjelenés vagy citoszkeleton által indukált összetartás áll ezen pszeudo-dimerek mögött (28, 29). Több közlemény is kimutatta, hogy dimerek léteznek a sejtek felszínén ligandum nélkül is, és ezen preformált dimerek preferenciálisan kötik meg a ligandumot (28, 30). Szintén a fenti egyszerű elképzelést árnyalja az, hogy az extracelluláris domén zárt konformációja nem elégséges a monomer állapot fenntartásához (31). Az eddigiek összefoglalásaként kijelenthetjük, hogy bár a ligand indukált dimerizáció sok észlelést megmagyaráz, a receptor asszociációk és aktiváció több apróbb részletét megmagyarázatlanul hagyja.

A legfontosabb ilyen „apróbb” részletek a klaszterizáció két aspektusát érintik:

- a. Egyrészt szinte bizonyosra vehető, hogy léteznek preformált, konstitutív homo- és heterodimerek is (28, 30, 32). Ezek lehetnek tranziensek (28, 30) vagy feltételezhetően hosszú élettartamúak (33). Másrészt ezen dimerek lehetnek tényleges fehérje-fehérje kölcsönhatás által összetartott komplexek, amelyek ligandum kötés hatására konformáció-változáson mennek keresztül (33, 34) vagy a fentebb már leírt pszeudo-dimerek. Jelentőségüket valószínűleg az adja, hogy „előasszociált” formában a receptor aktiváció gyorsabban következik be a növekedési faktor kötődése után. A preformált dimerek vonatkozásában saját eredményeink is relevánsak, amelyek a dolgozat „Eredmények és megbeszélés” fejezetében kerülnek ismertetésre (35, 36).
- b. Másrészt a dimereknél magasabb rendű asszociátumok is léteznek, melyek mérete több száz molekuláig terjedhet (37). Létezhetnek konstitutív módon (38) vagy ligand

által indukáltan (39, 40). A ErbB fehérjék jelentősen különböznek egymástól mind preformált dimer, mind nagyobb méretű klaszterizációs képességükben. Részben saját (35, 37), részben mások (41) eredményei alapján az ErbB2 és ErbB3 ilyen irányú hajlama jelentősen nagyobb, mint az ErbB1-é. A klasztereket összetartó erők valószínűleg indirektek: lehetnek citoskeletális eredetűek vagy közös lipid doménbe történő particionálás következményei. Mivel saját eredményeink jelentősen hozzájárultak ezen klaszter típus kvantitatív leírásához, lehetséges biológiai szerepüket részletesebben az „Eredmények és megbeszélés” fejezetben ismertettem (35, 36, 42, 43).

A receptorok által kialakított preformált dimerek és nagyobb méretű klaszterek ligandum hatására átrendeződnek, közvetlen molekuláris kontaktusok alakulnak ki a receptorok között, melyeket az extracelluláris, transzmembrán és kináz domének stabilizálnak. Ennek hatására a receptorok C-terminális részén keletkező foszfortirozin oldalláncok segítségével másodlagos jelátviteli folyamatok aktiválódnak, melyek áttekintésére a következő fejezetben kerül sor.

1.2. Az ErbB fehérjék biológiai jelentősége: transzmembrán jelátviteli folyamatok

Az ErbB fehérjék által generált transzmembrán szignál forrása, ill. alapegysége a receptorok által létrehozott homo- vagy heterodimer. A négy ErbB fehérje közül csak az ErbB1 és ErbB4 tekinthető teljesen átlagos receptor tirozin kináznak, amely mind ligandkötő, mind aktív kináz doménnel rendelkezik.

- a. Az ErbB2 struktúrájából következően képtelen ligandumot kötni (7), és mivel konstitutívan exponált dimerizációs karral rendelkezik, a többi ErbB receptor koreceptoraként viselkedik. Ezzel kapcsolatban fontos megjegyezni, hogy egy kisebbségi vélemény szerint az ErbB2 öngátolt konformációban van (16), amelyből csak valamilyen aktivációs hatás billentheti ki. Ilyen lehet pl. a MUC4 transzmembrán mucin EGF-szerű doménjével való kölcsönhatás, amely ebből a szempontból membránhoz kötött ligandumnak tekinthető (44).
- b. Az ErbB3 tirozin kináz doménje viszont inaktív (4, 25) vagy csak nagyon alacsony aktivitást mutat (45). A fentiek értelmében sem az ErbB2, sem az ErbB3 nem képes egymagában transzmembrán jelátvitelre, egymással heterodimerizálva viszont a

legaktívabb jelátviteli komplexet alkotják (46). Szemléletes hasonlaltal élve a süket és a vak funkcióképes párt alkot (15).

A dimerek összetételét egyrészt az aktiváló növekedési faktor, másrészt az adott sejt által kifejezett ErbB receptorok típusa és expressziós szintje határozza meg. Bár a négy receptorból elvileg létrehozható tízféle dimer mindegyikét kimutatták már, az ErbB1, ErbB4 homodimerek és az ErbB2-t magába foglaló heterodimerek a legjelentősebbek. A foszfortirozint kötő másodlagos jelátvivő molekulák szelektíven kötődnek az egyes ErbB fehérjék C-terminális doménjében keletkező foszforilált tirozin aminosavakhoz. Ez biztosítja azt, hogy az ErbB1 elsősorban a foszfolipáz-C, Cbl és MAPK útvonalak aktiválására képes, az ErbB2 több Shc kötőhelye miatt kimondottan erősen aktiválja a MAPK útvonalat, az ErbB3 elsősorban a foszfatidilinozitol 3-kináz (PI3K) serkentésében jár élen, míg az ErbB4 mind a MAPK, mind a PI3K útvonalakat bekapcsolja (47).

Az ErbB2 részvétele a heterodimerekben több szempontból javítja a jelátvitel hatékonyságát. Egyrészt a fent ismertetettek miatt az ErbB2 kiváló aktivátora a MAPK útvonalnak. Másrészt az ErbB2-t tartalmazó heterodimerek aktivációt követő endocitózisa gátolt (48) vagy az internalizációt követően lizoszómális degradáció helyett recirkulálnak a plazmamembránba (49, 50). Másrészt az ErbB2 emeli a vele asszociáló EGF vagy neuregulín receptorok (ErbB1, -3, -4) növekedési faktor iránti affinitását (51). Amennyiben figyelembe vesszük az ErbB3 kiváló PI3K aktiváló képességét, megérthetjük az ErbB2-3 heterodimer kimagasló onkogén potenciálját (46).

Mivel az ErbB2 ilyen fontos szerepet tölt be az egész ErbB szignalizációs hálózatban, az összes ErbB fehérje által mediált jelátviteli folyamatok központjának tekinthető. Rendszer biológiai szempontból a folyamat egy rendkívül komplex szabályozási hálózatot alkot, melynek fontos tulajdonsága a robosztusság. Ugyanakkor a rendszer kulcsfontosságú szabályozó elemeinek gátlásával az egész hálózat működése felborítható, ami a daganatok kezelésében hasznosítható (20).

A fentiekben ismertetett, konvencionálisnak nevezhető jelátviteli folyamatok mellett az ErbB fehérjék egyéb, nem „ortodox” folyamatokban is részt vesznek. Így pl. meggyőző bizonyítékok szólnak amellett, hogy az ErbB3 és az ErbB4 a sejtmagban is előfordul (52-54). Az ErbB4 presenilin függő intramembrán proteolízist szenved, pontosabban annak kizárólag a Jm-a izoformája, majd a sejtmagban többek között a STAT5A nukleáris chaperonjaként viselkedik (54-56). Ezenkívül az ErbB fehérjék nemcsak a hozzájuk kötődő növekedési

faktorok által kiváltott szignálokat integrálják, hanem több más jelátviteli folyamatban is részt vesznek (57). Így pl. a szolubilis tirozin kinázok közül a Src és a Jak képesek az ErbB fehérjét foszforilálni, így azok a receptorok (pl. növekedési hormon receptor, prolaktin receptor), amelyek ezeken az intracelluláris kinázokon keresztül hatnak, képesek transzaktiválni az ErbB rendszert (58). A G fehérjéhez kapcsolt receptorok ADAM proteázok aktiválásával a membránhoz kötött EGF-szerű növekedési faktorokból felszabadítják a szolubilis peptidet, amely közvetlenül képes az ErbB1 aktiválására (59-61). A CD44 többféleképpen vesz részt az ErbB szignalizációban. Egyrészt liganduma, a hialuronsav, által aktiválva serkenti az ErbB2 tirozin foszforilációját (62, 63), másrészt a HB-EGF prekursor és az azt processzáló MMP-7 proteáz megkötésével egy olyan komplexet hoz létre, amely az ErbB4 (és az ErbB1) aktiválásához vezet (64). A CD44 hialuronsav által történő aktivációja a jelátviteli folyamatok serkentése mellett (65) kiváltja a CD44 intramembrán proteolízisét is. Az így felszabaduló CD44 intracelluláris domén növeli a sejtek motilitását és így a daganatok metasztázis képzésében is szerepet játszhat (66, 67). A fenti folyamatokban tehát a CD44 és az ErbB receptorok szoros együttműködésben vesznek részt. Mivel ebben a vonatkozásban saját eredményeink is jelentősek, erre az „Eredmények és megbeszélés” fejezetben visszatérek (68).

Az ErbB receptorok aktivációja a már említett pozitív regulátorok mellett gátló hatásoknak is ki van téve. Ezek egyike a receptorok endocitózisa, melyet legrészletesebben az ErbB1 esetében tanulmányoztak (69). Valószínű, hogy ebben a receptor Cbl mediálta mono-ubikvitinációja játszik szerepet, amely végül lizoszómális degradációhoz vezet (70, 71). Azonban részletesebb vizsgálatok feltárták, hogy alacsony dózisú EGF stimuláció esetén a receptor mediált endocitózis klatrin függően következik be, míg magas dózisú növekedési faktor jelenlétében inkább a caveolin függő útvonal segítségével kerül endocitózisra a receptor (72). A receptor aktivációja is többszintű negatív reguláció alatt van. Egyrészt gátolható a receptor dimerizáció herstatin által, amely az ErbB2 szolubilis extracelluláris doménje, mely az ErbB2 alternatív splicing-ja révén jön létre. A RALT nevű fehérje a kináz doménnel képes kölcsönhatni és specifikusan gátolni azt. Két másik faktor, a decorin és az LRIG-1, az ErbB receptorok extracelluláris doménjével kölcsönhatva csökkenti a receptor expressziós szintjét (73). A Hsp90 pedig az ErbB2 heterodimerizációjának gátlásával éri el a szignalizáció gátlását (74). A negatív regulációs folyamatok nemcsak fiziológiás szempontból

jelentősek, hanem daganatokban sok esetben hiányoznak, ami a transzformált sejtek fokozott proliferációs és migrációs képességéhez vezet (75).

1.3. Az ErbB fehérjék onkológiai szerepe

Régóta ismert, hogy az ErbB fehérjék számtalan humán daganat kialakulásában szerepet játszanak (57). Fokozott aktiválódásuknak számtalan módja lehet. Történhet mutáció, pl. az ErbB1 kináz doménjében, amely aktiválja annak enzimatisz aktivitását (76) vagy deletálódhat az ErbB1 teljes extracelluláris doménje, amely egy konstitutívan aktivált ErbB1 fragmentet (EGFRvIII) hoz létre (77). A kináz domén mutáció elsősorban nem kissejtes tüdőrákban, míg az EGFRvIII főleg gliómákban elterjedt. Érdeemes megjegyezni, hogy kináz domén mutációt ErbB2-ben is leírtak, de ennek klinikai jelentősége még nem ismert (78). Trunkált ErbB2 molekulát viszont nemcsak találtak emlőtumorokban, hanem valószínűleg ezek expressziója fokozza a sejtek malignitását, és hozzájárul a receptor ellenes antitest terápiával szembeni rezisztenciához (79). Az ErbB receptorokat közvetlenül érintő genetikai módosulások mellett az ErbB szignalizációs hálózat transz-aktiválódhat más jelátviteli rendszerek, pl. G fehérjéhez kötött receptorok, által (57).

Az ErbB1, ErbB2 és ErbB3 fehérjék aktiválódásáról bebizonyosodott, hogy hozzájárulnak a daganatok keletkezéséhez és progressziójához, de az ErbB4-ről inkább azt feltételezik, hogy anti-onkogén hatású, hiszen sejtproliferációt gátló szignálok forrása lehet (80-82). Az ErbB fehérjék (ErbB1-3) többféle módon járulhatnak hozzá a rosszindulatú daganatok kialakulásához és progressziójához (57). Egyrészt fokozzák a sejtproliferációt (83, 84), gátolják az apoptózist (85) a PI3K-Akt túlélés útvonal aktiválásával (86). Ez utóbbiban mutat kimagasló hatásosságot az ErbB2-3 heterodimer (46, 57, 86). Ezeken kívül fokozzák a sejtek motilitását (87) és angiogenezist indukálnak (88). Fontos megjegyezni, hogy az ErbB fehérjék és membránhoz kötött ligandjaik fiziológiás körülmények között polarizált eloszlást mutatnak, tehát szelektíven expresszálódnak a sejtek apikális és bazolaterális doménjeiben. Ez a rendezett eloszlás megszűnik malignus sejtekben a sejt-sejt kontaktusok felbomlásával, így a receptor alegységek (pl. ErbB2 és ErbB3) vagy a membránhoz kötött ligandumok és a receptorok ugyanabban a membrán doménben fognak megjelenni, így aktivációjuk sokkal könnyebben bekövetkezhet (75, 89)

Mivel a dolgozat „Eredmények és megbeszélés” fejezetében különösen sok szó fog esni az ErbB2 emlőtumorokban betöltött szerepéről, erről érdemes egy kicsit részletesebben

szólni. Az ErbB2 az emlődaganatok kb. 30%-ban fokozott expressziót mutat, ami a daganat rossz prognózisával jár (90, 91). A prognosztikus marker szerep mellett az ErbB2 terápiás target is, ami hosszú évtizedek kutatásának eredménye (92). Az ErbB2-t és általában a receptor tirozin kinázokat kétféle módon lehet gátolni terápiás célzattal: az extracelluláris doménhez kötődő, általában humanizált antitestekkel és tirozin kináz inhibitorokkal (92, 93). A kináz inhibitorok közül elsőnek az ErbB1-re specifikusak jelentek meg (gefitinib (Iressa®), erlotinib (Tarceva®)), de elérhetőek már ErbB1-re és ErbB2-re ható ún. kettős specificitású reverzibilis (lapatinib, Tykerb®) és irreverzibilis gátló molekulák is (neratinib) (80, 94). Az ErbB ellenes antitestek közül az ErbB2-t felismerő trastuzumab (Herceptin®) volt az első, de más ErbB2 (Omnitarg®) és ErbB1 (cetuximab (Erbix®)) ellenes antitestek is elérhetőek már különböző daganatok kezelésére (80). Az antitestek hatásmechanizmusa többféle lehet (95). Egyrészt gátolhatják a növekedési faktor kötődését (pl. a cetuximab blokkolja az ErbB1 EGF kötő helyét), kiválthatják az immunrendszer aktiválódását (pl. ADCC vagy CDC reakció által) vagy gátolhatják a receptor aktivitását közvetlenül. Ez utóbbira különösen érdekes példa a pertuzumab, hiszen az antitest az ErbB2 dimerizációs karja által kiváltott receptor dimerizációt sztérikusán gátolja (9). A dolgozat témája szempontjából a trastuzumab hatásmechanizmusa és az ellene kialakuló rezisztencia módja különösen jelentős, amelyekről a következő fejezetben lesz szó.

1.4. A trastuzumab a receptor orientált kemoterapikumok prototípusa

Az ErbB2-t fokozottan kifejező emlőtumorkok kezelésére törzskönyvezett antitestek közül az első a trastuzumab volt. Eleinte monoterápiában alkalmazták előrehaladott, többszörösen előkezelt betegeken, majd az ErbB2-t fokozottan kifejező emlődaganatok standard terápiájává vált más kemoterapikumokkal kombinálva (96). Bár egyértelmű, hogy a trastuzumab szignifikáns javulást idéz elő a megfelelően szelektált (azaz ErbB2-pozitív) emlőtumoros betegek túlélésében (97), az ellene kifejlődő rezisztencia súlyos problémát jelent. Ezt vagy a rezisztencia mechanizmusának feltárásával vagy az emlődaganat molekuláris ujjlenyomatának és heterogenitásának ismeretében betegre szabott terápiával lehet megoldani (93).

A trastuzumab hatásmechanizmusával kapcsolatban két elmélet létezik. Az egyik szerint antitest függő celluláris citotoxicitási reakciót (ADCC) vagy komplement függő citotoxikus reakciót (CDC) vált ki, míg a másik elképzelés szerint a tumorelles hatása az

antitest közvetlen daganatsejtekre kifejtett gátló hatásából ered (98-100). Az utóbbi magába foglalja az ErbB2 és Src mediálta szignálok gátlását és a PTEN foszfatáz következményes aktiválódását (101) és az ErbB2-ErbB3 komplex felbontását, amely az Akt közvetített jelátviteli események blokkolásához vezet (102, 103). Ezen elsődleges hatások a sejtciklus G1 fázisban történő felfüggesztéséhez (104), apoptózis indukcióhoz (100) és a DNS szintézis, valamint az angiogenezis gátlásához vezetnek (105). Ezenkívül leírták, hogy a trastuzumab gátolja az ErbB2 ektodomén metalloproteázok általi hasítását, amely az extracelluláris doménjétől megfosztott, konstitutívan aktivált molekulát eredményez (106). A trastuzumab előnyei közé tartozik, hatásossága mellett, hogy relatíve kevés mellékhatással rendelkezik. Ezek közül a legjelentősebb a szívelégtelenséget kiváltó hatás, amely elsősorban doxorubicinnel is kezelt betegekben jelenik meg. Ennek valószínű oka az, hogy a trastuzumab gátolja a felnőtt szívben is aktív, ErbB2 mediálta szignalizációs folyamatokat, amelyek a szív szabadgyökök elleni védelmében és homeosztázisának fenntartásában játszanak szerepet (107, 108).

Mivel a trastuzumabot általában más kemoterápiás szerrel kombinációban alkalmazzák, az ellene kialakuló rezisztencia pontos megítélése problematikus. Ennek ellenére a hosszú kezelések alatt a terápia eredményessége szinte mindig csökken (109). Számtalan tényezőt hoztak már összefüggésbe a trastuzumab rezisztenciával. Ilyen pl. a PTEN hiányzó expressziója (101), a PI3K katalitikus alegységében kialakuló mutáció (103), más ErbB fehérjéken, Met onkogénen vagy IGF1R-on keresztüli, kompenzatórikusan fokozott jelátvitel (109) és a calpain-1 fokozott expressziója (110). Az előző paragrafusban, a trastuzumab hatásmechanizmusával kapcsolatban került említésre, hogy metalloproteázok konstitutívan aktív, trunkált ErbB2-t képesek generálni. Azonban ehhez hasonló trunkált ErbB2 a transzláció alternatív iniciációja révén is létre jöhet, és mivel dimert alkot egy párosítatlan szulfhidril csoport által létrehozott diszulfid kötés által, nagyon magas konstitutív aktivitással rendelkezik (111). Mivel az így keletkező ErbB2-nek nincs extracelluláris doménje, az ezt kifejező emlődaganatok rezisztensek a trastuzumabra (112). Egy új mechanizmus felfedezésével saját kutatási eredményeink is hozzájárultak a rezisztencia leírásához. Az „Eredmények és megbeszélés” fejezetben részletesen ismertetendő módon az ErbB2 molekula MUC4 vagy hialuronsav általi maszkírozása és a trastuzumab következményes gátolt kötődése szintén rezisztenciához vezet (113, 114).

1.5. A lipid membrán doménjei: a lipid tutajok szerkezete és biológiai funkciói

A membránreceptorok aktiválódása klaszterizációjuk megváltozásával jár együtt, ami a membrán lipid környezetében zajlik. Ezért nem meglepő, hogy a legtöbb transzmembrán jelátvitelben részt vevő receptort valamilyen mértékben befolyásolják a lipidek, a lipidek által alkotott klaszterek, ill. ezek legrészletesebben tanulmányozott típusai, a lipid tutajok (115), amelyekről Kai Simons eredeti felvetése óta szinte mindent és annak az ellenkezőjét is állították már (116, 117). A probléma forrását az jelenti, hogy azok a struktúrák, amiket lipid tutajokként különböző módszerekkel (pl. sejtbiológiai, biokémiai megközelítés, mesterséges membránokkal végzett kísérletek, dinamikus egyedi molekula mérések) vizsgálnak, szinte biztos, hogy nem ugyanazt a struktúrát takarják. Ebben a fejezetben röviden áttekintem ezen bizonytalanság eredetét és amit a lipid tutajok és a membránlipidek egyéb doménjeiről, ezek szerkezetéről és biológiai funkciójáról a dolgozat témája szempontjából tudni érdemes.

Az elmúlt évtizedek vizsgálatai egyértelművé tették, hogy a Singer és Nicolson által 1972-ben a sejtmembránra megfogalmazott folyékony mozaik modell több szempontból is revízióra szorul (118). Kiderült, hogy a lipidek nem struktúra nélküli tengert alkotnak, és hogy a fehérjék ebben nem véletlenszerűen oszlanak el és nem diffundálnak teljesen szabadon. Ezen szervezettség kialakulásában több tényező vesz részt.

1. Többkomponensű lipid membránokban a lipidek fiziko-kémiai tulajdonságaik miatt hajlamosak szeparált fázisokat vagy (mikro-)doméneket létre hozni. Ezek mérete és élettartama sokféle lehet, a koleszterin molekulák néhány nanoszekundumig létező klasztereitől (119) egészen a termodinamikailag stabil fázis szeparációig (120). A stabil domének közül a folyékony rendezett (L_o , liquid ordered) és folyékony rendezetlen (L_d , liquid disordered) domének jelentősek biológiai szempontból. A lipid tutajok fizikai-kémiai tulajdonságaik alapján az előbbinek felelnek meg. A lipidek klaszterizációját eltérő méretük (a zsírsav oldalláncok hossza), a zsírsavak flexibilitásának különbözősége és a lipidek között kialakuló hidrogén kötések váltják ki. Különösen jelentős ebből a szempontból a lipid tutajok fontos összetevői közül kettőnek, a koleszterinnek és a szfingolipideknek hidrogén kötésekben való részvételi hajlama (121). A koleszterin szerepe annyira fontos, hogy nélküle általában nem történik fázis szeparáció (120). A lipid tutajok két másik fontos komponense, a glikolipidek és a 2-hidroxilált zsírsavval rendelkező lipidek, szintén a hidrogén kötések kialakítása miatt játszanak fontos szerepet az L_o domének

kialakulásában (122, 123). A lipidek fizikokémiai tulajdonságai által vezérelt klaszterizáció és (mikro-)domén alkotás azért is jelentős, mert egyszerűen vizsgálható mesterséges, általában csak lipidet tartalmazó modellmembránokkal. A mesterséges membránokban megjelenő lipid domének általában sokkal nagyobbak és stabilabbak, mint élő sejtben megfigyelhető társaik (120). Ezért felmerül, hogy egyáltalán ezek megfelelnek-e a lipid tutajoknak. A válasz az, hogy bizonyos tekintetben igen, hiszen a lipid tutajok vélt tulajdonságai közül többet is rekapitulálnak. Ilyenek pl. a hideg, nem ionos detergensben való oldhatatlanság, a lipidek szorosabb rendeződése, rendezettsége, amit a telített zsírsav oldalláncok okoznak és a lassabb diffúzió (120, 121). Fontos megjegyezni, hogy a membrán fluiditás és rendezettség nem mindig változnak egymással összhangban. A fluiditás elsősorban a lipidek laterális és rotációs mobilitását befolyásolja, míg a rendezettség a membrán víz számára való hozzáférhetőségét, azaz a lipid molekulák hidratáltságát határozza meg elsősorban (124, 125).

2. A lipidek közötti fizikai-kémiai kölcsönhatások mellett a biológiai membránok esetében tekintetbe kell venni a membrán dinamikus változását, leginkább az állandóan folyó endo- és exocitotikus folyamatokat. Számítógépes szimulációk és kísérletek segítségével kimutatták, hogy ezek az aktív folyamatok egyrészt önmagukban is hozzájárulnak a klaszterek létrejöttéhez, ugyanakkor gátolják is túl nagy méretű aggregátumok létrejöttét. Ez lehet az egyik oka annak, hogy a biológiai membránokban sokkal kisebb mikrodomének jönnek létre, mint a mesterséges membránok nagy méretű doménjei (126, 127).
3. A biológiai membránokban a domének létrejöttének szabályozásában szintén nem lehet eltekinteni a fehérjék szerepének tárgyalásától. A lipid tutajokkal kapcsolatban elsősorban a glikozilfoszfatidil-inozitol (GPI) kötött fehérjéket kell megemlíteni, amelyek telített zsírsav tartalmuknak köszönhetően preferenciálisan a lipid tutajokban fordulnak elő (128). Ezenkívül a lipid módosított perifériás membránfehérjék is lehetnek lipid tutajokkal asszociáltak. Ezt elsősorban az aciláció, és nem a preniláció váltja ki (129). A transzmembrán (TM) fehérjék helyzete kicsit bonyolultabb. Míg a több TM szegmenssel rendelkező fehérjék (pl. G fehérjéhez kapcsolt fehérjék hét TM szegmenssel) elsősorban az L_d doménben találhatók (130), a membránt egyszer átívelő fehérjék megoszlását az L_d és L_o

domének között a TM hélix és a lipid környezet közötti hidrofób meg nem felelés („hydrophobic mismatch”) határozza meg, tehát a TM hélix és a membrán vastagságának eltérése (131). A TM hélixek jelenléte önmagában is domén szeparációt válthat ki azáltal, hogy a fehérje körül bizonyos lipidek preferenciálisan bedúsulnak (130, 132). Ebből a szempontból lényeges, hogy a koleszterin merev struktúrája miatt a TM fehérjék közvetlen közelében ritkán található (133). Más lipid molekulák viszont szinte stabilan asszociálódnak a fehérjék TM szegmesével. Ezt a szerveződést lipid héjnak („lipid shell”) nevezték el (134).

4. A citoszkeleton több ponton is beleszól a domének kialakulásának folyamatába. Egyrészt korlátozza a μm átmérőjű domének keletkezését (135), másrészt szubmembrán kerítések létrehozásával korlátozza a TM fehérjék diffúzióját és tranziensen néhány száz nanométer átmérőjű zónákba korlátozza azokat (136). Sőt, nemcsak a fehérjék, hanem a lipid molekulák is ilyen ugráló diffúziót („hop diffusion”) mutatnak (137). A citoszkeletonhoz rögzített TM fehérjék („karók”, „pickets”) hatása olyan drasztikus lehet, hogy teljesen le tudják állítani két membrán domén között a diffúziót (138).

A fenti összetett hatások eredményeképpen az élő sejtek membránjában is létrejönnek mikrodomének (lipid és fehérje molekulák aggregátumai), amelyek azonban termodinamikailag nem stabil fázisok, hanem dinamikusán változó képződmények. Méretük a néhányszor tíz nanométeres nagyságrendbe esik, és élettartamuk a néhány nanoszekundumtól másodpercekig terjed. Többen gondolják azt, hogy ami csak 1-2 nanoszekundumig stabil, az nem is létezik, és ezzel kétségbe vonják a lipid tutaj hipotézist (117, 133).

A fent körvonalazott sokféleségben többen próbáltak már rendet teremteni. Egyik próbálkozás szerint a membránban háromszintű doménszerkezet jön létre (133):

1. A citoszkeletonhoz kötött TM fehérjék által alkotott karók létrehozta domének, melyek átmérője a néhány száz nanométeres tartományba esik. Ez a szerveződési szint tehát anomálissá teszi a diffúziót azáltal, hogy a doméneken belül szabad diffúzió folyik, de a domének közötti határ átugrása („hop diffusion”) csak ritkán következik be.
2. A tulajdonképpeni mikrodomének, melyek megfelelnek a lipid tutajoknak. Átmérőjük a 10 nanométeres skálán van. A lipid tutajokban szfingolipidek,

glikolipidek, telített és hidroxilált zsírsavakat tartalmazó lipidek, koleszterin, GPI-kötött és bizonyos transzmembrán fehérjék találhatók. A diffúziót ez a szint is korlátozza, ill. anomálissá teszi, hiszen ezen doménekhez mind lipidek (elsősorban szfingolipidek), mind pedig fehérjék (elsősorban GPI-kötöttek) tranziensen kikötődnek, csapdába esnek („trapping”) (139). Érdemes megjegyezni, hogy ezt a jelenséget csak abban az esetben lehet észlelni, ha az optikai feloldóképesség a csapdák méretének nagyságrendjébe esik (139).

3. A kisméretű, *közvetlen fehérje-fehérje kölcsönhatások* segítségével kialakuló fehérje aggregátumok (általában dimerek). Ez a szint felel meg az előző fejezetekben említett ErbB fehérje dimereknek.

Érdemes megjegyezni, hogy a fenti, a szerveződési szinteket a lipidek oldaláról megközelítő modellhez hasonló elképzelést mi is javasoltunk, de javaslatunkat elsősorban a fehérje klaszterizációval alapoztuk meg (140). Ez részletesebben az „Eredmények és megbeszélés” fejezetben kerül tárgyalásra.

A legjelentősebb lipid mikrodomén, a lipid tutaj, számtalan biológiai folyamatban vesz részt. Egyrészt fontos szerepet játszik az endocitózisban. Ebben a folyamatban a lipid tutajok egy speciális alosztálya, a kaveola, vesz részt a legaktívabban. A kaveolák annyiban tekinthetők speciális lipid tutajoknak, hogy a fenti alkatrészekon kívül tartalmaznak egy caveolinnak nevezett fehérje komponenst is (75). Azonban az ún. klatrin és kaveolin független endocitózisban szintén lehet szerepe a nem kaveoláris lipid tutajoknak (75). A membránkörforgalomban történő szerepvállalás mellett a tutajok a transzmembrán jelátvitelben töltönek be kulcsfontosságú szerepet (115). Számtalan jelátviteli folyamat kötődik a lipid tutajokhoz, amelynek többféle mechanizmusa lehet. Elképzelhető, hogy egy receptor mind inaktív, mind aktív állapotában a lipid tutajokkal asszociált, de az is, hogy a ligand kötést kísérő konformáció-változás és klaszterizáció fokozza a fehérje lipid tutaj iránti affinitását, ami a receptor lipid tutajba való vándorlásában nyilvánul meg (pl. T sejt receptor esetében (141)). Az is lehetséges, hogy a lipid tutajok, ill. GPI-kapcsolt fehérjék keresztkötése indukálja a lipid tutajok aggregációját, ami elindítja a transzmembrán jelátviteli folyamatokat (142). Tehát általánosságban elmondható, hogy az aktiváció növeli a lipid tutajok méretét, és viszont: a lipid tutajok méretének növelése (pl. keresztkötés által) sokszor aktiválja a benne levő fehérjéket (115). Érdemes megjegyezni, hogy a fluoreszcens mikroszkópiában kolera

toxin A alegységgel (CTX-A) vagy antitestekkel megjelölt lipid tutajok nem egyedi tutajoknak, hanem azok keresztkötött aggregátumainak felelnek meg (143).

Az ErbB fehérjéknek is van közük a lipid tutajokhoz. Az ErbB1-ről kimutatták, hogy a lipid tutajok befolyásolják funkcióját. A lipid tutajok, ill. azok ganglioizid tartalma csökkenti az ErbB1 ligand iránti affinitását és dimerizációját (144, 145), és a caveolin gátolja az ErbB1 kináz aktivitását (146). Az ErbB1 aktivációját a lipid tutajok aggregációja kíséri (147), és ezzel párhuzamosan a receptor elhagyja a kaveoláris lipid tutaj frakciót (148). Mivel az ErbB2-ről mi mutattuk ki, hogy a tutajokkal asszociált (42), ezért a tutajok ErbB fehérjék globális jelátviteli képességeit befolyásoló hatásait az „Eredmények és megbeszélés” fejezetben tárgyalom részletesen.

1.6. A fehérjeasszociációk mérésének jelentősége és módszerei

A jelátviteli folyamatokban a különböző méretű fehérje klaszterek képződése alapvető jelentőségű, amit az ErbB receptorokkal kapcsolatban a „Bevezetés” eddigi fejezeteiben bemutattam. Hasonló klaszterek a másodlagos jelátvivő proteinek között is képződnek, mint pl. a KSR fehérje által alkotott platform a MAPK útvonalon (149). Ezért érthető, hogy a klaszterizáció mérése az e témával foglalkozó sejtbiológiai és biofizikai témájú projektekben központi jelentőségű. Ezen fejezet célja, hogy az egyes módszerek részleteinek bemutatása és minden eljárás felsorolása nélkül rendszerbe szervezze azokat, és rámutasson arra, hogy mik az egyes megközelítések előnyei és hátrányai. A módszerek közül azok, amelyek a tárgyalt cikkek szempontjából lényegesek, részletesebb bemutatásra kerülnek.

A módszereket célszerű két nagyobb csoportra osztani:

1. Kvalitatív-szemikvantitatív eljárások: Ezeket a módszereket alkalmazzák a legkiterjedtebben a sejt- és molekuláris biológiai kutatások során. Előnyük az egyszerűség, ill. az általános felszereltségű sejtbiológia laborban való megvalósíthatóság, de több potenciális hátrálótóval is rendelkeznek.
 - a. Koprecipitáció, kémiai keresztkötés, co-capping, Western blot: a legegyszerűbb és legáltalánosabban alkalmazott módszerek. Csak kvalitatívan tudják a fehérjék közötti kölcsönhatásokat jellemezni, és még ezeket az eredményeket is körültekintően kell értelmezni. Amennyiben a módszer antitesttel vagy kémiai ágenssel történő keresztkötést igényel, reális annak az esélye, hogy olyan molekulák is keresztbe kötődnek, amelyek az élő sejtben

nem álltak kölcsönhatásban egymással. A mesterségesen indukált kölcsönhatásokon kívül az a veszély is fenyeget, hogy az élő sejtéből történő kivonás során nemcsak ilyen arteficiális aggregátumok képződnek, hanem a gyengén kölcsönható klaszterek szétesnek (150).

- b. Proximitás ligációs vizsgálat (proximity ligation assay, PLA) (151, 152): Az eljárás alapja az, hogy a vizsgálandó két fehérjét megjelölik oligonukleotidhoz kapcsolt antitestekkel. Amennyiben a két fehérje egymás közelségében van, akkor a később hozzáadott lineáris összekötő oligonukleotidok segítségével cirkuláris DNS képződik, amely RCA (rolling circle amplification, gördülő cirkuláris amplifikáció) reakció segítségével sokszorozható. Ezen termékhez fluoreszcensen jelölt oligonukleotid hibridizálható, amely megjelöli a kölcsönható fehérjék helyzetet. A módszer kvantitatívításának hiányáról az „Eredmények és megbeszélés” fejezetben részletesen lesz szó (153).
 - c. Nagy áteresztőképességű szűrésre képes technikák (pl. élesztő két hibrid (yeast two hybrid, Y2H), bimolekuláris fluoreszcencia komplementáció (bimolecular fluorescence complementation, BiFC)) (154-156): Ezen módszerek alapja, hogy azt a fehérjét („csali”, „bait”), aminek az interakciós partnereit ki szeretnék mutatni, vagy egy transzkripció faktor (Y2H esetében) vagy egy fluoreszcens fehérje (BiFC esetében) egy fragmentumához kapcsolják, míg a keresett interakciós partnereket („áldozat”, „prey”) a transzkripció faktor vagy a fluoreszcens fehérje másik doménjével fuzionáltatják. Ha a csali és az áldozat kölcsönhat egymással, akkor a transzkripció faktor vagy a fluoreszcens protein két doménje is egymásra talál, és így helyre áll a két részre hasított protein aktivitása: a transzkripció vagy a fluoreszcencia detektálható. Felmerül annak a lehetősége, hogy a kölcsönhatást nem a „csali” és az „áldozat” fehérje hozza létre, hanem a transzkripció faktor két alegysége vagy a fluoreszcens fehérje N- és C-terminális doménje. További limitáció, hogy a fragmentálódott fluorofór vagy transzkripció faktor rekonstruálása irreverzibilis.
2. Kvantitatív eljárások: Míg az előző pontban említett módszerek esetében esély sincs arra, hogy a kölcsönhatást kvantitatívan jellemezzük, addig az itt tárgyalandó eljárások az interakció valamilyen aspektusát kvantitatív szempontból jellemzik.

Lényeges, hogy az egyes módszerek eltérő érzékenységet mutatnak a különböző méretű és stabilitású klaszterekre. Ez ellentmondó eredményekhez vezethet, amelyeket a módszerek korlátaik ismeretében fel lehet oldani. Általánosságban elmondható, hogy ezen módszerek kifinomult fizikai és matematikai mérőmódszereket vagy kiértékelési eljárásokat foglalnak magukba, amelyek gyakran elijeszti a sejt- és molekuláris biológiai közösség nagy részét (140, 154, 157, 158).

- a. Kolokalizáció mérése (159): Amennyiben két fluoreszcensen jelzett fehérje eloszlása fluoreszcens mikroszkópos felvételen hasonló (korrelál egymással), akkor feltételezhető, hogy a két molekula között valamilyen kölcsönhatás van. Számtalan tanulmány bizonyította már, hogy a kolokalizáció kvalitatív mérése (zölddel és vörössel jelölt fehérjék esetében sárga pixelek megjelenése) félrevezető lehet, ezért kizárólag a kvantitatív kiértékelés a mérvadó. Ennek széleskörűen elterjedt változata a Pearson féle korrelációs koefficiens, de sok más paraméter is számítható.
- b. Fluoreszcencia rezonancia energia transzfer (FRET) (160-162): A FRET, amelynek rövidítését néha leírója alapján Förster típusú rezonancia energia transzfernek értelmezik, egy fluoreszcens, ún. donor molekula és egy nem feltétlenül fluoreszcens akceptor molekula közötti dipól-dipól kölcsönhatás segítségével történő energia átadás (160, 163, 164). A folyamat során a gerjesztett donor molekula foton emisszió nélkül energiát ad át a környezetében levő akceptornak. A folyamat feltételei a következők: (1) a donor és az akceptor távolsága 2-10 nm legyen; (2) a donor és az akceptor orientációja megfelelő legyen, amit a κ^2 orientációs faktorról jellemeznek (165-167); (3) a donor normalizált emissziós és az akceptor abszorpciós spektrumai egymással kellő mértékű átfedést mutassanak, amit a J átfedési integrállal írnak le. A FRET sebességi állandóját a következő egyenlet adja meg:

$$k_{FRET} = konst \cdot J \cdot n^{-4} \cdot k_f \cdot R^{-6} \cdot \kappa^2 \quad (1)$$

ahol n a közeg optikai törésmutatója, R a donor-akceptor távolság és k_f a fluoreszcencia sebességi állandója. Az R és a κ^2 tagokon kívül a többi paraméter konstansnak tekinthető, azonban ahhoz, hogy a FRET valóban

távolság mérő módszerré válhasson, bizonyítani kell, hogy az orientációtól való függés elhanyagolható. A legtöbb esetben FRET-et molekuláris sokaságon mérnek, ahol a fenti k_{FRET} sebességi állandó átlagolódik. A konstans és a konstansnak tekinthető tagok (J , n , k_f) mellett a változó paraméterek átlagolódását kell megvizsgálni. Ezek átlagolódhatnak egymástól függetlenül ($\langle R^{-6} \rangle \langle \kappa^2 \rangle$) vagy szorzatként ($\langle R^{-6} \kappa^2 \rangle$). A legtöbb esetben jó közelítéssel a független átlagolódás következik be, amikor a donor és az akceptor gyors rotációs mozgást végez. Ebben az esetben a κ^2 átlagát, 2/3-ot helyettesítenek az egyenletbe (dinamikus átlagolódás), és a sebességi egyenlet a következőképpen egyszerűsödik (165-167):

$$k_{FRET} = konst \cdot R^{-6} \quad (2)$$

azaz a FRET sebességi állandója csak a távolságtól függ. A FRET kompetál a többi relaxációs mechanizmussal, a fluoreszcenciával és a többi, nem fluoreszcens átmenettel, melyek sebességi állandói rendre k_f és k_{nf} . A FRET hatásfok (E) megadja azon gerjesztett donor molekulák arányát, melyek FRET hatására relaxálódnak:

$$E = \frac{k_{FRET}}{k_{FRET} + k_f + k_{nf}} \quad (3)$$

A távolságfüggés világos kifejezése érdekében többször használják a következő egyenletet:

$$E = \frac{R_0^6}{R_0^6 + R^6} \quad (4)$$

ahol R_0 az ún. kritikus Förster távolság, amely egy adott donor-akceptor párra megadja azt a távolságot, ahol $E=50\%$.

A FRET gyakorlati alkalmazhatóságát meghatározza az, hogy milyen mérhető folyamatokban manifesztálódik. Ezt külön kell a FRET kétféle változata esetében tárgyalni.

(1) Hetero-FRET: konvencionális FRET-nek is szokták nevezni. Ebben az esetben a donor és akceptor molekulák spektroszkópiailag különböznek egymástól. Ebben az esetben a FRET a donor fluoreszcencia intenzitásának

csökkenéséhez (fluoreszcencia kioltás, „quenching”) és a donor fluoreszcencia élettartamának a csökkenéséhez vezet (dinamikus kioltás):

$$E = 1 - \frac{F_{DA}}{F_D} = 1 - \frac{\tau_{DA}}{\tau_D} \quad (5)$$

ahol F és τ a donor fluoreszcencia intenzitását és fluoreszcencia élettartamát jelentik, a D alsó index arra utal, hogy csak a donor van jelen, a DA pedig arra, hogy a donor mellett akceptor is van a rendszerben. A donor élettartamának csökkenése még több mérhető változáshoz vezet. (i) A donor rövidebb fluoreszcencia élettartama miatt kevesebb idő áll a donor rendelkezésére, hogy a gerjesztett állapotban forogjon, ezért kevésbé depolarizálódik a fluoreszcencia: nő a donor fluoreszcencia anizotrópiája (168):

$$E = 1 - \frac{\frac{r_0}{r_D} - 1}{\frac{r_{DA}}{r_D} - 1} \quad (6)$$

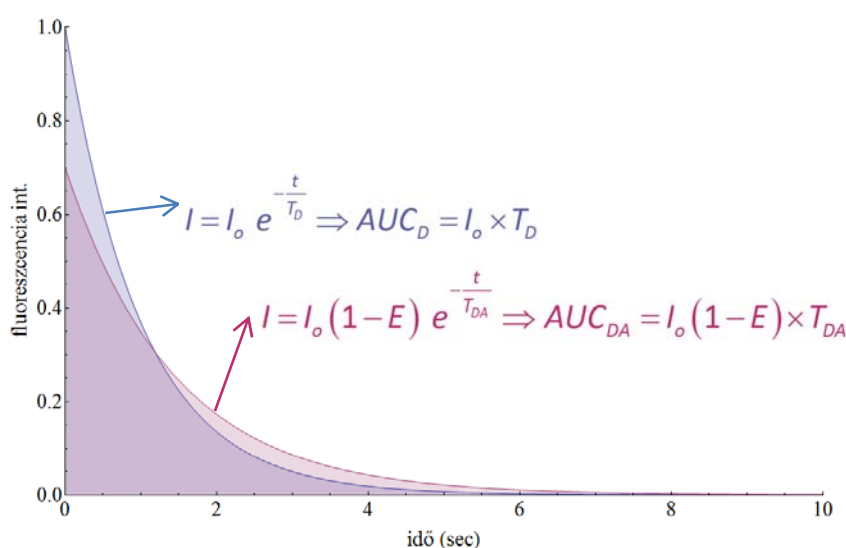
ahol r_0 a donor határanizotrópiája, r_D és r_{DA} pedig a donor anizotrópiája az akceptor nélkül és jelenlétében. A határanizotrópia az az anizotrópia érték, amely egy „befagyott” molekula esetében mérhető, amely nem végez forgást a fluoreszcencia élettartamnak megfelelő idő alatt. (ii) A donor kioltás egy másik detektálási módszere az akceptor fotoelhalványításán (photobleaching) alapszik. Ennek során megméri a donor fluoreszcencia intenzitását akceptor jelenlétében (F_{DA}), majd az akceptort magas intenzitású gerjesztő fénnel kiégetik. Mivel ezáltal megszűnik az akceptor abszorpciós képessége, az akceptor kiégetés után mért donor intenzitás megfelel az akceptorral nem jelölt minta fluoreszcencia intenzitásának (F_D), és a FRET az (5) egyenlet alapján kiszámítható (169, 170). (iii) A donor élettartamának csökkenése a donor fotoelhalványítási kinetikáját is lassítja (171, 172). Ennek oka az, hogy a fotoelhalványítás a gerjesztett állapotból kiinduló változás, amely a donor fluoreszcens tulajdonságainak elvesztéséhez vezet. Mivel a donor fluoreszcens élettartamának csökkenése miatt a donor kevesebb időt tölt a gerjesztett állapotban, a fotoelhalványítás sebessége lassul. Egy másik gondolatmenet is hasonló következtetésre vezet. A donor fotoelhalványodása

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előtt meghatározott számú fotont tud kibocsátani, amit a FRET nem befolyásol. Az emittált fotonok száma arányos a fluoreszcencia-idő grafikon alatti területtel. Ennek értelmében, ha a donor fotoelhalványítási idejét akceptor jelenlétében T_{DA} -val és nélküle T_D -vel jelöljük, a FRET hatásfok kiszámítható:

$$AUC_D = AUC_{DA}$$

$$I_0 T_D = I_0 (1-E) T_{DA} \Rightarrow E = 1 - \frac{T_D}{T_{DA}} \quad (7)$$



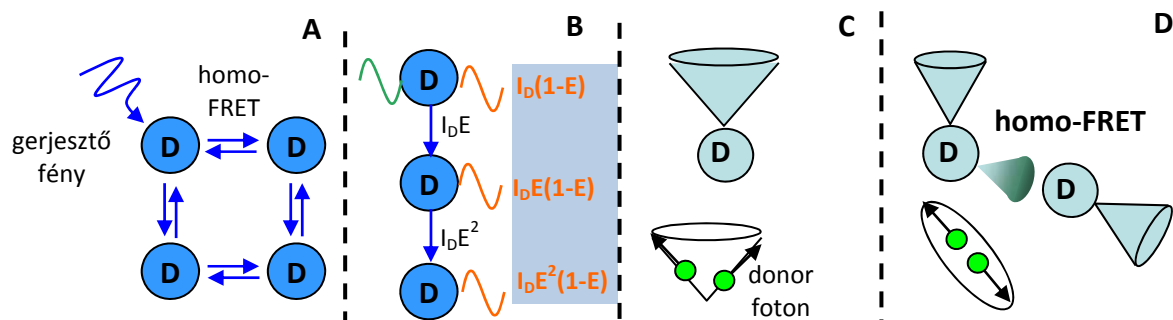
2. ábra. A donor fotoelhalványításon alapuló FRET mérés értelmezése a grafikon alatti területek (AUC – area under the curve) alapján. A kék görbe a donor fotoelhalványítási kinetikáját, míg a piros a donor akceptor jelenlétében mért fotoelhalványítási kinetikáját mutatja.

A hetero-FRET mérésének egy másik lehetőségét adja az akceptor fluoreszcenciájának növekedése (szenzitizált emisszió), amennyiben az akceptor fluoreszcens. Mivel a donor és az akceptor közötti szinte mindig van spektrális átfedés, ennek kiszámítása mindig az átvilágítások ismeretében kell, hogy történjen, és a műszertől, ill. a festékektől függ (161). Ennek vázlatos leírása az „Anyagok és módszerek” fejezetben található.

(2) homo-FRET: ebben az esetben a donor és az akceptor spektroszkópiailag megkülönböztethetetlen, azaz ugyanolyan kémiai szerkezetű és ugyanabban a környezetben levő molekula. A folyamatnak ugyanazok a feltételei, mint a fentebb felsoroltak, azzal a kiegészítéssel, hogy a J átfedési integrál itt egyazon molekula abszorpciós és normalizált emissziós

spektruma között értendő. Ennek van egy további fontos következménye. A donor molekula által homo-FRET-tel gerjesztett akceptor egy további homo-FRET kölcsönhatásban lehet donor, és így az energia szétterjed a klaszterben (3a. ábra). Ez lehetőséget teremt arra, hogy homo-FRET mérésekkel a klaszter méretét meghatározzuk. Az első molekula fluoreszcencia intenzitása $I_D(1-E)$, és I_DE energiát ad át a sorban következő molekulának, amely $I_DE(1-E)$ intenzitást emittál, és I_DE^2 mennyiségű energiát a harmadik molekulának ad. Általánosságban az n -dik molekula által kibocsátott intenzitás az $I_DE^{n-1}(1-E)$ képlettel írható le, és a molekuláris sokaság teljes intenzitását ennek a végtelen mértani sorozatnak az összege adja, ami I_D -vel egyenlő. Következésképpen nincs fluoreszcencia kioltás, a folyamat sem intenzitás, sem élettartam méréssel nem detektálható (3b. ábra). A homo-FRET egyetlen mérhető manifesztációja a donor fluoreszcencia anizotrópiájának csökkenése.

Egy fluoreszcens molekula anizotrópiáját polarizált fénnel történt gerjesztését követően az határozza meg, hogy a molekulák emissziós dipólusai mennyire maradnak rendezettek a fluoreszcencia élettartam alatt. Az emissziós dipólusokat egy kúppalásttal szokás reprezentálni. A rendezetlenséget a kúppalást nagyobb nyílásszöge mutatja, amit előidézhethet pl. rotációs mozgás. Mivel a homo-FRET segítségével olyan molekulák is gerjesztődnek, melyek emissziós dipólusa nem párhuzamos a donor molekuláéval, a molekuláris sokaság által kibocsátott fotonok rendezettsége, anizotrópiája csökken (3c-d. ábra). Mivel az anizotrópiát nemcsak a FRET határozza meg, ezért molekuláris asszociáció mértékével való korreláltatása problémás. Ennek kiküszöbölésére az anizotrópiát vagy időfüggően mérik (128) vagy a fluorofór koncentrációtól való függését elemzik. Ennek részletei az „Eredmények és megbeszélés” fejezetben kerülnek tárgyalásra.



3. ábra. (A) Polarizált fénnel történő gerjesztés hatására kialakult homo-FRET spektroszkópiailag azonos molekulák között. A kettős nyilak reprezentálják az oda-vissza is lejátszódó homo-FRET kölcsönhatást (D = donor). (B) Homo-FRET esetében a donorok sokasága által kibocsátott összes fluoreszcencia intenzitás nem csökken. (C-D) A fluoreszcencia anizotrópia FRET jelenléte nélkül (C) és homo-FRET esetében (D). A donor forgáskúpjának szélessége arányosan változik a molekula rotációs diffúziós mozgásával.

- c. Fluoreszcencia korrelációs spektroszkópia (FCS), „number and brightness” analízis (N&B) (173, 174): Az FCS esetében egy konfokális mikroszkóp detekciós térfogatában a fluoreszcencia fluktuációját mérik. Ha egy fehérjét jelölünk fluoreszcensen, akkor annak homoasszociációját lehet detektálni. FCS esetében a fluoreszcencia autokorrelációs függvényét számítják ki, ami több paraméter mellett függ az egy pixelben levő molekuláris egységek számától (N_D). Ha a diffúzió kívül más folyamat nem járul hozzá a fluoreszcencia intenzitás ($F(t)$) fluktuációjához, az autokorrelációs függvényt a következő egyenlet írja le két dimenzióra korlátozott mozgás esetén:

$$G(\tau) = \frac{\langle F(t)F(t+\tau) \rangle}{\langle F(t) \rangle^2} = 1 + \frac{1}{N_D} \frac{1}{1 + \frac{\tau}{\tau_D}} \quad (8)$$

ahol τ az időt, a τ_D a diffúziós korrelációs időt jelenti, míg a $\langle \rangle$ zárójel időbeli átlagolást jelöl. A molekuláris egység lehet egy darab fluorofór (monomer), de ha klaszterről van szó, akkor a dimer, trimer, stb. számít molekuláris egységnek, feltéve, hogy együttesen lépnek be és hagyják el a detektálási térfogatot. Ha ismerjük a detektálási térfogatban a detektálási idő alatt gyűjtött fotonok átlagos számát, akkor ennek és az N_D -nek a hányadosa megadja a molekuláris egység molekuláris fényességét (ε_M , a detekciós idő alatt a detektálási térfogatban emittált és detektált fotonok száma). Ha ismerjük egy fluorofór molekuláris fényességét (ε_F), akkor $\varepsilon_M/\varepsilon_F$ megadja a

homo-oligomerizáció fokát. Ezenkívül az autokorrelációs függvény illesztéséből kapott diffúziós korrelációs idő is függ az asszociáció mértékétől, de ebből a fentiekben leírt módon megszámlálni a klaszterben levő fluorofórok számát nem lehet (173).

A N&B mérések az FCS alapelveire épülnek (174). Az elv megértéséhez vizsgáljuk meg egy pixel fluoreszcencia intenzitásának varianciáját. Ennek két komponense van: (1) az egy pixelben levő molekulák számának fluktuációja (σ_N^2); (2) a foton detektálás Poisson statisztikája (σ_D^2). A teljes varianciát (σ^2) a két komponens összege adja meg:

$$\sigma^2 = \sigma_N^2 + \sigma_D^2 \quad (9)$$

Definiáljuk a látszólagos fényességet (B) a következő képlet szerint:

$$B = \frac{\sigma^2}{\langle I \rangle} = \frac{\sigma^2}{\varepsilon \langle N \rangle} = \frac{\sigma_N^2 + \sigma_D^2}{\varepsilon \langle N \rangle} \quad (10)$$

ahol $\langle N \rangle$ a molekuláris egységek átlagos száma a detektálási térfogatban, ε pedig a molekuláris fényességük. Statisztikai megfontolások alapján belátható, hogy

$$\sigma_N^2 = \varepsilon^2 \langle N \rangle, \quad \sigma_D^2 = \varepsilon \langle N \rangle \quad (11)$$

ezért

$$B = \frac{\sigma_N^2 + \sigma_D^2}{\varepsilon \langle N \rangle} = \frac{\varepsilon^2 \langle N \rangle + \varepsilon \langle N \rangle}{\varepsilon \langle N \rangle} = \varepsilon + 1 \quad (12)$$

Tehát egy pixelben a variancia és az átlagos fluoreszcencia intenzitás hányadosából ki lehet számítani a molekuláris egység molekuláris fényességét (ε), aminek a fluorofór molekuláris fényességével történő összehasonlítása megadja a homoasszociáció fokát. Megjegyzendő, hogy mind az FCS, mind a N&B módszernek létezik kétszínű változata, amellyel két különböző fluorofórral jelölt molekula heteroklaszterizációja mérhető kvantitatív módon (175, 176).

- d. Diffúzió mérésén alapuló módszerek (egyedi partikulum követés (single particle tracking, SPT), fluoreszcencia visszatérés fotoelhalványítás után (fluorescence recovery after photobleaching, FRAP)) (177, 178): Mindkét módszer esetében a jelölt (FRAP esetében fluoreszcensen, SPT esetében

fluoreszcensen vagy arany gömbbel) molekulák diffúzióját követjük, és a diffúziós állandó fentebb említett, asszociációtól (tehát hidrodinamikai sugártól) való függéséből lehet az oligomerizáció mértékére következtetni. Ez SPT esetében úgy történik, hogy az egyedi molekulák diffúziós pályáját kirajzolják, és az átlagos négyzetes elmozdulás ($\langle \Delta r^2 \rangle$) kiszámításával a következő egyenlet alapján meghatározzák a diffúziós állandót (D) (178):

$$\langle \Delta r^2 \rangle = 4Dt \quad (13)$$

A fenti egyenlet kétdimenziós diffúzió esetében használható. FRAP esetében egy megfigyelési területen egy erős lézerimpulzus segítségével elhalványítják (photobleaching) a fluorofórok fluoreszcenciáját, majd egy alacsony intenzitású lézer segítségével monitorozzák a fluoreszcencia visszatérését, amelynek illesztéséből a diffúziós állandó meghatározható (177). Mindkét diffúzió mérésén alapuló módszer esetében figyelembe kell venni azt, hogy a diffúzió a sejtmembránban nagyon gyakran anomális (l. 1.5 fejezet). Kétszínű SPT mérések esetében a két fluorofór pályájának követésével további információ is nyerhető. Egy fluorofór helyzete nanométeres pontossággal meghatározható (l. következő pont), és így a két vizsgált festék esetében megállapítható, hogy mikor kerülnek molekuláris távolságba egymástól. A folyamat statisztika analíziséből a dimerizáció kvantitatívan jellemezhető (28, 29).

- e. Lokalizáció mérésén alapuló módszerek (stimulált emisszió deplációs mikroszkópia (stimulated emission depletion microscopy, STED), sztochasztikus optikai rekonstrukciós mikroszkópia (stochastic optical reconstruction microscopy, STORM), fotoaktivációs lokalizációs mikroszkópia (photoactivation localization microscopy, PALM)) (179-182): Az itt röviden ismertetendő technikák azon a feltevésen alapulnak, hogy a molekuláris asszociációk jellemezhetők akkor, ha az egyedi molekulák pozícióját nanométeres pontossággal ismerjük. Ilyenkor két molekula akkor tekinthető egymással asszociáltnak, ha néhány nanométerre vannak egymástól. Hogy mindez fluoreszcens mikroszkóppal keresztülvihető legyen, át kell lépni az ún.

Abbé féle határt, ami a diffrakción alapuló leképező rendszerekben a feloldóképességet limitálja:

$$d = \frac{0.6\lambda}{n \sin \omega} \quad (14)$$

ahol d a két még elkülöníthető fluorofór távolsága, λ a hullámhossz (konfokális mikroszkópiában a gerjesztési), n a tárgy és az objektív közötti közeg törésmutatója, ω pedig az objektív félnyílásszöge (183). A három technika közül a STED esetében egy lézernyalábbal a diffrakció által meghatározott területen gerjesztik a fluorofórokat, majd egy fánk alakú nyaláb segítségével indukált emisszióval depletálják a gerjesztett energiaszintet. A depletáló nyaláb intenzitásának beállításával elérhető, hogy a gerjesztett molekulák egy néhány nanométeres átmérőjű területre korlátozódjanak (179).

A PALM és a STORM technikák közös, de a STED-től eltérő elven alapulnak. Mindkettő esetében azt használják ki, hogy egy fluorofór helyzete nanométeres pontossággal meghatározható, ha képe nem fed át más fluorofórok képével. Ehhez azt kell elérni, hogy a fluoreszcensen jelölt mintában egyszerre a fluorofórok töredéke legyen gerjeszthető állapotban. A PALM esetében ezt fluoreszcens proteinek fotoaktiválásával, míg a STORM esetében cianin típusú festékek fotokapcsolásával („photo-switching”) érik el (180-182).

A kvantitatív módszerek nyilvánvaló előnye, hogy számszerűsítik a protein klaszterizáció valamilyen aspektusát. Azonban tekintettel kell lenni arra, hogy a fent ismertetett módszerek érzékenységet jelentősen befolyásolja, hogy a vizsgált asszociátum milyen időskálán stabil és milyen méretű (hány protein van benne és hány nanométer az átmérője). Ennek jelentőségére több közlemény mellett mi is felhívtuk a figyelmet (140, 154, 157). Röviden, bizonyos módszerek tényleges molekuláris asszociátumokat mutatnak ki, amikor a vizsgált fehérjék között közvetlen kontaktus alakul ki. Ide leginkább a strukturális vizsgálmódszerek (röntgen krisztallográfia, NMR) tartoznak. A hetero-FRET a fehérjék 2-10 nm-es szeparációs távolsága esetén utal a kölcsönhatás jelenlétére, de itt figyelembe kell venni azt, hogy ha a vizsgálat ideje alatt a két fehérje távolsága fluktuál, de néha a FRET távolságon

belül vannak egymástól, akkor a kölcsönhatást ki lehet mutatni. Tehát a FRET jelezhet valódi molekuláris asszociációt vagy egybezártságot („co-confinement”) is, amikor a fehérjéket valami nem engedi messzire eltávolodni egymástól. A homo-FRET az energia klaszterben való szétterjedése miatt érzékenyebb a nagyobb méretű klaszterekre, mint a hetero-FRET. Az FCS és a hasonló elven működő eljárások (pl. N&B) szintén jelezhetnek valódi molekuláris asszociációt és egybezártságot is, de itt az egybezártság dimenzióját a mikroszkóp feloldóképessége határozza meg. Tehát ha a vizsgált fehérjék 100-200 nm-es kalitkába vannak zárva, akkor a kölcsönhatás észlelhető ezekkel az eljárásokkal, míg FRET (homo- és hetero-) esetében ez már nem valószínű. A diffúzió mérésén alapuló eljárások inkább az egybezártságot, semmint a közvetlen molekuláris interakciókat mutatják ki, bár az SPT esetében a molekulák diffúziós pályájának statisztikai elemzésével valódi molekuláris interakciók léteére sikerült bizonyítékot szolgáltatni (28, 29). A nem kvantitatív eljárásokat nehéz a fenti „térképre” ráhelyezni, de általánosságban az mondható el, hogy többségük az egybezártságot mutatja ki. A fentiek ismerete nélkülözhetetlen olyan ellentmondó kísérleti eredmények értelmezéséhez, amikor az egyik egy kölcsönhatás jelenlétére utal, míg a másik erre utaló bizonyítékot nem talál.

2. Célkitűzések

A receptor-klaszterizációval kapcsolatban a következő terveket tűztük ki:

- Új fluoreszcenciás módszereket kívántunk kifejleszteni a receptor klaszterizáció kvantitatív analízisére, hiszen a legtöbb alkalmazott módszer csak kvalitatív vagy szemikvantitatív leírást tesz lehetővé.
- Meg kívántuk vizsgálni, hogy léteznek-e dimernél nagyobb molekuláris klaszterek, és ha igen, akkor ezen klasztereknek mi az összetétele és a biológiai szerepe.
- Össze kívántuk hasonlítani az ErbB1 és ErbB2 fehérjéket klaszterizációs tulajdonságaik szempontjából.
- Meg kívántuk vizsgálni, hogy a lipid tutajok és más ErbB fehérjék lokális denzitása hogyan befolyásolja az ErbB2 homoklaszterizációját és szignalizációs tulajdonságait.
- Elemezni kívántuk, hogy az ErbB1 EGF indukálta internalizációját a többi ErbB fehérje hogyan befolyásolja.

Munkánk során az ErbB receptorokat és a lipid tutajokat terápiás felhasználhatóságuk szempontjából is meg kívántuk vizsgálni.

- A trastuzumab rezisztencia kialakulásának (egyik) molekuláris mechanizmusát kívántuk leírni a rezisztencia jelenségét mutató JIMT-1 sejtek segítségével. A vizsgálatokat mind *in vitro*, mint *in vivo* körülmények között el kívántuk végezni.
- Célul tűztük ki, hogy az ErbB receptorklaszterek és a velük kölcsön ható egyéb molekulák (pl. CD44) által alkotott asszociátumok biológiai szerepét vizsgáljuk a receptor orientált terápia szempontjából.
- Meg kívántuk vizsgálni, hogy az RNS interferencia felhasználható-e az ErbB1-et fokozottan kifejező sejtek elpusztítására.
- Az elisidepsin nevű experimentális kemoterápiás szer hatásmechanizmusát, ill. annak ErbB fehérjével való kapcsolatának vizsgálatát is célul tűztük ki.

3. Anyagok és módszerek

3.1. Sejtek, antitestek, növekedési faktorok

A kísérletek során felhasznált sejtek egy részét (SKBR-3, A431, HeLa) az ATCC-től (American Type Culture Collections, ATCC, Manassas, VA) szereztük be. A trastuzumab rezisztens emlőtumor sejtvonalat, a JIMT-1-et, finn kollaborációs partnerünk állította elő (184), és a sejtvonal elérhető a DSMZ-től (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Németország). Az A431 sejtek különböző ErbB fehérjék fluoreszcens proteinnel fuzionált változatával stabilan transzfektált alvonalait mi állítottuk elő (185). Röviden, az A4erbB1 sejtvonal az endogén ErbB1 mellett ErbB1-eGFP-t expresszál, az A4erbB2-be ErbB2-mYFP-t transzfektáltunk, míg az A4erbB3 ErbB3-citrine-t fejez ki. Az 1. táblázat tartalmazza a sejtvonalak ErbB expressziós profilját, amit mi határoztunk meg Qifikit segítségével (Dako, Glostrup, Dánia). A tranziensen transzfektált sejteket vagy Lipofectamine 2000 (plazmid esetében) vagy Oligofectamine (siRNS esetében) segítségével állítottuk elő (mindkettő az Invitrogen terméke), ill. bizonyos esetekben elektroporációt végeztünk a Lonza cég Nucleofector készülékével. A FRET kalibráláshoz használt, különböző hosszúságú linkerrel elválasztott CFP-YFP variánsokat expresszáló élesztő sejteket kollaborációs partnerünk hozta létre (186).

A növekedési faktorokat (EGF, heregulin- β 1) az R&D-től (Minneapolis, MN) szereztük be. Az EGFR455 antitestet a 455 számú hibridóma (European Collection of Cell Cultures, Wiltshire, UK), az Mab528 antitestet a HB-8509-es hibridóma (ATCC) felülúszójából izoláltuk protein A affinitás kromatográfiával. A trastuzumab (Herceptin®) antitestet a Roche Magyarország Kft-től vásároltuk. A 2C4, a pertuzumab (Omnitarg®) és a 7C2 a Genentech (South San Francisco, CA) ajándékai voltak. Az ErbB2 intracelluláris részét felismerő OP15 antitestet a Merck-től (Schwalbach, Németország), míg a foszforilált ErbB2 ellenes Ab18-at a LabVision-től (Fremont, CA) vettük. Az ErbB3 (H3.90.6, H3.90.12, H3.105.5), ErbB4 (H4.77.16) és IGF1R (Ab1-Clone 24-31) ellenes antitestjeinket szintén a LabVision-től szereztük be. A MUC4-et felismerő antitest Kermit Carraway (University of Miami, FL) ajándéka volt. A pan-CD44 ellenes antitestet, a Hermes-3-at, a HB-9480 számú hibridóma (ATCC) felülúszójából tisztítottuk. A CD44 intracelluláris doménje elleni anti-CD44cyto poliklonális antitestet kollaborációs partnerünk állította elő úgy, hogy nyulakat immunizáltak a CD44 intracelluláris doménjének megfelelő peptiddel (187). A tisztított hialuronsav fragmentek a Seikagaku Corporation (Tokyo, Japan) ajándékai voltak. A fluoreszcens kolera

toxin B alegységet (CTX-B) az Invitrogentől vásároltuk. A TMA-DPH-t (4'-(trimetilammónium)-difenilhexatrién), a Laurdant (6-dodecanoil-N,N-dimetil-2-naftilamin) és a 4-metilumbelliferont (4-MU) a Sigma-tól (St. Louis, MO) vettük. Az elisidepsint kollaborációs partnerünk állította elő és bocsátotta rendelkezésünkre. A hialuronsav jelöléséhez használt biotinált HABC kollaborációs partnerünktől, Markku Tammitól (University of Turku, Finnország) származott. Az antitesteket fluoreszcens festékekkel a gyártók előírásainak megfelelően jelöltük meg.

Sejtvonal neve	ErbB1	ErbB2	ErbB3
	expressziós szintek ($\times 10^3$)		
A431	2300	21	3
A4erbB1	2070 (endogén) 1020 (ErbB1-eGFP)	20	1,5
A4erbB2	1200	950 (endogén+ErbB2-mYFP)	1,5
A4erbB3	2000	20	800 (endogén+ErbB3-citrine)
CHO	0	~0	0
CHO-ErbB2	0	200	0
CHO-ErbB2-3	0	200	100
CHO-erbB2mYFP	0	300 (ErbB2-mYFP)	0
F1-4	600 (ErbB1-eGFP)	~0	0
F1-10	50 (ErbB1-eGFP)	~0	0
HeLa	50	30	1
HeLa-ErbB1GFP	250 (endogén+ErbB1-eGFP)	30	1
JIMT-1	160	620	10
SKBR-3	190	1100	42

1. táblázat. A kísérletek során használt stabil sejtvonalak ErbB1-3 expressziós profilja.

3.2. Xenograft tumorok létrehozása

A súlyos kombinált immundeficienciában (SCID) szenvedő C.B.-17-scid/scid egérpopuláció a Fox Chase Cancer Center-ből (Philadelphia, PA) származott és patogénmentes környezetben volt elhelyezve. Csak olyan egereket használtunk a kísérletekhez, amelyek szérum IgG szintje 100 ng/ml alatt volt. Hét hetes nőstény SCID egereket 150 μ l Hank's puffer és 150 μ l Matrigel (Basement Membrane Matrigel, BD Biosciences, Bedford, MA) keverékében szuszpendált, $5 \cdot 10^6$ db JIMT-1 sejttel oltottunk be szubkután. A tumor térfogatát a szélesség²×hossz/2 képlettel becsültük tolómérő segítségével hetente egyszer. A trastuzumabot 5 μ g/g-os dózisban heti intraperitoneális oltás formájában adtuk az egereknek. A kontroll állatoknak 100 μ l fiziológiás só oldatok injektáltunk intraperitoneálisan. A 4-MU-t 1% gumiarábikumban oldottuk fel, és 3 mg/g-os dózisban adtuk az állatoknak mindennap kétszer szájon keresztül. Az állatokat CO₂

inhalációval áldoztuk fel. A kísérleteket a Debreceni Egyetem Munkahelyi Állatkísérleti Bizottsága (DEMÁB) engedélyezte (engedélyek számai: 7/2002/DEMÁB; 13/2010/DEMÁB).

3.3. RNS interferencia

Az ErbB1 expresszió RNS interferenciával történő gátlásához három különböző siRNS-t terveztünk 3' túlnyúló TT dinukleotidokkal, melyeket Thomas Tuschl laboratóriumában szintetizáltattunk (Combinatorial Chemistry Group, Max Planck Institute for Biophysical Chemistry, Göttingen, Németország). A negatív kontrollként gyakran alkalmazott GFP-2 szekvenciát a GFP ellen szintén mi terveztük és a fenti laborban készítettük el (2. táblázat). A MUC4 ellenes siRNS-t szintén mi terveztük, és a Qiagen (Hilden, Németország) szintetizálta. A CD44 ellenes siRNS-t a Dharmacon (Chicago, IL) tervezte és szintetizálta. A két sikeresen használt CD44 siRNS anti-sense szálának szekvenciái: AUGUCUUCAGGAUUCGUUCUU (CD44 siRNA-4), UAUUCAAAUCGAUCUG CGCUU (CD44 siRNA-5). Az ErbB3 expresszió gátlásához a Sigma „MISSION shRNA” plazmidot használtuk, amely ErbB3 ellenes shRNS-t termel (TRCN0000009835, NM_001982.x-4705s1c1). A sejtek transzfekcióját vagy Oligofectamine-nal (Invitrogen) vagy a Lonza Nucleofector készülékével végeztük.

siRNS	Sense/anti-sense szálak szekvenciája	A célszekvencia kezdő pozíciója
ErbB1-1	5'-CUCUGGAGGAAAAGAAAGUTT TTGAGACCUCCUUUUCUUUCA-5'	316
ErbB1-2	5'-CGUAAAGGAAAUACAGGGTT TTGCAUUUCCUUUAGUGUCCC-5'	1436
ErbB1-3	5'-CACAGUGGAGCGAAUUCCTT TTGUGUCACCUCGCUUAAGGA-5'	527
GFP-2	5'-GAACGGCAUCAAGGUGAACTT TTCUUGCCGUAGUCCACUUG-5'	476
MUC4	5'-CGCAAGCAUCGGACUUCTT TTGCGUUCGUAGCCUGAAGUG-5'	-

2. táblázat. Az általunk tervezett siRNS-ek szekvenciái. A célszekvenciát az ErbB1 esetében a GI12002211, a GFP esetében a GI1373318 szekvenciában elfoglalt hely alapján adtuk meg.

3.4. Sejtek fluoreszcens jelölése antitestekkel szuszpenzióban és fedőlemezen

A sejteket rendszerint kamrás fedőlemezekre („chambered coverglass”, Nalge Nunc International, Rochester, NY) növesztettük. Amennyiben sejtfelszíni antigént jelöltünk, PBS-ben történő mosást követően az antitestek telítő koncentrációjával jelöltük a sejteket 30 percig jégen. Az antitesteket 0.1% BSA-t tartalmazó PBS-ben hígítottuk. Ha az elsődleges antitestek nem voltak fluoreszcensen jelzettek, kétszeri mosást követően a sejteket másodlagos antitesttel jelöltük szintén jégen 30 percig, majd kétszer PBS-ben történő mosást követően 1%-os formaldehidben fixáltuk őket. Amennyiben intracelluláris antigént jelöltünk, a procedura elején 3.7%-os formaldehidben fixáltuk a sejteket, majd kétszeri Tris pufferben történő mosást követően a jelölés a fentieknek megfelelően történt, kivéve, hogy az antitesteket 0.1% BSA-t és 0.1% Triton X-100-at tartalmazó PBS-ben hígítottuk. Amikor a fluoreszcensen jelölt sejteket áramlási citométeren vizsgáltuk, a jelölés előtt feltripszinezünk őket. A jelölés menete alapvetően megegyezett a fentiekkel.

A xenograft tumormetszetek jelölése előtt a tumort az elaltatott állatokból eltávolítottuk, Shandon Cryomatrix-ba (Thermo Electron Corporation, Waltham, MA) ágyaztuk be és folyékony nitrogénben tároltuk. Húsz μm vastag metszeteket készítettünk (Shandon AS-620E Cryotome, Thermo Electron Corporation), amiket szilanizált tárgylemezre helyeztünk és 4%-os formaldehiddel fixáltunk 30 percig. Kétszeri, 1%-os BSA-t tartalmazó PBS-ben történő mosást követően a jelölést a fenti protokoll szerint végeztük azzal az eltéréssel, hogy az elsődleges antitestekkel egész éjszakán keresztül, a másodlagos antitestekkel 1-2 órán keresztül jelöltünk. A konfokális mikroszkópos vizsgálat előtt a metszetekre 15 μl Mowiol-t öntöttünk, majd fedőlemezzel borítottuk. A hialuronsav jelöléséhez először a Molecular Probes „Endogeneous biotin blocking kit”-jével kezeltük a metszeteket, majd 5 $\mu\text{g/ml}$ biotinált HABC jelenlétében inkubáltuk a mintákat egész éjszakán keresztül 4 °C-on. A fluoreszcens avidinnel történő jelölés előtt a mintákat ötször mostuk PBS-BSA pufferrel.

3.5. Sejtek fluoreszcens jelölése Sfp transzferáz segítségével

Az Sfp foszforanteteinil transzferáz-His₆ enzimet (röviden Sfp transzferáz) a pET29-Sfp törzsből (Jun Yin, Harvard Medical School) izoláltuk az előírásoknak megfelelően (188). Az Atto565-KoA-t Atto565-meleimidből (Atto-Tec GmbH) és a KoA dilítium sójából (Sigma-Aldrich) szintetizáltuk publikált protokolloknak megfelelően (188, 189), majd C18-as

oszlopon tisztítottuk HPLC-vel acetonitril/ammónium-acetát gradiens segítségével. Az ACP címkével rendelkező ErbB2-t kifejező sejtek membránját 5 μM Atto565-KoA-val jelöltük meg 0.2 μM Sfp-transzferáz segítségével 10 mM MgCl_2 -ot tartalmazó Tyrode pufferben 20 percig szobahőmérsékleten, amit kétszeri mosás és N&B analízis követett.

3.6. Immunfluoreszcens és hetero-FRET mérések áramlási citometriával

A dolgozatban tárgyalt kísérleti eredmények során az áramlási citométeres méréseket Becton Dickinson FacsCalibur, FacsStar Plus, FacsVantage-Diva és FacsArray, ill. Coulter Epics Elite műszereken hajtottuk végre. A gerjesztési lézervonalak hullámhosszát, ill. az emissziós oldalon az optikai filtereket és dikroikus tükröket a használt fluorofóroknak megfelelően választottuk meg. Legalább tíz-húsz ezer sejt adatait lista módú fájlban mentettük el, és a kiértékelést vagy Reflex (190) vagy FCS Express (DeNovo Software) program segítségével hajtottuk végre. Az elemzés első lépéseként az előre irányú és oldal irányú fényszórás pontábrán kikapuzztuk az intakt sejteket tartalmazó populációt, és a fluoreszcencia intenzitás átlagát vagy mediánját határoztuk meg erre az alpopulációra. A korrigált fluoreszcencia intenzitások kiszámítása során a jelölt minták átlagából (vagy mediánjából) levontuk a jelöletlen vagy csak másodlagos antitesttel jelölt minták átlagát (vagy mediánját).

Az áramlási citometriás FRET mérések alapelve minden esetben az volt, hogy minden sejtről három fluoreszcencia intenzitást detektáltunk, melyek a donor, FRET és akceptor csatornáknak feleltek meg. A donor csatornában a gerjesztési és emissziós hullámhosszak a donornak feleltek meg, az akceptor csatornában az akceptor fluorofórnak, míg a FRET csatornában a gerjesztés a donor abszorpciós spektrumának megfelelően történt, az emissziót pedig az akceptor emissziós spektrumának megfelelő hullámhossztartományban mértük. A FRET hatásfok meghatározásához használt mintát mind donor, mind akceptor fluorofórral megjelöltük. Mivel az egyes csatornák szinte sosem tisztán a nevüknek megfelelő intenzitást detektálják, ezért szükség van a spektrális átfedések korrekciójára, amely az áramlási citométerektől és fluorofór kombinációktól függően más és más lehet. Egy tipikus egyenletrendszer a donor (I_1), FRET (I_2) és akceptor (I_3) csatornákra a következőképpen néz ki (161):

$$I_1 = I_D(1-E) + I_A \cdot S_4 + I_D \cdot E \cdot \alpha \cdot \frac{S_4}{S_2} \quad (15)$$

$$I_2 = I_D(1-E) \cdot S_1 + I_A \cdot S_2 + I_D \cdot E \cdot \alpha \quad (16)$$

dc_380_12

$$I_3 = I_D(1-E) \cdot S_3 + I_A + I_D \cdot E \cdot \alpha \cdot \frac{1}{S_2} \cdot \frac{\varepsilon_{\lambda_A}^D \cdot \varepsilon_{\lambda_D}^A}{\varepsilon_{\lambda_D}^D \cdot \varepsilon_{\lambda_A}^A} \quad (17)$$

ahol I_D a kioltatlan donor intenzitást, I_A pedig a közvetlenül gerjesztett akceptor intenzitást jelöli. Az S_1 a donornak a FRET csatornába, az S_3 a donornak az akceptor csatornába, az S_2 az akceptornak a FRET csatornába, az S_4 az akceptornak a donor csatornába történő átvilágítását jelöli, amelyeket egyszeresen (vagy csak donorral vagy csak akceptorral) jelölt mintákon határoztunk meg. E -vel a FRET hatásfokot, ε -nal a moláris abszorpciós koefficiens jelöltem. A moláris abszorpciós koefficiens felső indexében szereplő D vagy A arra utal, hogy a donor vagy akceptor fluorofór paraméteréről van-e szó, az alsó indexben szereplő λ_D vagy λ_A pedig arra, hogy a donor vagy az akceptor abszorpciós hullámhosszán kell-e értelmezni. Az α paraméter megadja egy gerjesztett akceptor molekula FRET csatornában mért intenzitásának és egy gerjesztett donor molekula donor csatornában mért intenzitásának a hányadosát. Az S és α paraméterek meghatározását a (161) referencia adja meg részletesen. A fenti egyenletrendszer általános megoldását E -re a következő egyenlet adja meg:

$$E = \frac{S_2 (I_2 - I_1 S_1 - I_3 S_2 + I_1 S_2 S_3 + I_3 S_1 S_4 - I_2 S_3 S_4)}{\alpha \left(\frac{\varepsilon_{635}^D \cdot \varepsilon_{488}^A}{\varepsilon_{488}^D \cdot \varepsilon_{635}^A} - 1 \right) (I_2 S_4 - I_1 S_2) + S_2 (I_2 - I_1 S_1 - I_3 S_2 + I_1 S_2 S_3 + I_3 S_1 S_4 - I_2 S_3 S_4)} \quad (18)$$

A legtöbb esetben azonban valamelyik spektrális átvilágítási korrekciós faktor (S) nulla vagy elhanyagolható, ami egyszerűsíti az egyenletrendszer megoldását (186).

3.7. Immunfluoreszcens és FRET mérések mikroszkópban

Az immunfluoreszcens méréseket Zeiss LSM 410, LSM510 vagy Olympus FV1000 konfokális mikroszkópokon hajtottuk végre. A gerjesztési és detektálási hullámhosszak kiválasztása a fluorofóroknak megfelelően történt. Három különböző megközelítést használtunk a FRET mérések végrehajtására: (1) Donor fotoelhalványítási kinetika analízise (42, 171, 172): az egyik fehérjét egy könnyen fotoelhalványítható festékkel (rendszerint fluoreszcein) jelöltük meg, míg a másik fehérjét egy a donornak megfelelő akceptor fluorofórral. Megmértük a donor fotoelhalványítási kinetikáját a fenti kettősen jelölt mintában és csak donorral jelölt sejteken. A fotoelhalványítási görbékre egy- vagy kétexponenciális egyenletet illesztettünk, és kiszámoltuk az effektív időállandót (T) az egyes exponenciális tagok időkonstansainak amplitúdóval súlyozott átlagából:

$$I = a_0 + \sum_i a_i e^{-\frac{t}{\tau_i}} \Rightarrow T = \frac{\sum_i a_i \tau_i}{\sum_i a_i} \quad (19)$$

A csak donorral, ill. a donorral és akceptorral kettősen jelölt minták effektív időállandóiból a (7) egyenlet alapján meghatároztuk a FRET hatásfokot. (2) Akceptor fotoelhalványítás mérése (42, 169): a mintát egy fotostabil donorral (pl. Cy3) és egy fotolabilis akceptorral (pl. Cy5) jelöltük meg. A donor csatornában felvettünk egy képet a mintáról az akceptor fotoelhalványítása előtt és után, és az (5) egyenlet alapján kiszámítottuk a FRET hatásfokot. Amennyiben a spektrális átfedések, az akceptor inkomplett fotoelhalványítása vagy a donor részleges fotoelhalványítása miatt korrekciók váltak szükségessé, az ImageJ programban elérhető plugin-t használtuk a kiértékeléshez (170). (3) Intenzitás arányon alapuló („ratiometric”) módszer (161): az eljárás elvi alapja azonos az áramlási citometriás FRET méréseknél fentebb leírtakkal. A kalibrációs faktorok (S , α) meghatározásában az áramlási citometriás módszerhez képest fennálló különbségeket egy korábbi publikációban írtuk le (191).

3.8. N&B mérések konfokális mikroszkópon

A N&B mérések kivitelezéséhez egy Olympus IX81 mikroszkópot használtunk FluoView FV1000 konfokális konfigurációval. A méréseket a Digman és mtsai által leírt módon hajtottuk végre (174). Az élő, fedőlemezre növesztett sejteket 10 mM glükózt és 0.1% BSA-t tartalmazó Tyrode pufferben vizsgáltuk szobahőmérsékleten, és a kísérleteket max. 30 percig folytattuk, hogy a klaszterek szerkezetében szobahőn bekövetkező átalakulásokat megelőzzük. A képeket a mikroszkóp pszeudo-fotonszámláló módjában vettük fel egy UPlanSApo 60× (NA=1.35) objektívvel. A pixelidő 10 μ s, a képidő 3.26 s volt, amikor transzfektált sejteket vizsgáltunk, és 2 μ s és 1.1 s, amikor az oldható monomer fluoreszcens proteineket analizáltuk. Az eGFP-t és az mYFP-t rendre 488 és 514 nm-en gerjesztettük. A lézerintenzitást ~ 70 μ W-ra állítottuk 488 nm-en, és ~ 90 μ W-ra 514 nm-es gerjesztés esetében. A fenti teljesítményeket az objektív fókuszpontjában mértük. Az Atto565-öt 543 nm-en gerjesztettük ~ 130 μ W-os teljesítménnyel. A felvett képek 512×512 pixel nagyságúak voltak, egy pixel x és y irányú kiterjedése 41 nm volt. Az analízishez a képek középső részét használtuk, hogy a pásztázás sebességének nemlineáris voltából eredő szél-artefaktumokat elkerüljük. A mérések során a vizsgált sejtek fedőlemezhez közeli rétegéről 50-100 képet

rögzítettünk, és a képsorozatokot egy általam fejlesztett, DiplImage (Delft University of Technology, Delft, Hollandia) függvényeket használó Matlab (Mathworks, Natick, MA) program segítségével értékeltük ki. A képeket először az oldalirányú elmozdulásra korrigáltuk a DiplImage „correctshift” parancsával, majd minden pixelnek meghatároztuk az átlagát és az időbeni varianciáját. Amennyiben az átlag több, mint 10%-ot csökkent a fotoelhalványítás vagy a mikroszkópasztal süllyedésének hatására, és ha a pixel variancia nem konvergált nullához a képszám növelésével, akkor az adott mérést nem vettük figyelembe. A pixelenkénti látszólagos fényesség értékeit a (12) képlet alapján határoztuk meg.

3.9. Antitestek internalizációjának mérése

Az antitestek internalizációját több esetben áramlási citometria segítségével határoztuk meg a dolgozatban tárgyalt cikkekben (42, 68, 113, 185). Legtöbbször az alábbi eljárást alkalmaztuk. A szuszpenzióban levő sejteket fluoreszcens antitesttel vagy EGF-fel jelöltük jégen, majd két mintát vettünk (A – kontroll, teljes fluoreszcencia intenzitás; B – savval mosott, internalizált fluoreszcencia). Ezt követően a mintát 37°C-on inkubáltuk, és a kívánt időpontokban ismét mintát vettünk belőle, amelyeket azonnal jégre helyeztünk. A kísérlet végén a B jelű és a különböző időpontokban vett mintákhoz 15-szörös térfogatnyi savas puffert adtunk („acid strip”: 0.5 M NaCl, 0.1 M Gly, pH 2.8) és 3 percig inkubáltuk jégen, míg az A jelű mintához ugyanakkora térfogat PBS-t adtunk. Mosás és PBS-ben történő reszuszpendálást követően a fluoreszcencia intenzitást áramlási citométeren határoztuk meg, és az internalizált hányadot (F_{int}) a következő egyenlet alapján számoltuk ki:

$$F_{int} = \frac{I_{t,B} - I_{0,A} F_{ext,pers}}{I_{0,A} (1 - F_{ext,pers})}, \text{ ahol } F_{ext,pers} = \frac{I_{0,B}}{I_{0,A}} \quad (20)$$

ahol $F_{ext,pers}$ a membránban levő fluoreszcencia intenzitás sav-rezisztens hányadát jelöli (tehát azt, amit a savas kezelés nem távolít el). Az $I_{0,A}$ és az $I_{0,B}$ a 0 percnél vett, PBS-sel, ill. savval kezelt minták fluoreszcencia intenzitásai, az $I_{t,B}$ pedig az egy adott időpontnál vett, savval kezelt minta fluoreszcencia intenzitása.

Más esetben nem egyszerűen az antitest által indukált internalizációt, hanem a sejtfelszíni fehérje leszabályozódását („down-regulation”) vizsgáltuk. A trastuzumab által indukált ErbB2 leszabályozódás esetében a sejteket jelöletlen trastuzumabbal kezeltük 37°C-

on, majd a sejtfelszínen maradt ErbB2-t egy a trastuzumabbal nem kompetáló ErbB2 ellenes antitesttel (pl. 7C2) jelöltük, és a fluoreszcencia intenzitást áramlási citométerrel mértük.

Az internalizáció kvantitatív meghatározására mikroszkópos eljárás is használható (68). A vizsgálandó membránfehérjét fluoreszcensen megjelöltük, majd a kezelést követően konfokális mikroszkópos képet készítettünk a sejtekről. A képeken a sejtmembrán azonosítását (l. 3.13 fejezet) követően a membránt jelentő vonaltól 5-10 pixelre levő területet hozzárendeltük a membrán maszkhoz. A membrántól való távolságot euklideszi távolság transzformációval mértük. Az így megvastagított membrán maszkon belüli területet az intracelluláris területként értelmeztük. A membrán és az intracelluláris maszkon belüli fluoreszcencia intenzitást háttér korrigáltuk és összegeztük. Az internalizáció jellemzésére az intracelluláris és membrán maszkokban levő összegzett, háttér korrigált fluoreszcencia intenzitások hányadosát használtuk.

3.10. Sejtek életképességének vizsgálata

A sejtek életképességét, ill. az elisidepsin életképességre gyakorolt rövidtávú hatását propidium jodid felvételen alapuló mikrofluorimetriás esszével mértük. A sejteket fekete falú, átlátszó aljú 96-lyukú edényben tenyésztettük, majd 25 mM Hepessel, 50 µg/ml propidium jodiddal és a vizsgált gyógyszer meghatározott koncentrációjával kiegészített médiumot adtunk a sejtekhez (pH 7.4), majd a propidium jodid felvételét 531 nm-es gerjesztés és 632 nm-es emissziós hullámhossz mellett követtük mikrofluoriméterrel. A méréseket a gyógyszer minden koncentrációjával négyszer végeztük el.

Az elisidepsin hosszú távú hatásait WST-1 reagenssel (Roche Applied Science, Mannheim, Németország) követtük. ~7000 sejtet tettünk 96-lyukú edény egy lyukába a kísérletet megelőző napon, majd az elisidepsin különböző koncentrációjával kezeltük a sejteket triplikátumban 2 óra hosszáig. Mosást követően 3 napra visszaraktuk a sejteket a sejttenyésztő inkubátorba, és a relatív sejtszámot a WST-1 reagens mintához adásával, az abszorbancia 450 és 620 nm történő leolvasásával határoztuk meg a gyártó előírásainak megfelelően. Az IC₅₀ értéket a következő képlet mérési pontokra történő illesztésével számítottuk ki:

$$A_{min} + \frac{A_{max} - A_{min}}{1 + 10^{n(\log(c) - \log(IC_{50}))}} \quad (21)$$

ahol A_{min} és A_{max} a legalacsonyabb és legmagasabb abszorbancia értékek, n a Hill koefficiens, c az elisidepsin koncentrációja. A log a 10-es alapú logaritmust jelöli.

3.11. Sejtciklus eloszlás meghatározása áramlási citométer segítségével

A sejteket feltripszineztek, majd 70%-os etanolban fixáltuk 4°C-on egy éjszakán át. Rehidratációt követően a sejteket 100 µg/ml propidium jodiddal (Sigma-Aldrich) és esetleg antitesttel jelöltük 100 µg/ml RNáz jelenlétében 60 percig 37 °C-on, majd Coulter Epics Elite áramlási citométeren analizáltuk. Az antitest fluoreszcens jelölését úgy választottuk meg, hogy az ne mutasson spektrális átfedést a propidium jodid spektrumával, így általában Cy5 vagy AlexaFluor647 fluorofórt használtunk. Az eredményeket egy alternatív jelölési protokoll segítségével megerősítettük, amely szerint a sejteket hipotónikus fluorokróm oldatban jelöltük (50 µg/ml propidium jodid 0.1% nátrium-citrátban, 0.1% Triton X-100) 4°C-on egy éjszakán át (192, 193). A sejtciklus analízist a Cylchred program (Department of Hematology, College of Medicine, University of Wales) segítségével végeztük.

3.12. Fluoreszcencia anizotrópia és generalizált polarizáció (GP) mérése fluoriméteren

A feltripszinezett sejteket 10⁷/ml-es koncentrációban vettük fel Hank's pufferben, és 2 µM TMA-DPH-val vagy 2.5 µM Laurdannel jelöltük meg szobahőmérsékleten 20 percig. A TMA-DPH-val történő jelölést követően a sejteket mosás nélkül kihígítottuk 10⁶/ml-es koncentrációra a fluoriméteres mérésekhez, míg a Laurdannel jelölt sejteket egyszer mostuk és Hank's pufferben szuszpendáltuk fel szintén 10⁶/ml-es koncentrációban. A méréseket egy Fluorolog-3 spektrofluoriméteren (Horiba Jobin Yvon, Edison, NJ) végeztük. A küvettaház hőmérsékletét egy cirkuláltatott vízfürdő segítségével 37°C-ra állítottuk. A TMA-DPH-t 352 nm-en gerjesztettük és 430 nm-en detektáltuk a fluoreszcenciáját. A fluoreszcencia anizotrópiát (r) L-formátumban határoztuk meg a következő képlet alapján (194, 195):

$$r = \frac{I_{vv} - GI_{vh}}{I_{vv} + 2GI_{vh}} \quad (22)$$

ahol I_{vv} és I_{vh} a vertikálisan polarizált fénnel gerjesztett fluorofór emissziójának vertikálisan és horizontálisan polarizált komponense, G pedig egy kalibrációs faktor, amely a műszer vertikálisan és horizontálisan polarizált fényre vonatkoztatott eltérő érzékenységét jellemzi:

$$G = \frac{I_{hv}}{I_{hh}} \quad (23)$$

ahol I_{hv} és I_{hh} a horizontálisan polarizált fénnel gerjesztett fluorofór emissziójának vertikális és horizontális komponense. A Laurdant 350 nm-en gerjesztettük, és emisszióját a spektrum rövidhullámhosszú tartományában 435 nm-en ($I_{kék}$) és a hosszúhullámhosszú tartományban 500 nm-en ($I_{vörös}$) mértük. A Laurdan generalizált polarizációját (GP) az alábbi képlet alapján számítottuk ki (125, 196, 197):

$$GP = \frac{I_{kék} - I_{vörös}}{I_{kék} + I_{vörös}} \quad (24)$$

3.13. Sejtmembrán azonosítása „watershed” algoritmus segítségével konfokális mikroszkópos képeken

Több közleményben (43, 68, 98, 114, 198) alkalmaztuk a manuálisan indított vízfeltöltéses algoritmust („manually seeded watershed”) a képek szegmentálása során, pontosabban a sejtmembrán azonosítására (199). A sejtekről egy konfokális szeletet vettünk fel, majd a képen a felhasználó minden sejt belsejébe egy pontot („seed”) helyez el. Az algoritmus a képet úgy kezeli, mintha egy háromdimenziós domborzati térkép lenne, ahol a magasságot az intenzitás értékek határozzák meg. Az algoritmus ezt a domborzati térképet képzetesen vízzel tölti fel, és a medencék (sejtek) közötti határok ott lesznek, ahol két, egymással határos medence között átcsap a víz. A felhasználó által elhelyezett pontok azért szükségesek, mert a teljesen automatikus vízfeltöltéses algoritmusról köztudott, hogy túlszegmentálja a képeket, tehát túl sok határvonalat azonosít. A fenti eljárást egy általam írt, DipImage függvényeket felhasználó Matlab programmal valósítottuk meg.

3.14. CD44 intramembrán proteolízisének kvantitatív analízise

A CD44 ellen kétféle antitest áll rendelkezésünkre. Az egyik, a Hermes-3, a CD44 extracelluláris doménjéhez, míg az anti-CD44cyto a CD44 intracelluláris részéhez kötődik (187). Konfokális mikroszkópos képeken a 2.9 pontban leírt módon azonosítottuk a sejtek intracelluláris terét és membránját, és összegeztük a háttérkorrigált anti-CD44cyto és Hermes-3 intenzitásokat mindkét területen. Az anti-CD44cyto/Hermes-3 hányadost használtuk a CD44 intramembrán proteolízisének jellemzésére. Ennek a paraméternek az értéke egy, amennyiben nem következett be intramembrán proteolízis (hiszen a teljes hosszúságú CD44-hez mind a Hermes-3, mind az anti-CD44cyto antitest kötődik), míg a

proteolízis esetén egy fölé nő az értéke (hiszen az intracelluláris fragmentumhoz a Hermes-3 nem fog kötődni, és így csökken a tört nevezője).

3.15. Proximitás ligációs vizsgálat (PLA)

A PLA kísérletekhez az oligonukleotiddal jelölt antitesteket egy publikált protokoll alapján készítettük (151). Röviden, az antitesteket 4 mg/ml-es koncentrációban 30-szoros moláris feleslegben levő szukcinimidil 4-hidrazinonikotinnal (SANH, ThermoScientific) konjugáltuk PBS-ben szobahőmérsékleten 2 órán keresztül. A nem kötődött SANH-t konjugáló pufferrel (100 mM MES, 150 mM NaCl, pH 4,8) ekvibrált Bio-Gel P6-os oszlopon (BioRad) történő gélszűréssel távolítottuk el. Az SANH-val konjugált antitestekhez 3,5-szeres moláris feleslegben „priming” és „non-priming” oligonukleotidokat adtunk, és három órán keresztül inkubáltuk őket szobahőmérsékleten. Az oligonukleotidok szekvenciája a következő volt: „priming”: 5'-aldehyde-AAA-AAA-AAA-ATA-TGA-CAG-AAC-TAG-ACA-CTC-TT-3'; „non-priming”: 5'-aldehyde-AAA-AAA-AAA-AGA-CGC-TAA-TAG-TTA-AGA-CGC-TTU-UU-3', U=2'-O-Metil-RNS). Az oligonukleotidokat a TriLink Biotechnologies-től szereztük be (San Diego, CA). A nem kötődött oligonukleotidokat Superdex-75 oszlopon történő HPLC-vel vagy Sephadex „superfine” G-100-as oszloppal, gélszűréssel távolítottuk el. A kiválasztott frakciók koncentrációját gél elektroforézissel és az oligonukleotiddal konjugált antitestekkel jelölt sejtek immunfluoreszcens festésével becsültük meg.

A sejtek PLA kísérletekhez történő jelöléséhez kb. 1 millió sejtet mostunk hideg PBS-ben, majd fixáltuk őket 1% formaldehidben 10 percig jégen. Kétszeres PBS-es mosást követően 30 percig blokkoló pufferben (1% BSA, 5 µg/ml szonikált lazac sperma DNS PBS-ben oldva) inkubáltuk őket. Mosást követően a sejteket az oligonukleotiddal jelölt ún. proximitás próbákkal és fluoreszcens antitestekkel inkubáltuk 1-2 órán át 37°C-on antitest hígító pufferben (250 µg/ml BSA, 2.5 µg/ml lazac sperma DNS PBS-ben). Ezt követően a sejteket kétszer mostuk TBST-ben (20 mM Tris, 150 mM NaCl, pH 7,4, 0.05% Tween-20), majd összekötő oligonukleotiddal inkubáltuk őket a „DuoLink Detection Kit” (Olink Bioscience, Uppsala, Svédország) hibridizációs törzsoldatában 15 percig 37°C-on. TBST-ben történő mosás után a mintákat a DuoLink ligációs oldattal inkubáltuk 37°C-on 15 percig, majd mostuk őket TBST-ben. Ezután DuoLink amplifikációs oldatban történő inkubáció következett 60 percig 37°C-on. Végül TBST-ben történő mosást követően az RCA reakció termékét 25 nM Cy5-tel jelölt detektáló oligonukleotiddal vizualizáltuk (5'-Cy5- CAG-TGA-

ATG-CGA-GTC-CGT-CT-3'), amit detekciós pufferben (0.1 µg/ml lazac sperma DNS, 250 ng/ml BSA, 0.05% Tween-20, 2× SSC) oldottunk fel. Az áramlási citometriás vizsgálat előtt a sejteket egyszer mostuk TBST-ben és fixáltuk őket 1% formaldehidben.

Az áramlási citometriás méréseket FacsCalibur-on végeztük. A Cy5-tel jelölt oligonukleotidok jelét a FacsCalibur FL4 csatornájában detektáltuk 635 nm-en történő gerjesztést követően. Az AlexaFluor488-cal jelölt antitestek jelét 488 nm-es gerjesztést követően a műszer FL1 csatornájában mértük. A kétdimenziós hisztogramokat (kontúr ábra) Matlabbal készítettük. A PLA és a fluoreszcens antitest jelének korreláltatása ún. trend vonalak számításával történt Matlabban. Röviden, az x változónak (fluorofórral konjugált antitest jele) megfelelő tengelyt 30 részre osztottuk, majd minden egyes osztáshoz kiszámítottuk a neki megfelelő átlagos intenzitást a Cy5 csatornában (Cy5-tel jelölt proximitás próbák jele).

3.16. FRAP mérések

Az eGFP fluoreszcencia intenzitásának monitorozásához egy Zeiss LSM510 mikroszkópon az Ar ion lézer 488 nm-es vonalának teljesítményét 5%-ra állítottuk, és a fluoreszcenciát egy 505 nm-es felüáteresztő szűrőn keresztül mértük. Az eGFP fluoreszcenciájának kiégetéséhez a lézer teljesítményét 100%-ra emeltük, és a sejtmembrán egy négyszögletes területén (2×2 µm) fotoelhalványítást végeztünk. A fluoreszcencia visszatérést az 5%-ra gyengített lézerteljesítménnyel követtük nyomon úgy, hogy ~150 sec-ig minden másodpercben képet vettünk fel. A képanalízist DiImage segítségével végeztük Matlab környezetben. A képregisztráció a DiImage „correctshift” parancsával történt, amit a kiégetett terület átlagos fluoreszcencia intenzitásának meghatározása követett az idő függvényében. Végül a fluoreszcencia visszatérést az alábbi egyenletre illesztettük:

$$a + (1 - a - b) \left(1 - e^{-\frac{t}{\tau}} \right) \quad (25)$$

ahol τ az empirikus visszatérési időállandó. Az immobilis fluoreszcens molekulák hányadát a következő képlettel számítottuk ki:

$$\frac{b}{1 - a} \quad (26)$$

4. Eredmények és megbeszélés

4.1. Áramlási citometriás kalibrációs módszer kidolgozása kvantitatív FRET mérésekhez fluoreszcens fehérjét expresszáló sejteken

Az áramlási citométeres FRET mérések elvi és gyakorlati alapjai az 1.6 és 3.6 fejezetekben kerültek bemutatásra. Ezen mérések kvantitatív tételének, tehát a (15)-(17) egyenletek megoldásának feltétele, az α kalibrációs faktor ismerete. Az α paraméter megadja egy gerjesztett akceptor molekula FRET csatornában mért intenzitásának és egy gerjesztett donor molekula donor csatornában mért intenzitásának a hányadosát, és a fluorofórok és a detektorok paramétereivel a következő egyenlettel lehet megadni:

$$\alpha = \frac{\Phi_A \eta_A(I_2)}{\Phi_D \eta_D(I_1)} \quad (27)$$

ahol Φ_D és Φ_A a donor és az akceptor kvantumhatásfokát, míg $\eta_D(I_1)$ és $\eta_A(I_2)$ a donor és akceptor fluoreszcencia detektálási hatásfokát adja meg az I_1 és I_2 csatornában. A fenti egyenlet praktikus szempontból nem hasznos, hiszen mindegyik benne szereplő paramétert nehéz meghatározni. Fluoreszcens monoklonális antitestekkel jelölt sejteken végzett FRET mérések esetében az α meghatározására a következő egyenlet használható:

$$\frac{I_{2,A}}{I_{1,D}} = \frac{\varepsilon_A L_A \Phi_A \eta_A(I_2)}{\varepsilon_D L_D \Phi_D \eta_D(I_1)} \Rightarrow \alpha = \frac{I_{2,A}}{I_{1,D}} \frac{\varepsilon_D L_D}{\varepsilon_A L_A} \quad (28)$$

Tehát két mintán megjelöljük ugyanazt a fehérjét egyrészt donor, másrészt akceptor jelölt antitesttel, és az akceptorral jelölt minta I_2 intenzitását ($I_{2,A}$) elosztjuk a donorral jelölt minta I_1 intenzitásával ($I_{1,D}$), és korrigáljuk a donor és akceptor moláris abszorpciókoefficienseinek hányadosával ($\varepsilon_D/\varepsilon_A$, ahol mindkét ε -t a donor gerjesztési hullámhosszán kell értelmezni) és a donorral, ill. akceptorral jelölt antitestek jelölési arányának hányadosával (L_D/L_A). A fenti megközelítés alapja, hogy legyen két olyan minta, amiben ugyanannyi fehérje van az egyik esetben donorral, a másik esetben akceptorral megjelölve. Ezt az egyszerű módszert nem lehet fluoreszcens fehérjékkel történő FRET mérésekben alkalmazni, mert lehetetlen két olyan transzfektált (stabil vagy tranziens) sejtet előállítani, amelyek közül az egyik pontosan ugyanannyi donor fluoreszcens fehérjét fejez ki, mint amennyi akceptort a másik. Amennyiben egy olyan fúziós fehérjét állítunk elő, amelyben a donor és az akceptor egy konstrukcióban található, akkor a problémát az jelenti, hogy a kettő között FRET léphet fel, ami a fenti módszer alkalmazhatóságát kizárja.

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Ezért a következő módszert dolgoztuk ki az α paraméter meghatározására. A módszer kidolgozása során használt áramlási citométerre (Becton Dickinson Facstar Plus) a FRET egyenletrendszer a következő alakban írható fel (a (15)-(17). egyenletek alkalmazásával):

$$I_1(\lambda_{D,ex}, \lambda_{D,em}) = I_D(1-E) \quad (29)$$

$$I_2(\lambda_{D,ex}, \lambda_{A,em}) = I_D(1-E)S_1 + I_A S_2 + I_D E \alpha \quad (30)$$

$$I_3(\lambda_{A,ex}, \lambda_{A,em}) = I_D(1-E)S_3 + I_A + I_D E \alpha \frac{S_3}{S_1} \quad (31)$$

A fenti egyenletrendszerből a FRET hatékonyság kifejezhető:

$$\frac{E}{1-E} = \frac{1}{\alpha} \left(\frac{I_2 - S_2 I_3}{\left(1 - \frac{S_2 S_3}{S_1}\right) I_1} - S_1 \right) = \frac{1}{\alpha} R_I \quad (32)$$

$$E = \frac{R_I}{\alpha + R_I} \quad (33)$$

A FRET hatásfok a szenzitizált emissziót felhasználó másik egyenletből is kiszámítható (200):

$$\frac{F_{AD}}{F_A} = 1 + \frac{\varepsilon_D C_D}{\varepsilon_A C_A} E = R_F \quad (34)$$

ahol F_{AD} és F_A rendre az akceptor intenzitása a donor jelenlétében és nélküle, C_D és C_A rendre a donor és az akceptor moláris koncentrációi. Az F_{AD} és F_A a (29)-(31) egyenletrendszer jelöléseit használva:

$$F_{AD} = I_2 - I_1 S_1 \quad (35)$$

$$F_A = I_A S_2 \approx I_3 S_2 \quad (36)$$

A fenti közelítés csak akkor igaz, amennyiben S_3 elhanyagolható, amely igaz volt az általunk alkalmazott kísérleti rendszerben. Ezen második megközelítés alapján a FRET hatásfok a következő egyenlettel fejezhető ki:

$$E = \frac{R_F - 1}{\frac{\varepsilon_D C_D}{\varepsilon_A C_A}} \quad (37)$$

A FRET hatásfok (33) és (37) egyenlet alapján történő meghatározása egy-egy ismeretlen változót tartalmaz, az első az α paramétert, a második az abszorpciós hányadost ($\varepsilon_D C_D / (\varepsilon_A C_A)$). Ezek meghatározására egy mintasorozatot használtunk, amelyekben donor és akceptor fluoreszcens fehérjék (CFP és YFP) különböző hosszúságú linkerrel voltak

összekötve, így várható volt, hogy eltérő FRET értékeket generálnak. Mivel a donor és az akceptor mindig 1:1 arányban fejeződött ki, ezért az abszorpciós hányados $\varepsilon_D/\varepsilon_A$ -ra egyszerűsödik. Amennyiben az első mintát referenciának tekintjük ($i=1$), és a többi minta (37) egyenlet alapján számolt FRET értékét elosztjuk a referencia minta FRET értékével, akkor elimináljuk az abszorpciós hányadost. Számoljuk ki ezeket a hányadosokat mindkét módszerrel kapott FRET értékekre, és a különbségüket összegezzük az összes mintára:

$$\sum_{j=2,3..n} \left(\frac{R_{F,1}-1}{R_{F,j}-1} - \frac{R_{I,1}}{\alpha + R_{I,1}} \frac{\alpha + R_{I,j}}{R_{I,j}} \right)^2 \quad (38)$$

Mivel a két módszerrel számolt FRET értékeknek meg kell egyeznie mérési hibahatáron belül, a fenti kifejezésnek minimuma van, amely az α értékének variálásával található meg. A kifejezés minimumát adó α értéket fogadjuk el az α paraméter legjobb közelítésének. Az abszorpciós hányados is kiszámolható, ha a (33)-as egyenlet alapján a fent kapott α -val számolt E értéket a (34)-es egyenletbe helyettesítjük, és az F_{AD}/F_A -t az E függvényében ábrázoljuk, a grafikon meredeksége az abszorpciós hányados lesz.

A (33)-as egyenletet a (34)-esbe helyettesítve az R_F és R_I közötti összefüggés levezethető:

$$R_F = 1 + \frac{\varepsilon_D C_D}{\varepsilon_A C_A} \frac{R_I}{\alpha + R_I} \quad (39)$$

A fenti egyenlet linearizált formája szintén használható az α paraméter meghatározására:

$$\frac{1}{R_F - 1} = \frac{\varepsilon_A C_A}{\varepsilon_D C_D} \left(1 + \frac{\alpha}{R_I} \right) \quad (40)$$

Ha az $1/(R_F-1)$ -et ábrázoljuk az $1/R_I$ függvényében, akkor az egyenes tengelymetszete $(\varepsilon_A C_A)/(\varepsilon_D C_D)$, meredeksége $\alpha \cdot \text{tengelymetszet}$ lesz.

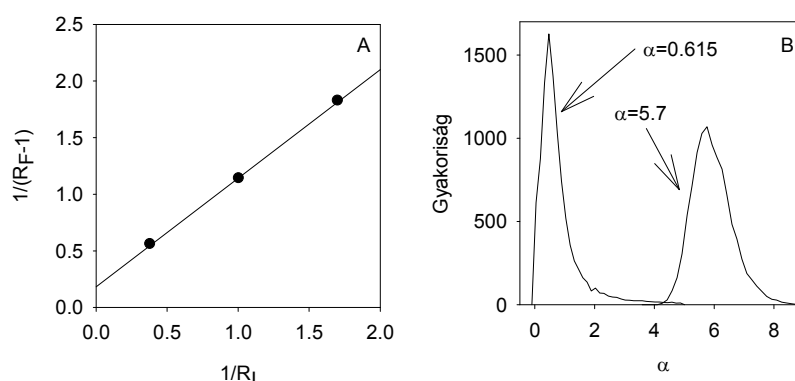
A fenti elmélet teszteléséhez három különböző CFP-YFP konstrukciót állítottunk elő, amelyben a donort és az akceptort három glicin, harminc prolin vagy egy tripszin szenzitív, huszonöt aminosavból álló linker választotta el egymástól. A fehérjéket expresszáló élesztő sejtek áramlási citometriás analízise során meghatároztuk az R_I és R_F intenzitás hányadosokat, majd a (38)-as egyenlet minimalizálásával az α paramétert és az abszorpciós hányadost (3. táblázat). A minimalizálást egy általam írt LabView (National Instruments, Austin, TX) programmal hajtottuk végre. Ezt követően a (40)-es egyenlettel végrehajtott lineáris regresszió segítségével kiértékeljük ugyanazon mérési eredménysort is, és az α

értékére 5,26, az abszorpciós hányadosra 5,48 adódott (4A. ábra). A két módszerrel meghatározott érték jó egyezést mutatott egymással. Ezt követően a mért intenzitások és a származtatott S faktorok értékeit felhasználva Monte Carlo szimulációt hajtottunk végre az α paraméter konfidencia intervallumának meghatározására. A 4B. ábra tanúsága szerint a kisebb α értékek esetében a meghatározás relatív hibája nagyobb, és a hisztogram aszimmetrikusan elnyúlik a magasabb α értékek felé.

linker	R_I	R_F	FRET (%)		$\alpha, \varepsilon_D/\varepsilon_A$
			R_I -ből	R_F -ből	
30 Pro	0,62	1,54	9,8	10,1	$\alpha=5,7$ $\varepsilon_D/\varepsilon_A=5,3$
25 aminosav	1,05	1,87	15,6	16,3	
3 Gly	2,82	2,79	33,1	33,5	

3. táblázat A FRET értékek kalibrálása három különböző CFP-YFP konstrukció alapján.

A háromféle CFP-YFP fúziós proteinre a kétféle módszerrel meghatároztuk az intenzitás hányadosokat (R_I és R_F), majd a (38)-as egyenlet minimalizálásával kiszámítottuk az α paramétert, majd az abszorpciós hányadost.



4. ábra. Fluoreszcens fehérjékkel végrehajtott FRET mérések kalibrálása és az eljárás megbízhatósága.

(A) Az abszorpciós hányados és az α paraméter meghatározása lineáris regressziós módszerrel. Az egyenes meredeksége 0,96, tengelymetszete 0,18 lett, amelyből az α értékére 5,26, az abszorpciós hányadosra 5,48 adódott.

(B) Az α paraméter konfidencia intervallumának meghatározása. A három CFP-YFP konstrukciót kifejező élesztősejt-sorozatot kétféle műszerbeállítás mellett mértük le, melyeknél az α paraméter értéke 0,615, ill. 5,7 lett. Ezen két esetben az I_1 - I_3 , S_1 és S_2 paraméterek átlag és szórás értékeit felhasználva 20000 normális eloszlású I_1 - I_3 , S_1 és S_2 változót állítottunk elő, amelyekből az α értékét meghatároztuk. A két α hisztogram alapján a 95%-os konfidencia intervallum 4,72 és 7,56 ($\alpha=5,7$), ill. 0,06 és 3,067 ($\alpha=0,615$) között volt.

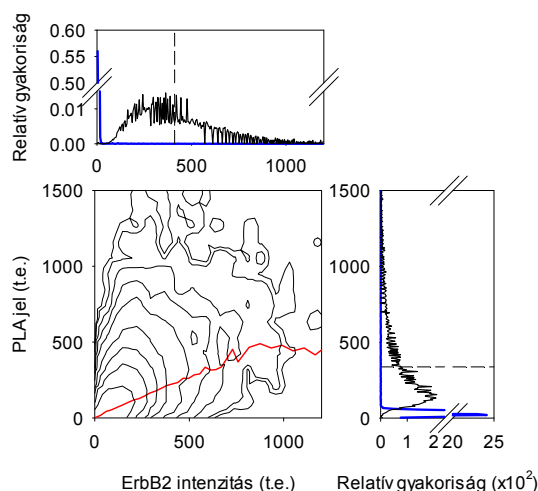
A fentiek alapján megállapíthatjuk, hogy a leírt módszer megbízhatóan alkalmazható a fluoreszcens proteinekkel történő FRET mérések kalibrálására. Elvileg a módszer alkalmazhatóságához minimum kétféle konstrukció szükséges, de a megbízhatóság jelentősen növekszik, ha több pontra történik a (40)-es egyenlet illesztése vagy több mérési pont alapján minimalizáljuk a (38) egyenletet. Az általunk kidolgozott módszer egyszerűbb változatát írták le Chen és mtsai, amiben csak két különböző donor-akceptor fúziós konstrukciót alkalmaztak, és ennek megfelelően a kalibráció pontossága kisebb volt (201). Az általunk kidolgozott módszer alapján kvantitatív FRET mérések végezhetők áramlási citométerrel fluoreszcens fehérjék felhasználásával. A kvantitatív FRET mérések lehetővé teszik a FRET hatások kiszámítását, amely pontos fizikai jelentéssel bír és műszertől független. Ezért véleményünk szerint előnyben részesítendő az irodalomban számtalan helyen leírt nem kalibrált FRET mérésekkel vagy fizikai jelentéssel nem bíró FRET paraméterekkel szemben (202, 203). *(A tárgyalt cikk a referencialistában: (186).)*

4.2. A proximitás ligációs vizsgálat (PLA) a protein klaszterizáció szemikvantitatív mérőmódszere

Bár a FRET hatások a proteinek asszociációjával kvantitatív viszonyban levő paraméter, kiszámítása a legtöbb esetben különböző bonyolultsági fokú egyenletek vagy egyenletrendszerek megoldását igényli, amely számos biológiai alkalmazás esetében hátrányos. Ezért is övezte nagy várakozás a PLA technikát, amelyről kidolgozói azt állították, hogy a PLA szignál arányos a protein asszociáció mértékével, és így hallgatólagosan azt feltételezték róla, hogy kvantitatív mérőmódszerről van szó (151, 152). A mi érdeklődésünk azért is fordult a PLA technika felé, mert a FRET mérések magas autofluoreszcenciával rendelkező mintákon (pl. szövet metszetek) nem vagy nehézkesen alkalmazhatók, a PLA módszer a magas jel/zaj arány miatt viszont kiválóan használható ilyen körülmények között is. Mivel az új technika kvantitatív képességeit még nem tesztelték, ezért célul tűztük ki, hogy ezt szisztematikus vizsgálatokkal elemezzük.

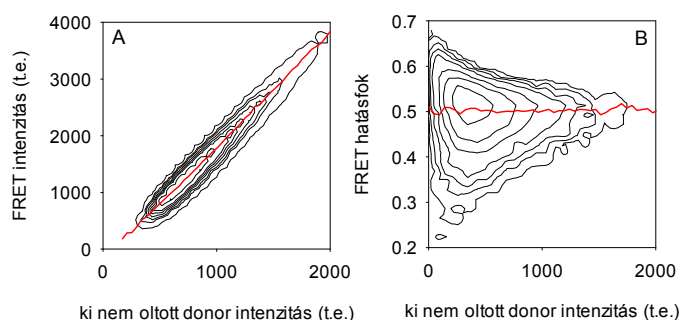
Először az ErbB2 két epitópját jelöltük meg „priming” és „non-priming” oligonukleotiddal konjugált antitesttel (pertuzumab-„priming”, trastuzumab-„non-priming”) SKBR-3 sejteken a PLA jel generálásához, amit Cy5-jelölt detektáló oligonukleotiddal vizualizáltunk. Célunk azt volt, hogy megvizsgáljuk, hogyan változik a PLA jel intenzitása az expressziós szint függvényében. Ehhez szükségünk volt egy, az ErbB2-t a fenti antitestekkel

nem kompetáló módon megjelölő harmadik antitestre, amely immunfluoreszcensen karakterizálta a fehérje kifejeződési szintjét. Ehhez AlexaFluor488-konjugált 7C2 antitestet használtunk. Áramlási citométeren megmértük a PLA jelet és az expressziós szintet jellemző antitest intenzitását, és kétdimenziós hisztogramon vizsgáltuk a kettő közötti összefüggést. Megállapítottuk, hogy magas expressziós szintek mellett a PLA jel határozott telítési effektust mutat (5. ábra). Ha ugyanezt az intramolekuláris modellt teszteltük FRET segítségével, teljesen más kép tárult elénk. Ugyancsak SKBR-3 sejteken megjelöltük az ErbB2-t donorhoz konjugált trastuzumabbal és akceptorhoz konjugált pertuzumabbal. Áramlási citometriás FRET mérésekkel meghatároztuk a FRET intenzitást ($I_D \cdot E \cdot \alpha$ a (16) vagy (30) egyenletekben) és ábrázoltuk a fehérje expressziós szintjének függvényében, amit a ki nem oltott (tehát FRET-et nem mutató) donor intenzitásával jellemeztünk (6A. ábra). Szigorú lineáris korrelációt kaptunk a két mennyiség között. Ezzel szemben a FRET hatásfok nem változott a fehérje expressziós szintjének függvényében (6B. ábra).



5. ábra. A PLA jel telítődik az ugyanazon molekula két különböző epitópja elleni proximitás próbák esetében. Az ErbB2 két epitópját pertuzumab-„priming” és trastuzumab-„non-priming” antitesttel jelöltük meg a PLA jel méréséhez, amelyet Cy5-jelölt detektáló oligonukleotiddal mértünk. Ugyanakkor az ErbB2 egy harmadik epitópját AlexaFluor488-7C2 antitesttel jelöltük az ErbB2 expressziós szint detektálásához. A vörös trendvonalak a kétdimenziós hisztogramon az átlagos PLA jelet mutatják az ErbB2 expressziós szint függvényében. A kontúrszinteket logaritmikusan definiáltuk. A szélső ábrákon a fekete hisztogramok az AlexaFluor488-7C2 intenzitás (fent) és a PLA szignál (jobbra) eloszlását mutatják. A szaggatott referenciavonalak a fekete hisztogramok átlagát jelölik. A kék hisztogram a felső ábrán a jelöletlen sejtek eloszlását mutatja az AlexaFluor488-as csatornában, míg a jobb oldali ábrán a PLA szignált jeleníti meg negatív kontroll sejteken, amelyeket csak trastuzumab-„non-priming” antitesttel jelöltünk meg.

dc_380_12

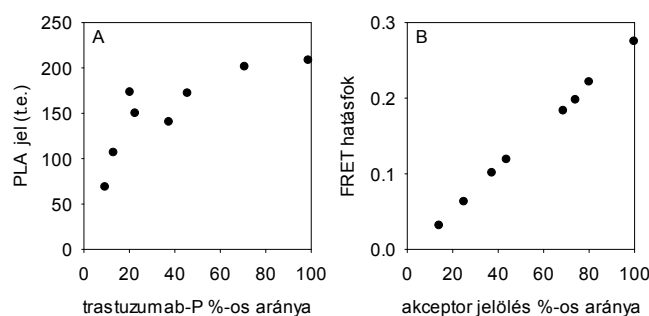


6. ábra. Az intramolekuláris FRET hatások konstans, míg a FRET intenzitás lineárisan változik az expressziós szint függvényében. SKBR-3 sejteket donor-trastuzumab és akceptor-pertuzumab antitestekkel jelöltünk meg az ErbB2 két epitópja közötti intramolekuláris FRET áramlási citometriás méréséhez. A ki nem oltott donor intenzitást, a FRET intenzitást és a FRET hatásfokot sejtenként számoltuk ki. A kontúr szinteket logaritmikusan definiáltuk mindkét hisztogramon. A vörös trend vonal az átlagos FRET intenzitást (A) és az átlagos FRET hatásfokot (B) mutatja az expressziós szint függvényében.

A fenti eredmények arra utalnak, hogy a PLA jel nem arányosan nő az asszociációt mutató fehérjék mennyiségével, és ez a telítési effektus olyan fehérje kifejeződési szinteknél jelentkezik, amelyek daganatos sejtekben nem ritkák. A FRET intenzitás ($I_D \cdot E \cdot \alpha$) várhatóan a klaszterizálódó fehérjék mennyiségével arányos, így a FRET intenzitás és az expressziós szint közötti lineáris összefüggés ezt a feltevést és a FRET megbízható használhatóságát támasztja alá. Szintén emellett szól az is, hogy az intramolekuláris FRET értékek nem változtak az expressziós szint függvényében.

Hogy a PLA jel telítési hajlamát egy másik független és meggyőző módon demonstráljuk, szisztematikusan variáltuk a proximitási próbák denzitását a sejtfelszínen. Ehhez a sejteket pertuzumab-„non-priming” antitesttel jelöltük meg, és a másik epitópot trastuzumab-„priming” és fluoreszcens trastuzumab antitestek keverékével jelöltük meg. Többféle mintát készítettünk, amelyekben a kétféleképpen jelölt trastuzumab antitest relatív arányát változtattuk, és így a fluoreszcens trastuzumabbal leszorítottuk a PLA jel generálásához szükséges trastuzumab-„priming” antitestet. Az eredmények újból meggyőzően arra utaltak, hogy az oligonukleotiddal jelölt proximitás próbák denzitásának emelésekor a PLA jel telítésbe fut (7A. ábra). Ezen mérésekkel párhuzamosan ugyanolyan típusú sejteket donorral konjugált pertuzumabbal jelöltünk, és az akceptorral konjugált trastuzumabot leszorítottuk jelöletlen trastuzumabbal. Szintén egy mintasorozatot készítettünk, amiben a kétféle trastuzumab antitest aránya fokozatosan változott. A

várákozásnak és az előző eredményeknek megfelelően a FRET hatások szigorúan lineárisan változott a fluorofórral jelölt antitestek denzitásának függvényében (7B. ábra).



7. ábra. Telítési effektus PLA-ban az antitest denzitás függvényében.

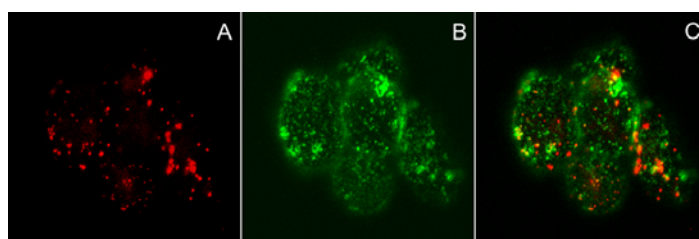
(A) SKBR-3 sejteket pertuzumab-„non-priming” antitesttel változó arányban összekevert trastuzumab-„priming” és AlexaFluor488-trastuzumab antitestekkel jelöltünk meg. Az intramolekuláris PLA jelet a pertuzumab-„non-priming” és trastuzumab-„priming” antitestek között Cy5-detektáló oligonukleotiddal mértük. A trastuzumab kötő epitóp trastuzumab-„priming” antitest általi százalékos telítettségét úgy számoltuk ki, hogy az AlexaFluor488 csatornában mért intenzitást kivontuk a csak AlexaFluor488-trastuzumabbal jelölt sejtek intenzitásából, és ezt normalizáltuk az utóbbi intenzitásra. (B) A pertuzumab kötő epitópot donorral konjugált pertuzumabbal telítettük, a trastuzumab kötő epitóphoz viszont változó mennyiségben jelöletlen és akceptorral konjugált trastuzumabot kötöttünk. Ezt követően megmértük a donor-pertuzumab és az akceptor-trastuzumab közötti FRET-et 20000 sejten áramlási citometriával, és az így meghatározott FRET értékeket ábráztuk az akceptorral konjugált antitest relatív denzitásának függvényében (100%=akceptor intenzitás a kompetáló antitest hiányában).

Tehát megállapítottuk, hogy a PLA módszer telítési effektust mutat, és ezért véleményünk szerint csak szemikvantitatív megközelítésként használható a protein asszociációk mérésére. Ezzel szemben a FRET az elméleti jóslatoknak megfelelően viselkedett kísérleteink során. Ez annak köszönhető, hogy a FRET jelenség szilárd fizikai alapokon nyugszik. Sejtes rendszerben a FRET hatások nem egyszerűen a donor-akceptor távolságtól függ a (2) egyenlet szerint, hanem a molekuláris asszociátumok sztöchiometriájától és a klaszterben levő molekulák arányától. A fenti komplexitás ellenére számtalan olyan modellt alkottak már, többek között mi is, amely kvantitatív jóslatokat tesz a FRET mérések alapján a fehérje klaszterizációra vonatkozóan (35, 43, 204, 205).

Mivel a PLA szignál és a kísérleti körülmények közötti kvantitatív viszony ismeretlen, csak találgatni lehet a telítési effektus okaival kapcsolatban. Véleményünk szerint a sűrűn elhelyezkedő proximitási próbák által létrehozott sztérikus gátlás megakadályozhatja, hogy a PLA jelet generáló enzimek hozzáférhessenek az oligonukleotidokhoz. Ezenkívül a PLA jel

keletkezésében az RCA reakció során amplifikáció játszódik le, ami nem-lineáris hatások megjelenéséhez vezethet. A feltételezést, mely szerint sztérikus gátlás és nem-lineáris folyamatok játszódnak le a PLA reakcióban az amplifikációs fázis során, alátámasztja az a megfigyelés, hogy a PLA jel megjelenése „kvantált”, tehát fluoreszcens mikroszkópos képen nem homogén jel, hanem egységes méretű és fényességű foltok jelennek meg még intramolekuláris mérések során is (8. ábra). Bár nem várható, hogy egy molekula két epitópja közötti távolság heterogenitást mutat egy sejt felszínén, a PLA szignál mégsem egyenletes eloszlású. Ha az amplifikációs folyamat sztérikus okok miatt nem indulhat be, akkor a PLA jel teljesen hiányzik. Ha azonban beindul, akkor a PLA szignál egy teljesen kifejtett folttá érik.

Összegzésképpen elmondható, hogy a PLA folyamat rendkívüli jel/zaj arányáért valószínűleg ugyanaz a folyamat, az amplifikáció, felelős, mint ami a telítési effektust létre hozza. Ezért a PLA mérések esetén kapott eredményeket körültekintéssel kell értelmezni. (A tárgyalt cikk a referencialistában: (153).)



8. ábra. Reprezentatív képek a PLA jel sejteken tapasztalt eloszlására.

SKBR-3 emlőtumor sejteket trastuzumab-„priming”, pertuzumab-„non-priming” és AlexaFluor488-7C2 ErbB2 ellenes antitestekkel jelöltünk meg. A PLA jelet Cy5 jelölt detektáló oligonukleotiddal tettük láthatóvá (A). Az AlexaFluor488-7C2 (B), ill. a PLA és az immunfluoreszcens jel egymásra rétegzése (C) is látható.

4.3. Áramlási citometriás homo-FRET módszer kidolgozása a membránfehérjék homoklaszterizációjának kvantitatív jellemzésére

Az előző fejezetben elmondottak értelmében a FRET szilárd fizikai alapjai lehetővé teszik olyan kvantitatív modellek létrehozását, amelyek nem egyszerűen arra adnak választ, hogy van-e kölcsönhatás két molekula között, ill. ez hogyan változik egy kísérlet során, hanem a receptorklaszterek sztöchiometriájára is rávilágítanak. A dimernél nagyobb méretű homoklaszterek vizsgálatára a homo-FRET kimondottan alkalmas, hiszen az energia szétterjed az egész klaszterben, és így a homo-FRET egyetlen manifesztációja, a fluoreszcencia anizotrópia, a klaszter méretére (tehát a benne levő fehérjék számára)

érzékeny (l. 1.6. fejezet, 3. ábra). A megoldandó problémát az jelenti, hogy a fluoreszcencia anizotrópiát nemcsak a homo-FRET befolyásolja, ezért egyetlen anizotrópia értékből nem lehet a klaszterméretre visszakövetkeztetni. Ennek megoldására több megközelítés is létezik (l. 1.6. fejezet). Mivel áramlási citometriára még nem állt rendelkezésre ilyen eljárás, ennek megalkotását tűztük ki célul.

Megközelítésünk alapja, hogy egy olyan mintasorozatot állítunk elő, amelyben változik a vizsgált fehérjét jelölő fluoreszcens antitestek denzitása. Ezt úgy érjük el, hogy ugyanazon antitest fluoreszcensen jelölt és jelöletlen változatát keverjük össze, és fokozatosan változtatjuk arányukat, miközben a teljes antitest koncentráció konstans marad, és ügyelünk arra, hogy az antitestkeverék telítse a kötőhelyeket. Ebben az esetben a fluoreszcens antitest által elfoglalt epitópok aránya (szaturáció, s) egyenlő a fluoreszcens antitest moláris arányával. Az antitestek pontatlan keverése és koncentrációjuk bizonytalan meghatározása miatt a szaturációt a kísérletek során az adott minta fluoreszcencia intenzitásának és a csak fluoreszcens antitesttel jelölt minta intenzitásának hányadosaként becsültük meg. A modellben azt tételeztük fel, hogy a fehérjék egy része monomer (arányuk mon), míg az adott típusú fehérje többi része N -mert alkot, ezek aránya: $1-mon$. A kísérleti eredmények értelmezése szempontjából lényeges, hogy minden fehérjét, amely nem képes homo-FRET kölcsönhatásban részt venni, monomernek tekintünk, tehát pl. egy fluoreszcensen jelölt fehérje, amely heterodimert alkot egy másik, nem jelölt fehérjével. A binomiális eloszlás felhasználásával annak valószínűsége, hogy egy N -meren belül k darab fehérjéhez kötődik fluoreszcens antitest, amikor a jelölt antitestek aránya s :

$$P_{s,k,N} = \binom{N}{k} s^k (1-s)^{N-k} \quad (41)$$

Runnels és Scarlata szerint egy ilyen homoklaszter fluoreszcencia anizotrópiáját a következő egyenlet írja le:

$$r_k = r_1 \frac{(1+d^6)}{1+k d^6} + r_{FRET} \frac{(k-1)d^6}{1+k d^6}, \quad d = \frac{R_0}{R} \quad (42)$$

ahol r_1 és r_{FRET} rendre az első gerjesztett fluorofór anizotrópiája és a FRET-tel gerjesztett fluorofórok anizotrópiája. Azt tételeztük fel, hogy minden FRET által gerjesztett fluorofór teljesen depolarizált fluoreszcenciát emittál, tehát $r_{FRET}=0$ (206). Mivel a fluoreszcensen jelzett antitestek és a rajta levő fluorofórok rotációs mobilitása elég szabad, ill. relatív orientációjuk random, a fenti feltételezés valószínűleg helyes vagy közel jár az igazsághoz.

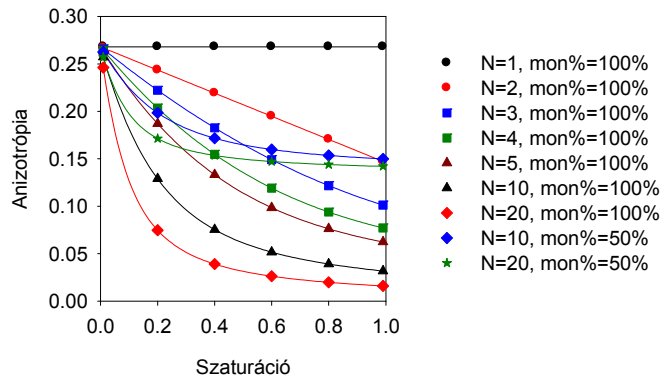
Amennyiben az $r_{FRET}=0$ feltételezés nem igaz, az a monomer% felülbecsléséhez és a klaszterméret alulbecsléséhez vezet. Egy monomerekből és N-merekből álló keverék fluoreszcencia anizotrópiáját az anizotrópiák intenzitással súlyozott átlaga adja meg (195):

$$r_{s,k,N} = \frac{(1-mon) \sum_{k=0}^N P_{s,k,N} \frac{k}{N} r_k + s \cdot mon \cdot r_1}{(1-mon) \sum_{k=0}^N P_{s,k,N} \frac{k}{N} + s \cdot mon} \quad (43)$$

ahol k/N egy korrekciós faktor, amely a monomerek és N-merek fluoreszcencia összegét egyre normálja. A (41)-(43) egyenletek kombinálásával adódik az eredő anizotrópia:

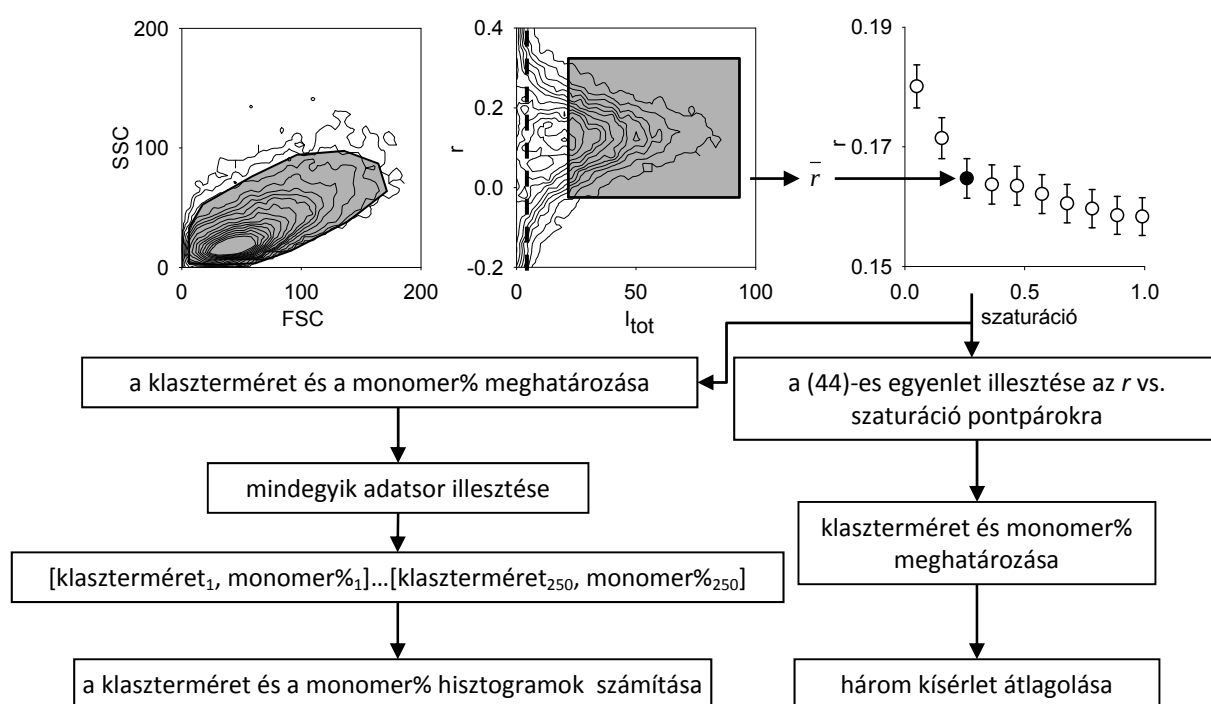
$$\begin{aligned} r_{s,N} &= \frac{(1-mon) \sum_{k=0}^N \left[\binom{N}{k} s^k (1-s)^{N-k} \frac{k}{N} \left(r_1 \frac{(1+d^6)}{1+k d^6} + r_{FRET} \frac{(k-1)d^6}{1+k d^6} \right) \right] + s \cdot mon \cdot r_1}{(1-mon) \sum_{k=0}^N \binom{N}{k} s^k (1-s)^{N-k} \frac{k}{N} + s \cdot mon} = \\ &= \frac{(1-mon)}{Ns} \sum_{k=0}^N \left[\binom{N}{k} s^k (1-s)^{N-k} k \left(r_1 \frac{1+d^6}{1+k d^6} + r_{FRET} \frac{(k-1)d^6}{1+k d^6} \right) \right] + mon \cdot r_1, \quad (44) \\ \text{ha } r_{FRET} = 0 &\Rightarrow r_{s,N} = \frac{(1-mon)}{Ns} \sum_{k=0}^N \left[\binom{N}{k} s^k (1-s)^{N-k} k \left(r_1 \frac{1+d^6}{1+k d^6} \right) \right] + mon \cdot r_1 \end{aligned}$$

A fenti egyenlet a 9. ábrán látható jöslatokat teszi az anizotrópiára a monomer% és a klaszterméret függvényében. Látható, hogy monomerek jelenlétében az anizotrópia állandó, dimer esetében lineárisan csökken a szaturáció mértékével, nagyobb méretű oligomer esetében a csökkenés egyre meredekebb.



9. ábra. Az anizotrópia függése a klasztermérettől és a monomerek arányától. A (44) egyenlet alapján számított görbékhez $r_1=0,27$ anizotrópiát tételeztünk fel az elsődlegesen gerjesztett fluorofór esetében.

A kísérletek során meghatározott anizotrópia-szaturáció párokra a (44)-es egyenletet illesztettük, amelyből meghatároztuk a monomerek arányát és a klaszterméretet. Ezek után Monte Carlo szimulációt hajtottunk végre, és az anizotrópia átlagának és szórásának ismeretében 250 véletlen adatsort állítottunk elő. Ezekre az anizotrópia-szaturáció szimulált adatsorokra szintén a (44)-es egyenletet illesztettük, így 250 darab monomer% és klaszterméret értéket állítottunk elő, amelyek histogramjai kerülnek bemutatásra a következő alfejezetekben. Az egész algoritmus folyamatábráját a 10. ábra foglalja össze.



10. ábra A homo-FRET mérések algoritmus.

Áramlási citométerrel lementünk 50000 sejtet, amelyeket jelölt és jelöletlen antitestek keverékével jelöltünk meg. Az analízis során csak az intakt sejteket vettük figyelembe, melyeket az előre irányú (FSC) vs. oldal irányú fénysszórás (SSC) pontábrán azonosítottunk. A következő kapuzási lépés során csak a magas fluoreszcencia intenzitású (I_{tot}) sejteket vettük figyelembe. Az ábrán a vastag szaggatott vonal a jelöletlen sejtek átlagintenzitását mutatja. A mintasor mindegyik mintáján meghatároztuk az anizotrópia-szaturáció értékeket, és a pontsorra illesztettük a (44)-es egyenletet. Az anizotrópia átlagának és szórásának ismeretében 250 véletlen adatsort állítottunk elő, amelyeket ugyanúgy elemeztünk, mint a valódi kísérleti adatokat. Az így kapott 250 klaszterméret és monomer arányból histogramot számoltunk.

A (44)-es képlet alkalmazhatóságához még két paraméter értékét kellett meghatározni, az r_1 határanizotrópiát és az R_0/R hányadost. A határanizotrópia meghatározásához a Perrin

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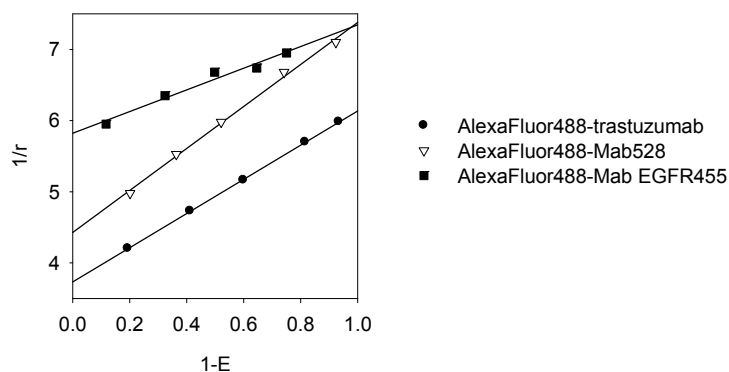
egyenletet használtuk fel, amely leírja egy fluorofór anizotrópiájának változását a rotációs korrelációs idő és a fluoreszcencia élettartam függvényében:

$$\frac{1}{r} = \frac{1}{r_0} \left(1 + \frac{\tau_{fl}}{\tau_{rot}} \right) \quad (45)$$

ahol τ_{fl} és τ_{rot} rendre a fluoreszcencia élettartam és a rotációs korrelációs idő. Mivel a hetero-FRET a fluoreszcencia élettartamot csökkenti, Perrin ábrát lehet szerkeszteni a hetero-FRET hatásfok változtatásával:

$$\tau_{fl} = (1-E)\tau_D \Rightarrow \frac{1}{r} = \frac{1}{r_0} \left(1 + \frac{(1-E)\tau_D}{\tau_{rot}} \right) \quad (46)$$

ahol τ_D a donor élettartama FRET hiányában. Az $1/r$ vs. $(1-E)$ ábrára illesztett egyenes tengelymetszete a határanizotrópia reciproka. A hetero-FRET hatásfok variálásához a sejteket donorral jelölt antitesttel jelöltük meg, majd változó koncentrációban akceptorral jelölt másodlagos antitesttel rájelöltünk. Mind a hetero-FRET hatásfokot, mind az anizotrópiát megmértük, és a kapott Perrin ábrákra egyenest illesztettünk, amelyek tengelymetszetéből meghatároztuk a határanizotrópiát (11. ábra, $r_{0,A488-trastuzumab}=0,268$; $r_{0,A488-trastuzumab Fab}=0,289$; $r_{0,A488-Mab528}=0,226$; $r_{0,A488-Mab EGFR455}=0,170$).



11. ábra. A határanizotrópia meghatározása.

A sejteket a feltüntetett antitestek telítő koncentrációjával jelöltük meg, majd akceptor-konjugált (Cy3 vagy AlexaFluor546) másodlagos antitesttel rájelöltünk változó koncentrációban. Meghatároztuk mind a hetero-FRET hatásfokot, mind az anizotrópiát, és a Perrin ábrán az y-tengelymetszetből kiszámítottuk a határanizotrópiát.

A Perrin analízis alapján meghatározott határanizotrópiáról (r_0) azt tételeztük fel, hogy azonos az izolált, homo-FRET kölcsönhatásban nem álló fluorofór anizotrópiájával (r_1). Mivel a Perrin ábrán a nagyon rövid élettartamok (magas FRET értékek) nem voltak

reprezentálva, ezért könnyen elképzelhető, hogy az ilyen gyors forgások okozta depolarizációt nem vettük figyelembe, ezért a valódi határanizotrópia valószínűleg magasabb. De véleményünk szerint ez egy szerencsés félrebecslés, hiszen a homo-FRET analízis során nem az izolált, immobilis fluorofór anizotrópiáját (kanonikus határanizotrópia) kell figyelembe venni, hanem azt az anizotrópiát, amit a rotáció már csökkentett, de a homo-FRET még nem befolyásolt. Ezért az általunk meghatározott „határanizotrópia” jól használható a homo-FRET mérések esetében az r_1 becslésére.

Az (R/R_0) meghatározásához először az R_0 értékét határoztuk meg két AlexaFluor488 közötti homo-FRET kölcsönhatásra az alábbi egyenlet szerint (200):

$$R_0 = 8.8 \cdot 10^{-18} \left(J \cdot \Psi_D \cdot n^{-4} \cdot \kappa^2 \right)^{1/6} \quad (47)$$

Az átfedési integrált az Invitrogen honlapján elérhető spektrumokból becsültük meg az alábbi képlet szerint:

$$J = \int_0^{\infty} f_D(\lambda) \varepsilon_D(\lambda) \lambda^4 d\lambda \quad (48)$$

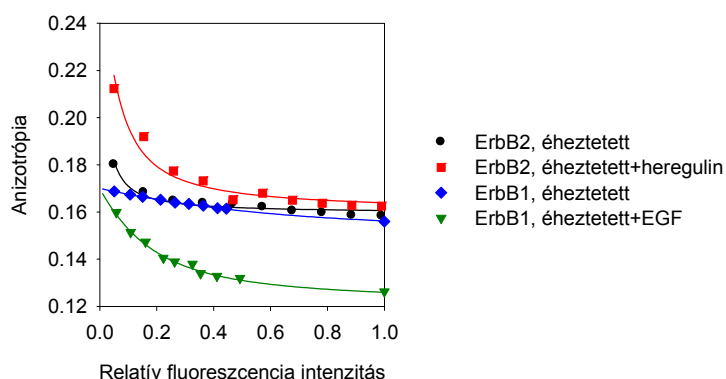
ahol f_D a fluorofór normalizált emissziós spektruma, ε_D a fluorofór moláris abszorpciós koefficiense. Az AlexaFluor488 kvantumhatásfokát (Ψ_D) korábban 0,6-nak mérték ki (207). A törésmutató (n) és az orientációs faktor (κ^2) értékeire rendre 1,4-et és 2/3-ot tételeztünk fel, és így az AlexaFluor488 homo-FRET kölcsönhatására az R_0 érték 4,8 nm-nek adódott. Az Cy3-trastuzumab és Cy5-trastuzumab közötti FRET érték ErbB2 homoasszociációra 20% volt korábbi mérések szerint (208). Mivel a Cy3-Cy5 közötti R_0 érték 5 nm (208), a fenti FRET érték 6,29 nm donor-akceptor távolságnak felel meg az alábbi egyenlet alapján:

$$R = R_0 \sqrt[6]{\frac{1-E}{E}} \quad (49)$$

Ezért két trastuzumab közötti távolság az AlexaFluor488 homo-FRET-re vonatkoztatott R_0 értékekben $6,29/4,8=1,3 \times R_0$, amely reciproka megadja a (42)-es egyenletben a d -t. Hasonló megközelítést alkalmazva két EGFR455 antitestre a távolság $1,1 \times R_0$, míg két Mab528-ra $1,4 \times R_0$.

Egy, a fent leírt módon mért és kiértékelt mérést mutat a 12. ábra, amely alapján levonható az a következtetés, hogy az általunk kidolgozott módszer megbízhatóan alkalmazható a homo-FRET mérésére és a klaszterizáció kvantitatív jellemzésére. Mivel áramlási citometriás méréseken alapszik, statisztikailag megbízható. Az általunk alkalmazott

egyszerűsítő feltevések miatt az illesztett paraméterek száma alacsony, így gyakorlati szempontból is alkalmazható megközelítést jelent, szemben más publikált módszerekkel (204). (A tárgyalt cikk a referencialistában: (35).)



12. ábra. Az anizotrópia modell illesztése kísérleti eredményekre.

Éheztetett vagy növekedési faktorról stimulált sejteket megjelöltünk jelölt és jelöletlen antitestek keverékével. Az ErbB2 jelölésére trastuzumabot, az ErbB1 jelölésére EGFR455 antitestet használtunk. A szaturáció függvényében megmértük és ábrázoltuk az anizotrópiát (szimbólumok), és a (44)-es egyenletet illesztettük rá (folytonos vonalak).

4.4. Az ErbB1 és ErbB2 homoklasztareinek vizsgálata áramlási citometriás homo-FRET módszerrel

A 3.3. pontban leírt módszerrel először az ErbB2 homoklasztareit vizsgáltuk meg SKBR-3 sejteken. Nyugalomban lévő sejteken, melyeket 0,1% szérumban tartalmazó médiumban éhezettünk egy éjszakán át, az ErbB2 ~60%-a monomer volt, de a fennmaradó hányad nagyméretű klasztereket alkotott, melyekben átlagosan ~110 ErbB2 fehérje volt (4. táblázat, 13A. ábra). A sejtek EGF-fel vagy heregulinval történő stimulációja a monomer%-ot nem változtatta jelentősen, de a klaszterek méretét szignifikánsan csökkentette. A változás heregulin esetében volt a nagyobb mértékű.

Feltevésünk szerint a növekedési faktor által indukált ErbB2 homoklaszter méret csökkenést az okozta, hogy az ErbB2-t az EGF által aktivált ErbB1 vagy a heregulin által stimulált ErbB3 kiszakította a homoklaszterekből. Az így létrejövő vegyes ErbB2-3 vagy ErbB1-2 klaszterekben az ErbB2 nem monomer, hanem ott is homooligomer, ezért nem nő a monomer%. A fenti elképzelés igazolására a kísérletet elvégeztük úgy, hogy a sejteket a növekedési faktor stimuláció előtt pertuzumabbal előkezeltük. A pertuzumab antitestről

tudott, hogy az ErbB2 heterodimerizációját gátolja (9). A pertuzumab önmagában is csökkentette az ErbB2 homoklaszterek méretét, ami arra utal, hogy az ErbB2 homoasszociációra is gátló hatást fejt ki. A heregulin által kiváltott ErbB2 homoklaszter méretcsökkenést a pertuzumab előkezelés nagymértékben gátolta (4. táblázat, 13A. ábra). Ez a kísérleti eredmény meggyőzően alátámasztotta, hogy az ErbB2 homoklaszterekből a növekedési faktor által aktivált heregulin receptor (ErbB3) ErbB2 molekulákat szakít ki.

Annak kizárására, hogy az ErbB2 nagyméretű klasztereit az antitestek által okozott keresztkötés hozza létre, a kísérleteket megismételtük monovalens Fab felhasználásával, és a bivalens antitestekkel kapott eredményekhez teljesen hasonló következtetésre jutottunk (eredmények nincsenek bemutatva).

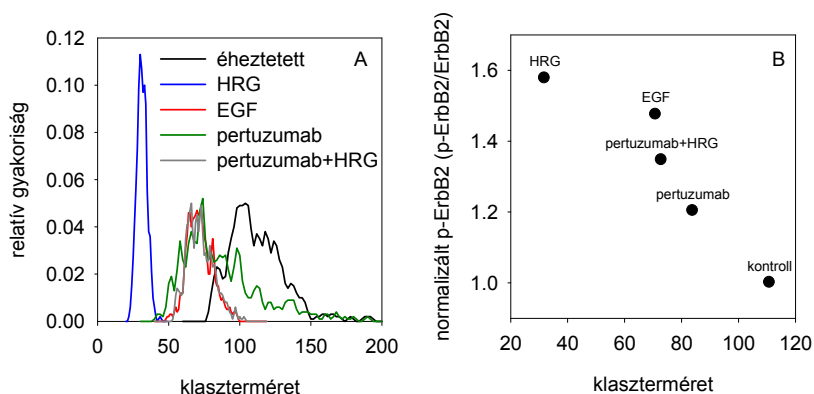
A fenti eredmények alapján úgy tűnik, hogy a stimulálatlan sejteken az ErbB2 klaszterek mérete nagyobb, mint a növekedési faktor által stimuláltakon. Ez arra utal, hogy az ErbB2 klaszterméret fordítottan arányos a sejtek, ill. valószínűleg az ErbB2 aktiváltsági szintjével. E feltételezés közvetlen ellenőrzésére nyugalomban levő és stimulált sejteken megmértük mind az ErbB2 homoklaszterek méretét, mind az ErbB2 tirozin foszforiláltsági szintjét. Az utóbbit a foszforilált ErbB2 és a totál ErbB2 arányával jellemeztük áramlási citometria segítségével. A két paraméter negatív korrelációt mutatott egymással (13B. ábra). Ezen eredmények szintén megerősítik azt a feltevést, mely szerint a nagyméretű ErbB2 homoklaszterek inaktív ErbB2-t tartalmaznak, melyekből a növekedési faktorok által aktivált más ErbB fehérjék kiszakítják az ErbB2-t.

	ErbB1		ErbB2	
	klaszterméret	monomer%	klaszterméret	monomer%
kontroll	4±1	88±4	111±12	60±5
EGF	11±2	71±3	71±6	61±4
heregulin	–	–	32±4	59±5
pertuzumab	–	–	84±7	70±4
pertuzumab + heregulin	–	–	73±6	58±3

4. táblázat. Az ErbB1 és ErbB2 klaszterméret és monomer arányok éheztetett és stimulált sejteken. Az ErbB1-et EGFR455 antitesttel, az ErbB2-t trastuzumabbal jelöltük. Az átlag±SEM értékeket három független mérésből számítottuk.

Az ErbB1 receptort A431 sejteken jellemeztük, mivel ezen a sejten expresszálódik ez a fehérje kellően magas szinten a homo-FRET mérések elvégzéséhez. Nyugalomban levő

sejteken az ErbB1 ~90%-a monomer volt, míg a fennmaradó hányad viszonylag kisebb méretű klasztereket alkotott, amelyekben átlagosan ~4 ErbB1 volt (4. táblázat). EGF stimuláció hatására az ErbB1 monomer%-a csökkent, és az átlagos klaszterméret növekedett. Ez a változás ellentétes az ErbB2 fentebb leírt viselkedésével, de összhangban áll az irodalmi adatokkal, melyek szerint az ErbB1 EGF-fel történő stimulációja dimerizációhoz vagy nagyobb méretű oligomerek képződéséhez vezet. A két ErbB fehérje jelentősen eltérő viselkedése felhívja a figyelmet arra, hogy a tankönyvi dogmaként tárgyalt elmélet, mely szerint az aktiválatlan receptor monomer, amely aktiváció hatására asszociálódik, nem tartható. A különféle módszerek alkalmazása alapján az ErbB receptorok hierarchikus asszociációjára felállított modellünk a 4.9 fejezetben kerül ismertetésre. (A tárgyalt cikk a referencialistában: (35).)



13. ábra. Az ErbB2 homoklaszterek jellemzése.

(A) Az ábrán feltüntetett körülmények között meghatároztuk az ErbB2 homoklaszterek méretét, és a konfidencia intervallumot Monte Carlo szimulációval becsültük meg. Az ábrán az előállított 250 szimulált adatsorra történt illesztés során kapott klaszterméret eloszlása látható (HRG=heregulin). (B) Különböző körülmények között megmértük az ErbB2 homoklasztereinek méretét és az ErbB2 normalizált tirozin foszforiláltsági szintjét. A két paraméter negatív korrelációt mutatott egymással.

4.5. FRET-szenzitizált akceptor fotoelhalványítás (FSAB) a fehérjék heteroklaszterizációjának kvantitatív jellemzésére

A heteroklaszterek tanulmányozására a fent kidolgozott homo-FRET módszer nem alkalmas. Bár több eljárás is képes arra, hogy a heteroklaszterizációt kvantitatív módon jellemezze (pl. kétszínű FCS (176)), ezek általában nem alkalmazhatók hagyományos konfokális mikroszkópon. Ezért célul tűztük ki egy ilyen módszer kifejlesztését és tesztelését.

Az 1990-es években Mekler és mtsai leírtak egy jelenséget, amelyet ők a szenzitivitás fotokémiai növelésének („photochemical enhancement of sensitivity”) neveztek (209, 210). Eszerint az akceptor tetszőleges fotokémiai folyamatát hetero-FRET segítségével elő lehet segíteni, hiszen a donor ugyanúgy gerjesztett (és így reakcióképes) állapotba hozza az akceptort FRET révén, mint az akceptor közvetlen gerjesztése tenné. A szerzők véleménye szerint módszerükkel nagyon alacsony FRET hatásfok is megbízhatóan mérhető, ha az akceptor azon a hullámhosszon, ahol a donort gerjesztik, minimális abszorpciót mutat. Az akceptor vizsgált fotokémiai reakciója lehet a fotoelhalványítása is. Ebben az esetben a FRET abban nyilvánul meg, hogy az akceptor fotoelhalványítási kinetikája felgyorsul a donor jelenlétében, ha a gerjesztést a donor abszorpció maximumán végezzük. A jelenség akkor mérhető könnyen, ha fotolabilis akceptor mellé fotostabil donort választunk. A folyamat nemcsak a FRET hatásfok, hanem a donorral komplexben levő akceptorok arányának meghatározására is felhasználható az alábbiak szerint. Mivel a FRET kölcsönhatásban részt vevő akceptorok preferenciálisan szenvednek fotoelhalványítást, ezért ki lehet számolni a fotoelhalványított akceptor populáció relatív hozzájárulását a FRET-hez a FRET hatásfok és a szenzitizált akceptor emisszió időbeli változásának összehasonlításával (210). Bennünket ezen kinetika végpontja érdekelt, tehát amikor az összes FRET kölcsönhatásban részt vevő akceptor kiégett. Ekkor a FRET hatásfok nullára esik, és az akceptor intenzitás relatív csökkenéséből ki lehet számolni ezen akceptor populáció hányadát. Hangsúlyozandó, hogy a módszer a FRET által megnövelt akceptor fotoelhalványításon alapszik, elneveztük FRET szenzitizált akceptor fotoelhalványításnak („FRET-sensitized acceptor bleaching”, FSAB).

A módszer matematikai bevezetése során feltételeztük, hogy a donorral való kölcsönhatás szempontjából kétféle akceptor populáció létezik: 1) donorhoz kötött akceptor molekulák, tehát amelyek FRET távolságon belül vannak a donoroktól (A_{bound}); 2) szabad akceptorok (A_{free}). Mivel praktikusán minden esetben a szabad akceptorok egy része is kiég a donor gerjesztési hullámhosszán, ezért ezt korrekcióba kell venni. A szabad akceptorok kiégett hányadát *BCF*-nek („bleaching correction factor”) neveztük. A *BCF* értékét abban a pillanatban kell meghatározni, amikor a kötött akceptorok 100%-a kiég, tehát amikor a FRET hatásfok nullára csökken. A fentiek értelmében fel lehet írni a következő egyenletrendszer:

$$F_{\text{bleached}} = \frac{A_{\text{free}} BCF + A_{\text{bound}}}{A_{\text{free}} + A_{\text{bound}}} \quad (50)$$

$$A_{bound} + A_{free} = A_0 \quad (51)$$

ahol $F_{bleached}$ a kiégett akceptorok hányadát jelenti. Ezek alapján a kötött akceptorok arányát a következőképpen lehet megadni:

$$\frac{A_{bound}}{A_0} = \frac{F_{bleached} - BCF}{1 - BCF} \quad (52)$$

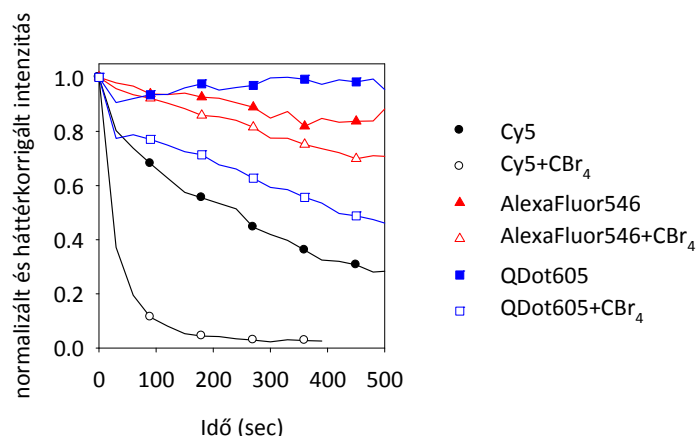
Mivel a BCF értéke antitestenként változott, ezért minden antitestre külön meg kellett határozni. Mivel az akzeptornak alkalmazott Cy5 (l. később) festék fotoelhalványítási kinetikája függ a fluorofórok denzitásától (211), ezért a BCF -et olyan sejteken határoztuk meg, amelyek fluoreszcencia intenzitása hasonló volt, mint amilyeneket a FRET mérések során használtunk. Néhány esetben tapasztaltunk olyat, hogy a kötött akceptor nem teljes egészében égett ki. Ebben az esetben az (50) és (52) egyenleteket a következőképpen kell módosítani:

$$F_{bleached} = \frac{A_{free} BCF + A_{bound} BCF_{FRET}}{A_{free} + A_{bound}} \Rightarrow \frac{A_{bound}}{A_0} = \frac{F_{bleached} - BCF}{BCF_{FRET} - BCF} \quad (53)$$

ahol BCF_{FRET} a kötött akceptorok kiégethető hányada. A BCF_{FRET} értékét olyan mintán lehet meghatározni, amelyen biztosan mindegyik akceptor FRET távolságon belül van a donoroktól. Ilyet elő lehet állítani úgy, hogy akceptor fluorofórral konjugált elsődleges antitesttel megjelölt sejthez donorral konjugált másodlagos antitestet adunk. Részletes számításokkal kimutattuk, hogy ha a donor fotoelhalványítási kinetikája jelentősen lassabb, mint az akceptoré, akkor ez nem befolyásolja jelentősen a kötött akceptor mennyiségének meghatározását (43). Ezért a módszer alkalmazhatóságának igazolására először egy olyan párt kellett találnunk, amelyben a donor sokkal fotostabilabb, mint az akceptor.

A legtöbb jelenleg elérhető fluoreszcens festék fotostabil, hiszen ez a fejlesztők egyik legfontosabb célja. A Cy5 közismerten relatíve gyors fotoelhalványítási kinetikája és az a tény, hogy Mekler és mtsai is cianin típusú festékeket használtak, arra utalt, hogy ez a fluorofór jó választás lesz akzeptornak. Mivel azonban a Cy5 kiégése praktikus szempontból még így is túl lassú, CBr_4 -ot használtunk a kiégetés felgyorsítására, hiszen a CBr_4 a cianin festékekkel töltésátviteli komplexet („charge transfer complex”) alkotva felgyorsítja azok fotoelhalványítását (209, 210). Ezért a donor kiválasztásánál a CBr_4 jelenlétében mutatott fotostabilitás volt a döntő. Mivel a fluoreszcens QDot-okról köztudott, hogy rendkívül fotostabilak (212), ezért kézenfekvő választásnak tűntek donornak. Tapasztalatunk szerint a donorként szóba jövő QDot-ok fotoelhalványítási kinetikáját a CBr_4 jelentősen felgyorsítja,

ezért más lehetőség után kellett néznünk (14. ábra). Az AlexaFluor festékek szintén fotostabilitásukról nevezetesek, bár ebből a szempontból elmaradnak a QDot-ok mögött (213, 214). Kísérleteink szerint jó fotostabilitását az AlexaFluor546 CBr₄ jelenlétében is megőrzi, ezért az FSAB kísérletekhez az AlexaFluor546-Cy5 donor-akceptor párt használtuk (14. ábra).

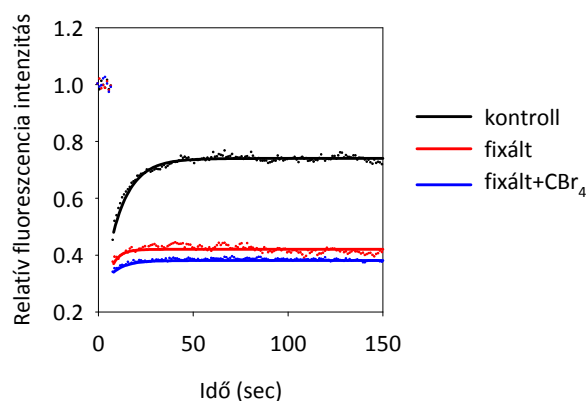


14. ábra. A CBr₄ hatása potenciális donorok és akceptorok fotoelhalványítási kinetikájára. SKBR-3 sejteket az ábrán feltüntetett fluorofórral konjugált trastuzumabbal jelöltük meg, majd abszorpciós maximumuk környékén (AlexaFluor546, QDot605 – 543 nm; Cy5 – 633 nm) történő gerjesztés mellett vizsgáltuk fotoelhalványítási kinetikájukat CBr₄ jelenlétében és nélkül. A gerjesztéshez a két különböző hullámhosszon hasonló lézerteljesítményeket használtunk, és ez hasonló volt az FSAB kísérletek során használt teljesítményhez. A kiégetést 30 másodpercenként megszakítottuk, hogy gyengébb lézerteljesítménnyel képeket vegyünk fel. A szimbólumokat csak minden harmadik mérési pontnál tüntettem fel.

Mivel az FSAB kísérletek során az akceptor kiégetésére több száz másodpercet kell várni, felmerül a kérdés, hogy ilyen hosszú időtartam alatt a vizsgált membránfehérjék mozognak-e vagy „befagyottnak” tekinthető a rendszer. Mivel a kísérleteket 1%-os formaldehidben fixált sejteken végeztük, meg kellett vizsgálni a membránfehérjék laterális mobilitását ilyen körülmények között. FRAP kísérleteket végeztünk ErbB1-eGFP-vel stabilan transzfektált A4erbB1 sejtekkel. Eredményeink szerint az ErbB1-eGFP immobilis hányada 55±3% volt nem fixált sejteken, és ez 92±1%-ra, ill. 94±2%-ra nőtt 1% formaldehidben fixált sejteken CBr₄ nélkül és jelenlétében (15. ábra). Bár néhány korábbi publikáció szerint az enyhe formaldehides fixálás nem módosítja jelentősen a transzmembrán fehérjék mobilis hányadát (215-217), kísérleteink meggyőzően és reprodukálhatóan bizonyították, hogy az általuk alkalmazott rendszerben ez másképp van. A fenti eredmények alapján az FSAB

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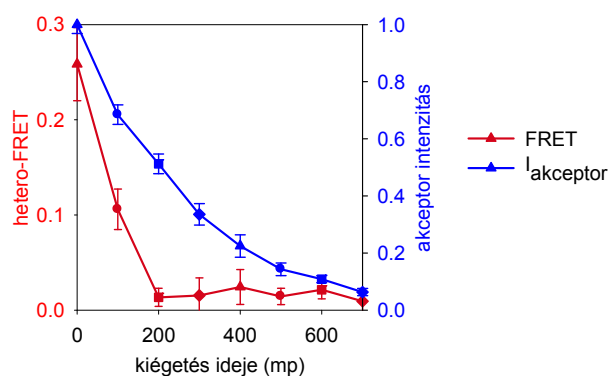
kísérletek során a donorok csak a közvetlen közelükben levő akceptor molekulákat fogják fotoelhalványítani, hiszen diffúzió révén nem képesek távoli akceptorokat elérni.



15. ábra. A formaldehid fixálás immobilizálja az ErbB1-eGFP-t.

A4erbB1 sejteken vizsgáltuk a fluoreszcencia visszatérés mértékét egy erős kiégető lézerimpulzus után a feltüntetett körülmények között. A pontok a mérési eredményeket, a folytonos vonal az illesztést mutatja a (25)-ös egyenlet szerint.

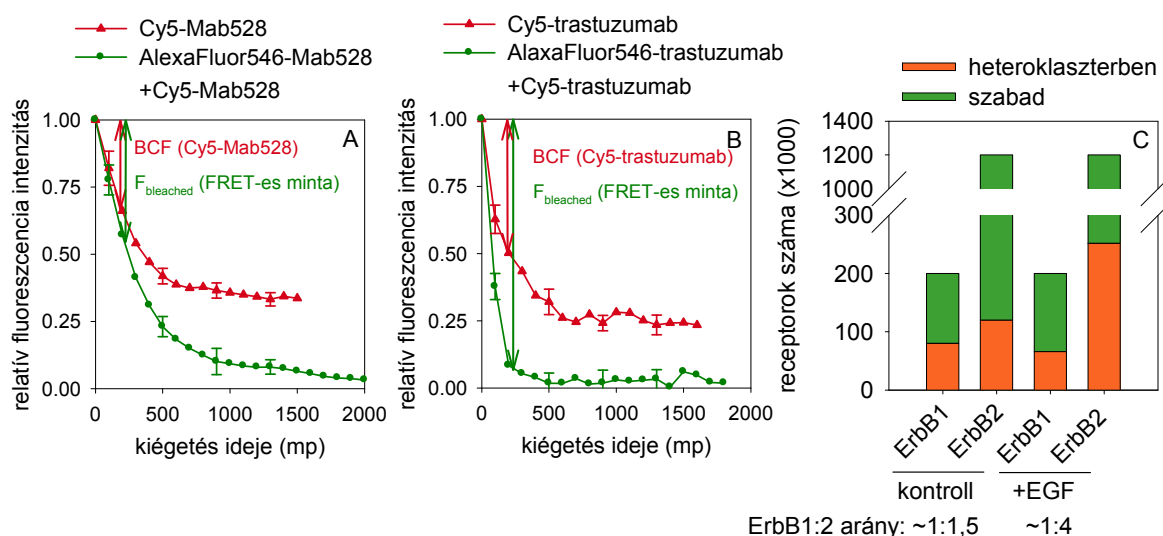
Az FSAB módszer biológiai alkalmazása előtt igazolni kellett, hogy az elv tényleg működőképes, tehát a donorhoz közeli akceptorok preferenciálisan kiégnek a donor gerjesztése esetén. A 16. ábra tanúsága szerint ez tényleg így történt. Ezen kísérletből még azt is meghatároztuk, hogy ~200 sec szükséges ahhoz, hogy a donoroktól FRET távolságra levő akceptorok teljesen kiégjenek, hiszen ekkorra csökkent a FRET hatásfok nullára.



16. ábra. Az FSAB módszer elvének igazolása.

SKBR-3 sejteket megjelöltünk AlexaFluor546-trastuzumab és Cy5-trastuzumab antitestek keverékével, majd az FSAB módszerrel vizsgáltuk az akceptor fotoelhalványodását és a FRET hatásfok csökkenését a donor gerjesztése közben.

Még mielőtt az FSAB technikát az ErbB1-2 heteroklaszterek vizsgálatára használtuk volna, ellenőriztük, hogy a 4.3-4.4. fejezetekben kidolgozott és alkalmazott homo-FRET mérésekhez hasonló eredményeket szolgáltat-e az ErbB1 és ErbB2 homoklasztereire. A 17A-B. ábrák összehasonlításából azonnal látható, hogy az FSAB módszer szerint is az ErbB2 nagyobb aránya alkot homoklasztereket, hiszen az akceptorral jelölt ErbB2 ellenes antitest kiégését a donorral jelölt ErbB2 ellenes antitest jelentősen felgyorsította, míg a donorral jelölt ErbB1 ellenes antitestnek elhanyagolható hatása volt az akceptorral jelölt ErbB1 ellenes antitest fotoelhalványítási kinetikájára. (A tárgyalt cikk a referencialistában: (43).)



17. ábra. Az ErbB1 és ErbB2 homo- és heteroklasztereinek jellemzése FSAB módszerrel. (A-B) Szérum éhezettetett SKBR-3 sejteket az ábrán jelölt antitestekkel jelöltünk meg, és megmértük az ErbB1 (A) és az ErbB2 (B) homoklasztert formáló hányadát. A homoasszociátumot alkotó arány meghatározásához a 200 sec-nál mért intenzitásokat helyettesítettük az (52)-es egyenletbe (ennek magyarázatát l. 16. ábra). Így megállapítottuk, hogy az ErbB1 13%-a, míg az ErbB2 83%-a van homoklaszterekben. A mérések hibáját (SEM) az áttekinthetőség kedvéért csak minden ötödik mérési pontnál tüntettem fel. (C) A heteroklasztert formáló ErbB1 és ErbB2 receptorok száma stimulálatlan és EGF kezelt SKBR-3 sejteken. Az ábra mind a szabad (nem heteroklaszterben levő), mind a heteroasszociált molekulák számát feltünteti. Az így kapott számok alapján megbecsültük az ErbB1-2 heteroklaszterek átlagos összetételét kezeletlen és EGF stimulált sejteken.

4.6. Az ErbB1 és ErbB2 heteroklaszterizációjának kvantitatív jellemzése FSAB módszerrel

Az előző fejezetben bemutatott FSAB módszer segítségével kvantitatív analízisnek vetettük alá az ErbB1 és ErbB2 heteroklaszterizációját nyugalomban levő és EGF stimulált

SKBR-3 sejteken. Olyan sejtekben, amelyeket egy éjszakán át alacsony szérumszintű médiumban tenyésztettünk, az ErbB1 ~40%-a alkotott heteroasszociátumot az ErbB2-vel, míg az ErbB2 ~10% volt heteroklaszterben az ErbB1 molekulákkal (17C, 18. ábra). Ahhoz, hogy a fenti arányszámokat receptor számokká tudjuk konvertálni, szükség volt arra, hogy az analizált sejteken meghatározzuk az ErbB1 és ErbB2 proteinek számát. Ehhez két mintát készítettünk SKBR-3 sejtekből, az egyiket donorral konjugált trastuzumabbal, a másikat akceptor konjugált trastuzumabbal jelöltük meg. Meghatároztuk a háttérkorrigált fluoreszcencia intenzitás átlagát mindkét mintában ~100 sejt lemérése alapján (ezeket $\langle I_d \rangle$ -vel, ill. $\langle I_a \rangle$ -val jelöljük). Mivel a két mintában ugyanannyi sejtfelszíni fehérje lett megjelölve, ezért a két átlagos intenzitás jelölési aránnyal korrigált hányadosa megadja a donor és akceptor csatornában azonos számú fluorofór által generált fluoreszcencia intenzitások hányadosát ($R_{a/d}$):

$$R_{a/d} = \frac{\langle I_a \rangle L_d}{\langle I_d \rangle L_a} \quad (54)$$

ahol L_d és L_a rendre a donor és akceptor jelölt antitestek jelölési aránya. Az FSAB kísérletek során a donor kép intenzitását megszoroztuk az $R_{a/d}$ fakttorral, hogy biztosítsuk, hogy a donor és akceptor képek azonos skálán legyenek. A fluoreszcencia intenzitások receptor számokká történő konvertálásához meghatároztuk az SKBR-3 sejtek átlagos ErbB1 és ErbB2 expressziós szintjét áramlási citometriás mérésekkel Qifikit segítségével. Ezt követően az egyedi sejtek fluoreszcencia intenzitását a következő képlet segítségével konvertáltuk receptor számmá:

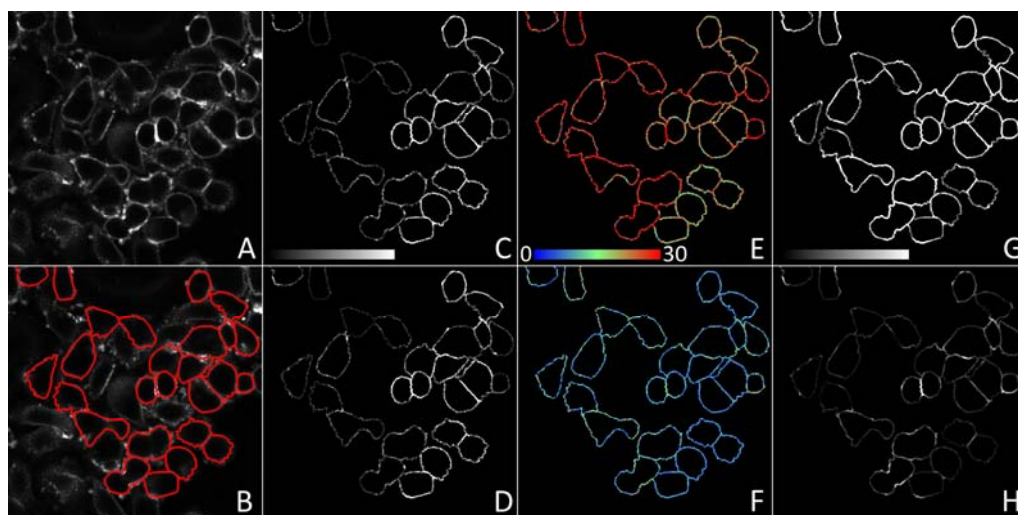
$$N_i = \frac{I_i}{\langle I \rangle} N_{Qifi} \quad (55)$$

ahol N_i és N_{Qifi} rendre a receptor szám az adott sejten és az átlagos receptor szám a populációban, I_i és $\langle I \rangle$ pedig rendre az adott sejt fluoreszcencia intenzitása és a sejtpopuláció átlagos fluoreszcencia intenzitása. Ezen kalibráció felhasználásával kaptuk a 18C. ábrán látható szabad és kötött receptor számokat.

A nyugalomban levő sejtek után megvizsgáltuk az ErbB1-2 heteroasszociációt EGF stimulált sejteken. Az ErbB2 heteroklaszterizációt mutató hányada ~2-szeresére növekedett EGF kezelés hatására, míg az ErbB1 heteroasszociáló hányada nem változott jelentősen. Értelmezésünk szerint ennek oka az, hogy az EGF nemcsak ErbB1 homoasszociációt, hanem ErbB1-2 heteroasszociációt is kivált, ezért az ErbB1 heteroasszociáló hányada nem változik. A 4.4 fejezetben ismertetett homo-FRET mérések szerint az ErbB2 nyugalomban tekintélyes

méretű homoasszociátumokat alkot, melyekből EGF stimuláció hatására a növekedési faktor által aktivált ErbB1 proteinek ErbB2-t szakítanak ki. Ezt a feltételezést az FSAB mérések alátámasztották, hiszen az ErbB2 ErbB1-gyel heteroklasztert formáló hányada tényleg szignifikánsan nőtt EGF stimulációt követően.

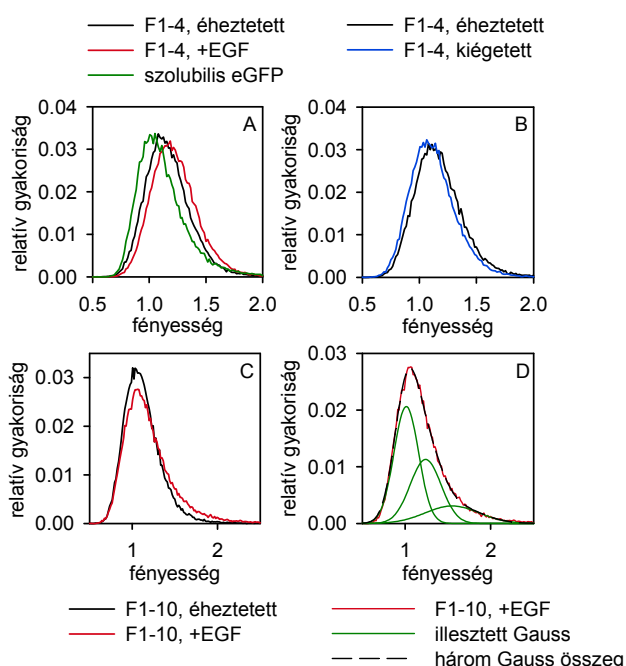
Az FSAB módszerrel lehetőségünk nyílt arra is, hogy a heteroklaszterek átlagos relatív összetételét, sztöchiometriáját megbecsüljük. Eszerint stimulálatlan sejtekben az ErbB1-2 heteroklaszterekben az ErbB1 és ErbB2 arány $\sim 1:1,5$, és ez $\sim 1:4$ -re nő EGF stimuláció hatására. Összegzésképpen megállapíthatjuk, hogy az FSAB módszer betekintést nyújt a receptorok által alkotott heteroklaszterek összetételébe, és az általa szolgáltatott eredmények a homo-FRET kísérletekkel összhangban vannak. (A tárgyalt cikk a referencialistában: (43).)



18. ábra. Reprezentatív képek az ErbB2 heteroasszociáció hányadának meghatározására. SKBR-3 sejteket AlexaFluor546-Mab528 és Cy5-trastuzumab antitestek keverékével jelöltünk meg. Az akceptor csatornában (A) felvett képen manuálisan indított vízfeltöltéses algoritmust használtunk a sejtmembrán azonosítására. A membránt a piros maszk jelöli a B képen. (C-D) A ki nem oltott donor intenzitás a kiégetés előtt (C) és 200 sec-os kiégető impulzus után (D). (E-F) A FRET hatásfok a kiégetés előtt (E) és után (F). A FRET hatásfok színskódolt 0-30%-ig. (G-H) A közvetlen gerjesztett akceptor intenzitás a kiégetés előtt (G) és után (H). A fluoreszcens képek (C, D, G és H) kontraszt fokozáson („contrast stretch”) mentek át, ezért a donor (C, D) és akceptor (G, H) képek intenzitásai nem összehasonlíthatóak, de a két donor és a két akceptor kép külön-külön azonos skálán vannak.

4.7. Az ErbB1 homoasszociációjának vizsgálata konfokális mikroszkópos N&B módszerrel

Az 1.6. fejezetben leírtak szerint a különböző, a fehérje asszociációkat karakterizáló technikák a klaszterizáció különböző dimenzióit jellemzik. Ezért bár a 4.4. fejezetben részletesen leírtuk az ErbB1 és ErbB2 receptorok homoasszociációit, egy másik módszerrel, a N&B analízissel is szeretnénk volna megerősíteni, ill. más távolságtartományban jellemezni ezeket a klasztereket. Másrészt többféle sejtvonal segítségével arra is választ szeretnénk volna kapni, hogy a fehérjék expressziós szintje hogyan befolyásolja az asszociátumokat.



19. ábra. Az ErbB1 homoklaszterizációja nyugalmi és stimulált sejteken.

(A) Szérum éheztetett, EGF stimulált F1-4 sejtek és szolubilis monomer eGFP fényesség („brightness”) hisztogramjai. A sejteket három percig stimuláltuk 100 nM EGF-fel. (B) Szérum éheztetett és kiégetett F1-4 sejtek fényesség hisztogramja. A sejtek kiégetését addig végeztük, míg a maradék intenzitás a kezdetinek ~20%-a lett. (C) Szérum éheztetett és 3 percig EGF stimulált F1-10 sejtek fényesség hisztogramjai. (D) Az EGF stimulált F1-10 sejtek fényesség hisztogramját három Gauss görbével illesztettük, melyek összege tökéletesen illeszkedik a mért adatokra.

Az ErbB1 fehérje homoklasztereinek vizsgálatához ErbB1-eGFP-vel stabilan transzfektált sejtvonalakat használtunk. Először a magas expresszióval rendelkező daganatsejtek modelljének tekinthető F1-4 sejtet vizsgáltuk, amely $\sim 6 \cdot 10^5$ ErbB1 fehérjét fejez ki. A stimulálatlan, szérum éheztetett sejtek molekuláris fényesség értéke magasabb volt, mint a szolubilis, monomer eGFP-jé, ami arra utal, hogy az ErbB1 fehérje már

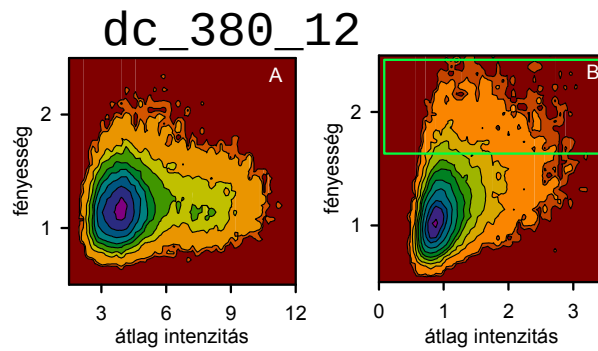
stimulálatlan állapotban sem tisztán monomerként van jelen F1-4 sejtek membránjában (5. táblázat, 19. ábra). Bár a molekuláris fényességből nem lehet arra következtetni, hogy egy pixelben monomerek és dimerek vagy monomerek és magasabb rendű asszociátumok keveréke van-e jelen, de a molekuláris fényesség monomer és dimer eGFP közötti értéke arra utal, hogy jelentős mennyiségben biztos nincsenek jelen nagyméretű ErbB1-eGFP klaszterek stimulálatlan F1-4 sejteken. Hogy a preformált asszociátumok létezésére egy másik bizonyítékkal is szolgáljunk, a szérum éheztetett F1-4 sejteken az ErbB1-eGFP fluoreszcenciájának ~80%-át kiégettük, aminek hatására az ErbB1-eGFP molekuláris fényessége a szolubilis, monomer eGFP-re jellemző értékre csökkent (5. táblázat, 19. ábra). Ez arra utal, hogy az F1-4 sejtek felszínén valóban preformált ErbB1-eGFP klaszterek voltak jelen. Ezt követően megvizsgáltuk az EGF stimuláció hatását. 100 nM EGF a molekuláris fényességet a dimerekre jellemző értékre emelte, miszerint a növekedési faktor a teljes receptor populáció dimerizációját kiváltotta (5. táblázat, 19. ábra).

		szolubilis eGFP	F1-4			F1-10		HeLa-ErbB1-eGFP	
			éheztetett	kiégetett	+EGF	éheztetett	+EGF	éheztetett	+EGF
mol. fényesség $\left(\frac{db. mol.}{klaszter}\right)$	0,118± 0,002	0,158±0,002 (1,3)	0,128±0,002 (1,0)	0,217±0,003 (1,8)	0,122±0,001 (1,0)	0,175±0,003 (1,5)	0,126±0,005 (1,0)	0,172± 0,005 (1,5)	
		szolubilis mYFP	A4erbB2				CHO-erbB2mYFP		
			éheztetett	+EGF	pertuzumab	pertuzumab+EGF	éheztetett	pertuzumab	
mol. fényesség $\left(\frac{db. mol.}{klaszter}\right)$	0,063±	m.k.	0,4±0,02 (6,3)	0,283±0,025 (4,5)	0,35± 0,023 (5,6)	0,33± 0,017 (5,2)	0,91± 0,056 (14,4)	0,75± 0,053 (11,9)	
	0,01	m.k-en kívül	0,182±0,008 (2,9)	0,164± 0,019 (2,6)	0,16± 0,021 (2,5)	0,14± 0,023 (2,2)	0,28± 0,037 (4,4)	0,28± 0,03 (4,4)	

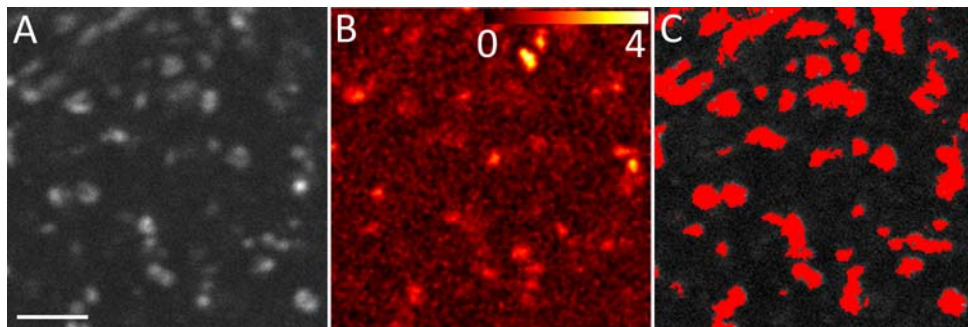
5. táblázat. Az ErbB1-eGFP és ErbB2-mYFP molekuláris fényesség értékei.

A szérum éheztetett és EGF stimulált sejtek, valamint a monomer eGFP és mYFP molekuláris fényesség értékeit ($\pm$ SEM) mutatja a táblázat a különböző sejtvonalakban. A molekuláris fényesség után zárójelben a homoklaszterben található molekulák átlagos számát (db. mol./klaszter) is megadtuk. Az ErbB2 esetében külön került megadásra a makroklaszterben (m.k.) és a makroklasztereken kívül levő (m.k-en kívül) ErbB2-mYFP molekuláris fényessége. A feltüntetett értékek 5-10 mérés átlagolásából lettek számolva.

Ezt követően két alacsonyabb ErbB1-eGFP expresszióval rendelkező sejten (F1-10, HeLa-ErbB1eGFP) végeztük el a N&B analízist, amelyek jobban modellezik a fiziológias ErbB1 expressziós szinteket. Az F1-10 és HeLa-ErbB1eGFP sejtek $\sim 5 \cdot 10^4$ és $\sim 2 \cdot 10^5$ ErbB1-eGFP-t fejeznek ki. Mindkét sejtvonal szérum éheztetése után az ErbB1-eGFP molekuláris fényessége arra utalt, hogy a fehérje tisztán monomerként volt jelen. EGF stimuláció hatására az ErbB1 receptor asszociáció mértéke szignifikánsan növekedett (5. táblázat, 19-21. ábra). Stimulált F1-10 sejteken a molekuláris fényesség eloszlása aszimmetrikus volt, ami arra utalt, hogy egyes pixeleken nagyobb molekuláris fényességgel rendelkező klaszterek is



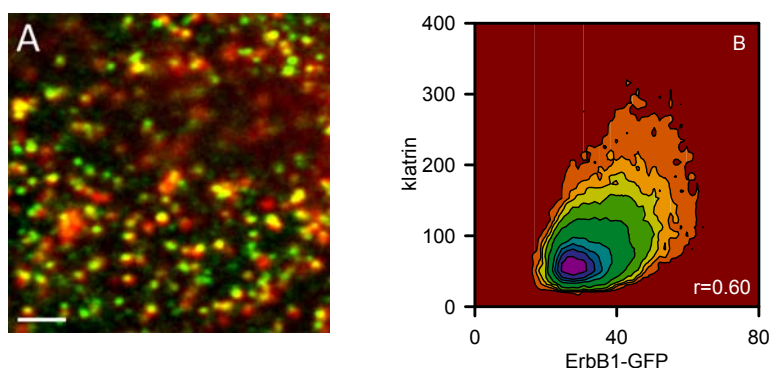
20. ábra. Nyugalmi és stimulált F1-10 sejtek fényesség-intenzitás hisztogramjai. Szérum éheztetett (A) és 100 nM EGF-fel három percig stimulált (B) F1-10 sejtek fényesség-intenzitás kontúrábráit konfokális mikroszkópos N&B mérések alapján készítettük el. A jobb oldali ábrán a zöld téglalap által bekerített területnek megfelelő pixelek pirossal jelöltek a 21. ábra C részén.



21. ábra. Az ErbB1 molekula átlagos fluoreszcencia intenzitás és fényesség képei EGF stimulált F1-10 sejteken.

F1-10 sejteket EGF-fel stimuláltunk, és N&B analízist hajtottunk végre rajtuk. Kiszámítottuk a rögzített konfokális mikroszkópos szeletek átlagát (A) és a pixelenkénti fényesség értékeket (B). A 20. ábra B részén zöld téglalappal bekeretezett, magas fényességű területnek megfelelő pixelek az ábra C részén pirossal vannak jelölve. (Fehér vonal: 2 μ m)

jelen voltak. Az EGF stimulált F1-10 sejtek fényesség eloszlását három Gauss görbével tudtuk illeszteni, és a legjobboldalibb csúcs átlagos fényessége pentamerek jelenlétére utalt (19. ábra). Ezek a magasabb molekuláris fényességgel rendelkező klaszterek olyan pixelekben voltak, amelyeknek a fluoreszcencia intenzitása is magasabb volt, mint a környező területeké, hiszen a magas molekuláris fényességű pixelekre kapuzva a magas intenzitású membránterületeket jelöltük ki (20-21. ábra). A kolokalizációs analízis azt mutatta, hogy ezen magas intenzitású és molekuláris fényességű pixelek eloszlása a klatrinéval korrelál (22. ábra). (A tárgyalt cikk a referencialistában: (36).)



22. ábra. Az ErbB1 nagy fényességű klaszterei kolokalizálnak a klatrinnal EGF stimulált F1-10 sejteken.

F1-10 sejteket 100 nm EGF-fel stimuláltunk 3 percig, majd fixálást és permeabilitálást követően immunfluoreszcensen festettük a klatrin könnyű láncát (vörös csatorna). Az ErbB1-eGFP intenzitása zöld színnel jelenik meg. A két fehérje közötti korreláció a *B* részen látható kontúrábra alapján $r=0,6$. Vonás=2 μm.

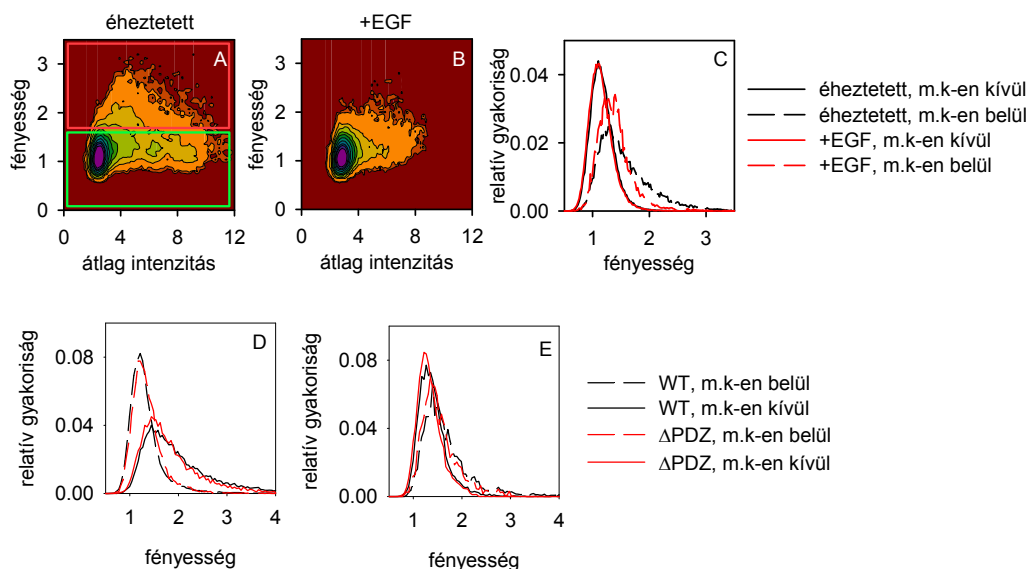
4.8. Az ErbB2 homoasszociációjának vizsgálata konfokális mikroszkópos N&B módszerrel

Az ErbB2 homoklaszterének vizsgálatát szérumban éheztetett A4erbB2 sejtekkel kezdtük, melyek $\sim 1,2 \cdot 10^6$ ErbB1-et és $\sim 9,5 \cdot 10^5$ ErbB2-mYFP-t fejeznek ki. Stimulálatlan A4erbB2 sejtek membránjában az ErbB2 mind mikroszkópos, mind molekuláris szinten jelentős klaszterizációs hajlamot mutatott (23-24. ábra). A mikroszkópos képen látható klasztereket, a molekuláris asszociátumoktól elkülönítendő, makroklasztereknek neveztük el. Az ErbB2-mYFP molekuláris fényessége szignifikánsan magasabb volt a makroklaszterekben, mint azokon kívül, de mindkét helyen arra utaltak a mérések, hogy az ErbB2 nem monomerként található (5. táblázat). Az ErbB1 homoasszociációs hajlamához képest az ErbB2 nagyobb méretű asszociátumokat mutatott. Ez a tendencia összhangban van a homo-FRET mérésekkel kapott eredményekkel. Egy olyan sejtvonalon (CHO-erbB2mYFP), amely csak ErbB2-mYFP-t fejez ki ($\sim 3 \cdot 10^5$ molekula/sejt), az ErbB2 szintén jelentős homoklaszterizációs hajlamot mutatott, amely még az A4erbB2 sejteken tapasztaltnál is nagyobb volt (5. táblázat). Ennek valószínű magyarázata az, hogy az ErbB2 mellett más ErbB fehérjét is kifejező sejtekben a többi ErbB fehérje heteroasszociáció révén csökkenti az ErbB2 homoklaszterizációját.

	szolubilis mYFP		WT ErbB2- short-mYFP	Δ VPV-ErbB2- short-mYFP
mol. fényesség $\left(\frac{\text{db. mol.}}{\text{klaszter}}\right)$	0,063 $\pm$ 0,01	makroklaszteren belül	0,89 $\pm$ 0,06 (14,1)	0,96 $\pm$ 0,08 (15,2)
		makroklaszteren kívül	0,38 $\pm$ 0,05 (6,0)	0,40 $\pm$ 0,04 (6,4)
	szolubilis Atto565		WT ACP-ErbB2	Δ VPV-ACP-ErbB2
mol. fényesség $\left(\frac{\text{db. mol.}}{\text{klaszter}}\right)$	0,19 $\pm$ 0,01	makroklaszteren belül	0,81 $\pm$ 0,04 (7,8)	0,76 $\pm$ 0,04 (7,3)
		makroklaszteren kívül	0,44 $\pm$ 0,03 (4,2)	0,39 $\pm$ 0,04 (3,7)

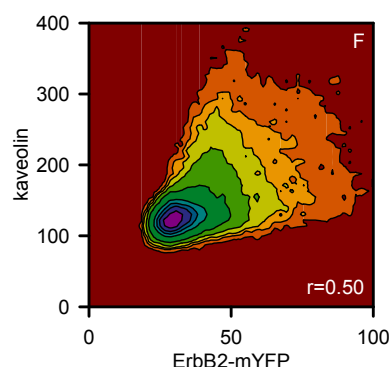
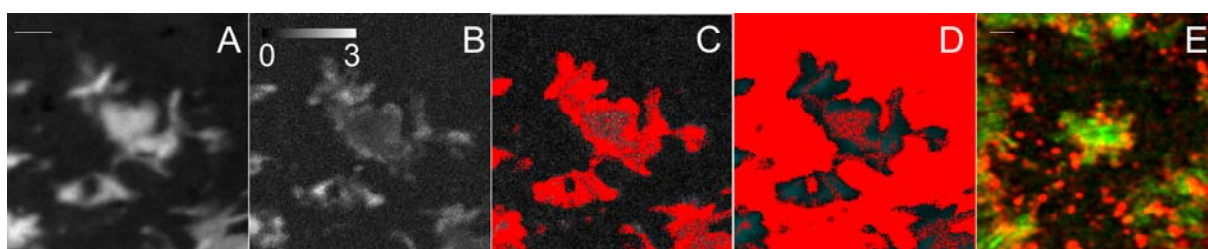
6. táblázat. Az ErbB2 fúziós konstrukciók molekuláris fényessége tranziensen transzfektált HeLa sejteken.

A tranziensen transzfektált sejteket két nappal a transzekció után vizsgáltuk. Az ACP jel esetében Sfp transzferáz segítségével Atto565-tel jelöltük a fehérjét. A feltüntetett molekuláris fényesség az átlag $\pm$ SEM értékeket jelenti min. 3 mérésből számítva. A zárójelekben az egy klaszterben levő ErbB2 átlagos számát tüntettük fel. Mivel a HeLa sejt ~ 30000 „sötét”, endogén ErbB2-t fejez ki, ezért az egy klaszterben levő molekulák átlagos számát erre korrigálni kellett. A transzfektált ErbB2 sejtenkénti száma $\sim 5 \cdot 10^5$ és $3 \cdot 10^5$ volt az mYFP- és az ACP-jelölt fehérjék esetében, ezért az egy klaszterben levő molekulák számát 0,94-dal, ill. 0,9-del osztottuk a két esetben. Az ACP-fúziós fehérjék esetében még egy korrekciós lépésre volt szükség, ami figyelembe veszi az ACP-jel nem teljes jelölését Sfp transzferázzal. Mivel méréseink szerint az általunk alkalmazott jelölési körülmények mellett az ACP jel $\sim 60\%$ -a jelölődik, ezért az egy klaszterben levő molekulák számát 0,6-dal el kellett osztani.



23. ábra. ErbB2 homoklaszterek N&B analízise.

(A-B) Szérum éheztetett és EGF stimulált A4erbB2 sejtek fényesség-átlag intenzitás kontúrabrója. Az A részen pirossal és zölddel jelölt téglalapokkal rendre a magas és alacsony fényességű pixeleket szelektáltuk, amelyek a 24. ábra C és D részén vannak pirossal feltüntetve. (C) Az A és B ábrák alapján készített fényesség hisztogramok a makroklaszteren (m.k.) belüli és kívüli pixelekre. (D-E) Tranziensen transzfektált Hela sejteken vizsgáltuk az ErbB2-short-mYFP (D) és az ACP-ErbB2 (E) fehérjék vad típusú (WT) és PDZ kötő domén deletált (Δ PDZ) változatának molekuláris fényességét makroklasztereken kívül és belül.



24. ábra. ErbB2 molekuláris fényessége eltérő a makroklasztereken kívül és belül.

Szérum éheztetett A4erbB2 sejtek N&B analízise során 100 konfokális képet vettünk fel, aminek kiszámoltuk az átlagát (A) és az ErbB2-mYFP fényességét (B). A magas (C) és alacsony (D) fényességet mutató pixeleket pirossal jelöltük a 23A. ábrán jelölt kapuknak megfelelően, és ezt a maszkot rárétegeztük az átlagos intenzitást mutató képre. (E) Fixált A4erbB2 sejteket permeabilizáltunk és a kaveolint immunfluoreszcensen jelöltük (vörös csatorna). Az ErbB2-mYFP fluoreszcenciája a zöld csatornában látható. (F) A kaveolin és az ErbB2-mYFP kolokalizációs analízise.

Számtalan oka lehet az ErbB2-mYFP ErbB1-hez képest magasabb homoasszociációs hajlamának: (i) a C-terminális PDZ kötő régió (218); (ii) az mYFP-t az ErbB2-höz kapcsoló linker szekvencia (185); (iii) az mYFP csoport (219). Ezen lehetőségek megvizsgálásához négy új fúziós fehérjét állítottunk elő: ErbB2-short-mYFP, amelyben az mYFP és az ErbB2 között egy dipeptid linker van; ACP-ErbB2, amelyben az ErbB2-höz egy Sfp transzferázzal jelölhető ACP szekvenciát kötöttünk C-terminálisan (tehát extracellulárisan); valamint mindkét szekvencia VPV deléciós mutánsát. A VPV szekvencia felelős a PDZ doménnel rendelkező fehérjék megkötéséért. Az ErbB2-short-mYFP konstrukciót HeLa sejtekbe transzfektálva ugyanolyan klaszterméreteket kaptunk, mint A4erbB2 sejteken a N&B analízissel meghatározott molekuláris fényesség és a makroklaszterek jelenléte szempontjából is (23. ábra, 6. táblázat). Az Atto565-tel kovalensen jelölt ACP-ErbB2 ugyanolyan klasztereket alkotott, mint az ErbB2-mYFP (23. ábra, 6. táblázat). Végül összehasonlítottuk a Δ VPV mutánsok és a vad típusú ErbB2-short-mYFP és ACP-ErbB2 fehérjék klaszterizációs hajlamát, ami szintén nem mutatott semmilyen különbséget (23. ábra, 6. táblázat). Ezért arra következtettünk, hogy az ErbB2 jelentős homoklaszterizációs hajlama a molekula saját tulajdonsága, amelyben a PDZ kötő domén nem játszik szerepet.

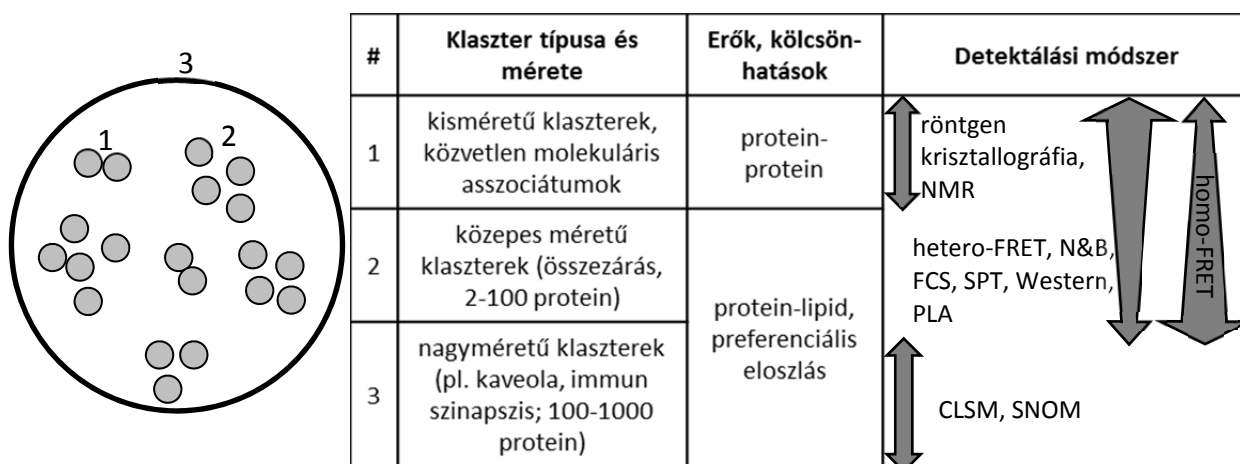
Ezt követően megvizsgáltuk, hogy az ErbB2 makroklaszterek milyen más membránfehérjével mutatnak kolokalizációt. Az egyetlen pozitív eredményt szolgáltató

fehérje a caveolin volt, amellyel az ErbB2 közepesen erős korrelációt mutatott ($r=0.5$, 24. ábra). Bár kvantitatív analízissel nem igazoltuk, feltűnő volt, hogy a caveolin rendszerint az ErbB2 makroklaszterek perifériáján helyezkedett el (24. ábra).

Végezetül megvizsgáltuk, hogy A4erbB2 sejtek EGF stimulációja milyen hatást gyakorol az ErbB2 homoklaszterekre. Eredményeink szerint az EGF hatására a makroklaszterekben szignifikánsan csökkent az ErbB2 homoklaszterek mérete, míg a makroklasztereken kívül nem volt jelentős változás (23. ábra, 5. táblázat). Pertuzumabbal előkezelt sejtekben az EGF indukálta változások nem jelentek meg, ami arra utal, hogy az EGF által aktivált ErbB1 heterodimerizációja hatására csökken az ErbB2 homoklaszterek mérete (5. táblázat). Ezek az eredmények is összhangban vannak az ErbB2 homo-FRET-tel történt karakterizálásával. (A tárgyalt cikk a referencialistában: (36).)

4.9. Az ErbB fehérjék hierarchikus klaszterizációjának modellje

A 4.3-4.8. fejezetekben az ErbB1 és ErbB2 klaszterizációjának számtalan aspektusa került tárgyalásra, amely alapján megalkotható egy modell, amely a receptorok asszociációját több hierarchikus szinten tárgyalja. A modell legfontosabb üzeneteit a 25. ábra foglalja össze.



25. ábra. A membránfehérjék és a vizsgáló eljárások hierarchikus modellje.

A klaszterizáció egyes szintjeinek bővebb magyarázatát lásd a szövegben (összezárás=„co-confinement”). A detektálási módszerek oszlopban a nyilak vastagsága az egyes módszerek adott klaszterizációs szint kimutatására való érzékenységevel arányos.

A fehérje klaszterizáció legalacsonyabb szintjét a közvetlen molekuláris interakciók jelentik. Ezeket az asszociátumokat közvetlen fehérje-fehérje kölcsönhatások tartják össze, és legfontosabb és legelterjedtebb képviselőjük a dimer, de minden valószínűség szerint nagyobb méretű oligomerek is léteznek még ezen osztályon belül. Az átmenet a közepes méretű klaszterek felé folytonos. A klaszterek ezen osztályát már inkább lipid mediálta interakciók, de kisebb részben még a közvetlen protein-protein kölcsönhatások is fenntartják. A lipid mediálta interakciókban a proteinek elsősorban a transzmembrán doménjeikkel vesznek részt. Ezenkívül ide kell még sorolni az összezárt („co-confined”) proteineket is (28, 29). Az ErbB2 molekula homo-FRET és N&B módszerrel kimutatott „nagy méretű” klaszterei ebbe az asszociációs szintbe tartoznak. Az ErbB1 EGF által kialakított dimerei a kisméretű klaszterek közé tartoznak, de preformált asszociátumairól ez nem mondható el bizonyossággal. Elképzelhető, hogy a preformált ErbB1 klaszterek ugyanolyan szerkezetűek, mint az EGF által aktiváltak, azaz az extracelluláris rész és az intracelluláris kináz domének által alkotott dimerek. Ilyen asszociátumok ligandtól független létrejöttére vannak kísérletes bizonyítékok (30). Ugyanakkor elképzelhető, hogy az ErbB1 preformált asszociátumait más jellegű, pl. lipid mediált interakciók tartják össze.

A fehérje asszociátumok kísérletes jellemzésére használt módszerek eltérő érzékenységet mutatnak az egyes klaszterizációs szintekre. Ez az 1.6. fejezetben került részletesebb bemutatásra. Itt konkrétan csak arra szeretnék magyarázatot adni, hogy a homo-FRET és N&B mérések miért mutattak eltérő nagyságú klasztereket. Bár a FRET jelensége molekuláris távolságskálán jön létre, mégsem szükséges tényleges fehérje-fehérje kölcsönhatás ahhoz, hogy két protein között FRET legyen detektálható. Az 1.6. fejezetben elmondottak alapján a homo-FRET kimondottan érzékeny a nagyobb méretű asszociátumokra, ezért lehet az, hogy a homo-FRET nagyobb klasztereket mutatott ki mind ErbB1, mind ErbB2 jelenlétében, mint a N&B analízis. Meg kell azonban jegyezni, hogy a N&B analízissel detektált asszociátumok sem feltétlenül molekulárisak (protein-protein interakció által összetartottak), hiszen ha két fehérjét valamilyen indirekt (lipid vagy citoszkeleton mediált) erő egy pixelnél kisebb egységbe fog, akkor mindenfajta korrelációs alapú eljárással asszociáció lesz kimutatható.

A klaszterizáció harmadik hierarchikus szintjét a nagyméretű asszociátumok alkotják. Ezek már fénymikroszkóppal is látható objektumok, és több száz vagy ezer fehérjét tartalmaznak. Érdeemes megjegyezni, hogy Kusumi és mtsai a plazmamembrán

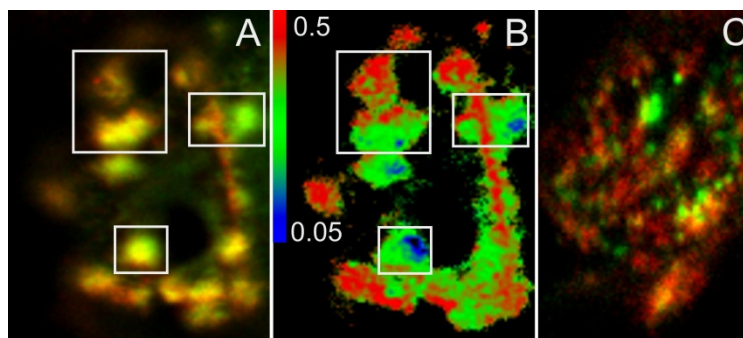
doménszerkezetének elemzése kapcsán az itt javasolt háromszintű klaszterizációs modellhez sokban hasonló elképzelést vázoltak fel (133).

Az egyes asszociációs szintek biológiai szerepei eltérőek. A kisméretű klaszterek (főleg dimerek) egyértelműen a receptor aktiváció alapegységeinek tekinthetők, míg a közepes és nagyméretű klaszterek inkább platformot teremtenek a kisméretű asszociátumok létrejöttének finomhangolására. Így pl. a közepes és nagyméretű klasztereken belül megnő a receptorok denzitása, ami elősegíti a dimerizációt és az aktivációt. Az ErbB2 esetében ennek inkább ellenkezője igaz, hiszen a közepes méretű ErbB2 klaszterek inaktív ErbB2 molekulák rezervoárjaként működnek. *(A tárgyalt cikkek a referencialistában: (35, 36, 43, 140).)*

4.10. A lipid tutajok és az ErbB3 hatása az ErbB2 homoklaszterizációjára

Az előzőekben ismertetett eredmények feltárták, hogy az ErbB fehérjék milyen klasztereket alakítanak ki. A 4.9. fejezetben körvonalazott modell alapján ezek létrejöttében nemcsak közvetlen fehérje-fehérje kölcsönhatások, hanem a lipid környezet hatásai is szerepet játszanak. Ezért megvizsgáltuk, hogy az ErbB2 esetében ebben van-e szerepe a lipid tutajoknak. Először azt kellett eldönteni, hogy az ErbB2 receptor a lipid tutajokban helyezkedik-e el. Erre a kolera toxin B alegységének (CTX-B) fluoreszcensen jelölt változatával történő vizsgálatokat választottuk (143). Eredményeink szerint az ErbB2 és a lipid tutajokat jelölő CTX-B fluoreszcenciája korrelált egymással (26A. ábra). A kolokalizáció kvantitatív jellemzésére a Manders koefficienszt használtuk (159). Eszerint az ErbB2 és a lipid tutajok közötti átfedés $77 \pm 6\%$ -os volt akár az ErbB2-t, akár a CTX-B jelet használtuk referenciának. A képek egyértelműen bizonyították azt is, hogy az ErbB2 mikroszkóppal is látható klasztereket alkot, amelyek megfelelnek a N&B mérések során látott makroklasztereknek, és a korábban pásztázó közeli mező optikai mikroszkóppal („scanning near-field optical microscopy”, SNOM) látott asszociátumoknak (37). Mivel ezekben a makroklaszterekben az ErbB2 lokális denzitása sokkal nagyobb, mint a plazmamembrán többi részén, a tömeghatás törvénye alapján az várható, hogy a makroklaszterekben az ErbB2 homoasszociációjának mértéke nagyobb lesz. Ezt a feltételezést FRET mérések segítségével kívántuk ellenőrizni, de a makroklasztereken kívül a fluoreszcencia intenzitás túl alacsony volt megbízható mérések elvégzéséhez. A jel/zaj arány növelésére két módszert használtunk. Egyrészt az egymás mellett levő pixeleket szummáltuk („pixel pooling”), másrészt nagyméretű ROI-kat jelöltünk ki a makroklasztereken kívül és belül. Mindkét módszer arra utalt, hogy a klaszterekben

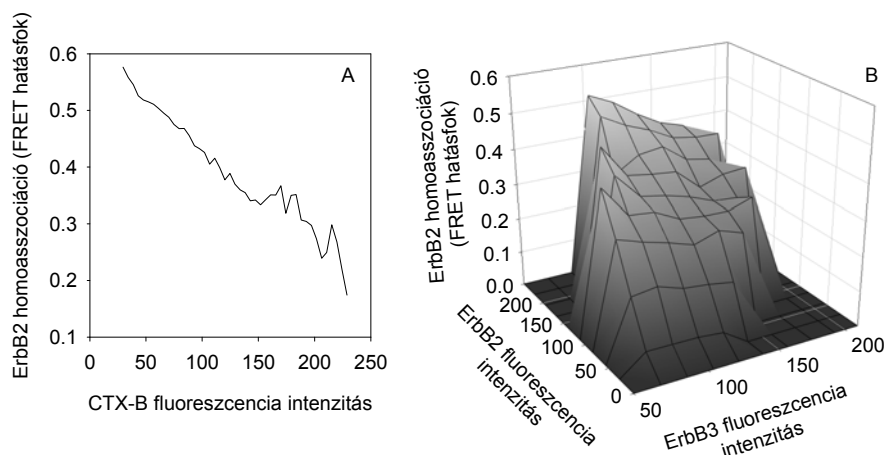
található magas ErbB2 denzitás ellenére nem nagyobb az ErbB2 homoasszociáció mértéke a klasztereken belül, mint kívül (pixel pooling: FRET a klasztereken kívül: $18\pm3\%$, klasztereken kívül: $16\pm3\%$; ROI-k segítségével: FRET a klasztereken kívül: $16\pm2\%$, klasztereken belül: $17\pm2\%$).



26. ábra. Az ErbB2 eloszlásának és asszociációjának viszonya a lipid tutajokhoz.

(A) SKBR-3 emlőtumor sejteken a lipid tutajokat FITC-CTX-B-vel jelöltük (zöld csatorna). Az ErbB2-t Cy3-4D5 Fab és Cy5-4D5 Fab keverékével jelöltük FRET mérésekhez. A Cy3 fluoreszcenciája a vörös csatornában látható. A jelölést jégen hajtottuk végre, hogy a CTX-B által indukált oligomerizációt és internalizációt meggátoljuk. A sárga szín megjelenése kolokalizációra utal. (B) Akceptor fotoelhalványítás segítségével az A részen feltüntetett sejten meghatároztuk a FRET hatásfokot, amelyet kéktől vörösig tartó színskálán tizedes törtben tüntettünk fel. A fehér téglalappal jelölt területeken jól látható az inverz korreláció a zöld intenzitás (CTX-B által jelölt GM1 gangliozid) és az ErbB2 homoasszociációja között. (C) SKBR-3 sejteket 37°C -on jelöltünk FITC-CTX-B-vel, majd jégen Cy3-4D5 Fab-vel. A kép színskálája ugyanaz, mint az A részen.

Ezen eredmények arra utaltak, hogy a makroklaszterekben valamilyen tényező gátolja az ErbB2 homoasszociációját. Ezért akceptor fotoelhalványításon alapuló FRET segítségével megmértük a pixelenkénti FRET-et ErbB2 homoasszociációra. Tapasztalatunk szerint az ErbB2 homoasszociáció mértéke negatív korrelációt mutat a tutajokban található, a CTX-B által megjelölt GM1 gangliozidok denzitásával (26B, 27A. ábra). Ez összhangban van a gangliozidok ErbB1 asszociációra gyakorolt negatív hatásával (145) és a homo-FRET mérések alapján felállított azon hipotézisünkkel, hogy a közepes vagy nagyméretű ErbB2 homoklaszterekben az ErbB2 inaktív formában van jelen. Az ErbB2 magas denzitása konstitutív asszociációra és aktivációra vezet (18). A lipid tutajok lipid környezetének ErbB2 homoasszociációt gátló hatása ezt a konstitutív aktivációt gátolja vagy csökkenti, biztosítva azt, hogy az ErbB2 homoklaszterekben levő molekulák ne aktiválódjanak. Az aktiváció majd csak akkor következhet be, amikor ligandum által aktivált más ErbB fehérjék kiszakítják az ErbB2-t a homoklaszterekből és heteroasszociátumokat képeznek egymással.

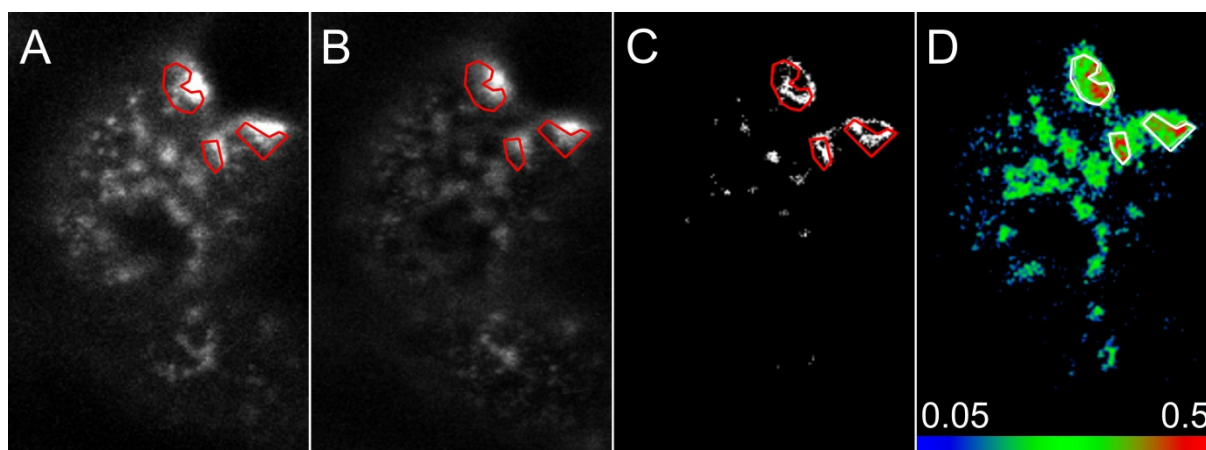


27. ábra. Az ErbB2 homoasszociáció negatívan korrelál a CTX-B és az ErbB3 lokális denzitásával.

(A) SKBR-3 sejteket megjelöltünk FITC-CTX-B-vel, ill. Cy3- és Cy5 konjugált 4D5 Fab keverékével jégen, majd megmértük az ErbB2 homoasszociációt akceptor fotoelhalványításos FRET segítségével, és ezt korreláltattuk a FITC-CTX-B intenzitásával. (B) SKBR-3 sejteken az ErbB2-t FITC- és Cy3 konjugált 4D5 Fab keverékével jelöltük, az ErbB3-at H3.90.6 monoklonális antitest másodlagos jelölésével Cy5 konjugált másodlagos Fab-vel. Az ErbB2 homoasszociációt donor fotoelhalványításos FRET segítségével mértük meg a makroklasztereken belül, és ezt korreláltattuk az ErbB2 és az ErbB3 lokális denzitásával.

A lipid környezet hatásán kívül minden olyan fehérje, amely az ErbB2-vel kölcsönhat, képes lehet arra, hogy kompetíció révén gátolja az ErbB2 homoasszociációját. Az SKBR-3 sejtek az ErbB2 legfontosabb heterodimerizációs partnerét, az ErbB3-at is kifejezik. Ezért megvizsgáltuk, hogy az ErbB3 lokális denzitása milyen hatást gyakorol az ErbB2 homoklaszterizációjára. Az ErbB2-t donorral konjugált és akceptorral konjugált antitestek keverékével jelöltük meg, míg egy harmadik fluoreszcens festékkel az ErbB3-t jelöltük, majd pixelenként megmértük a két fehérje expressziós szintjét és az ErbB2 homoasszociációját FRET mérésekkel. A kiértékelést csak a makroklasztereken belül végeztük el, és ez arra utalt, hogy az ErbB2 homoasszociációja arányos az ErbB2 lokális expressziós szintjével, és negatív korrelációt mutat az ErbB3 lokális denzitásával (27B, 28. ábra). Mindkét összefüggés a tömeghatás törvényének érvényesülését bizonyítja az ErbB2 homoasszociációja esetében.

A lipid tutajokban többféle különböző típusú fehérje fordul elő. Ezek közül az egyik a GPI-horgonyzott proteinek osztálya, melyekről kimutatták, hogy a pentamer CTX-B által okozott lipid tutaj keresztkötés hatására nagyobb méretű foltokba koncentrálnak a CTX-B-vel együtt (143). Megvizsgáltuk, hogy ez a stabil lipid tutaj asszociáció az ErbB2 esetében is felismerhető-e. SKBR-3 sejteket kezeltünk fluoreszcensen jelölt CTX-B-vel 37°C-on, majd



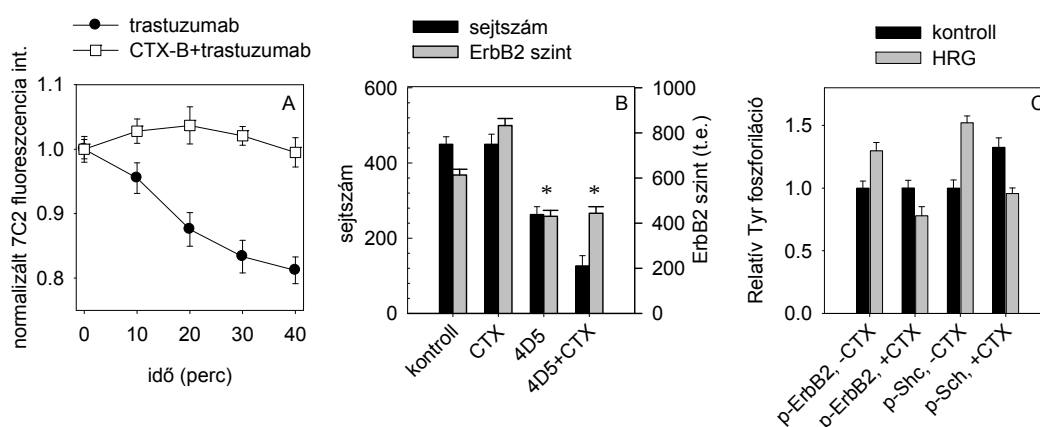
28. ábra. Az ErbB2 homoasszociációja az ErbB2 és ErbB3 expressziós szintjei függvényében. SKBR-3 emlőtumor sejteken az ErbB2-t FITC-4D5 és Cy3-4D5 antitestekkel, az ErbB3-at H3.90.6 antitesttel, majd Cy5-konjugált másodlagos Fab-vel jelöltük meg. Az A kép a Cy3-4D5 (ErbB2), míg a B kép a Cy5-konjugált másodlagos Fab (ErbB3) intenzitását mutatja. A C képen azok a pixelek vannak fehérrel jelölve, amelyek magas ErbB2 és alacsony (de a küszöbnél magasabb) ErbB3 intenzitást mutatnak. Donor fotoelhalványítás kinetikájának mérésén alapuló FRET-tel megmértük az ErbB2 homoasszociációját, ami a D képen látható. Az A-D képeken pirossal, ill. fehérrel bekerített területek a legmagasabb FRET értékeknek megfelelő területeket mutatják, amelyek egybeesnek a magas ErbB2, de relatíve alacsony ErbB3 expressziót mutató területekkel.

megjelöltük őket fluoreszcens anti-ErbB2 antitestekkel. A konfokális mikroszkópos felvételek tanúsága szerint a keresztkötött lipid tutajok hátrahagyták az ErbB2-t, hiszen a két fluoreszcens jel nem mutatott jelentős átfedést (26C. ábra). A kvantitatív analízis szerint a Manders koefficiens a CTX-B kezelés $28 \pm 5\%$ -re csökkentette. Eszerint a GPI-kötött fehérjékkel ellentétben az ErbB2, és elképzelhető, hogy más transzmembrán fehérjék is, kevésbé statikusan kötöttek a lipid tutajokhoz, hiszen a GM1 gangliozid CTX-B általi keresztkötése az ErbB2 lipid tutajbeli lokalizációját megszünteti. (A tárgyalt cikk a referencialistában: (42).)

4.11. Lipid tutajok CTX-B-vel való keresztkötésének hatása az ErbB2 biológiai funkcióira

Mivel a lipid tutajok CTX-B-vel történő keresztkötése jelentősen módosította az ErbB2 klaszterek eloszlását, ezért célszerűnek tűnt megvizsgálni, hogy ez milyen hatásokat váltott ki az ErbB2-höz köthető biológiai válaszokra. A várakozást, hogy valamilyen eltérést látni fogunk, arra alapoztuk, hogy a lipid környezet jelentősen befolyásolja a receptor tirozin kinázok működését (145). Először az ErbB2 trastuzumab által indukált internalizációját és leszabályozódását („down-regulation”) vizsgáltuk meg. Az eredmények szerint CTX-B

előkezelte sejteken mind az ErbB2 internalizációja (be nem mutatott eredmény), mind leszabályozódása jelentősen lelassult (29A. ábra). Mivel a trastuzumabot az ErbB2-t fokozottan expresszáló emlőtumorsejtek kezelésére használják, érdekesnek tűnt antiproliferatív hatását is megvizsgálni CTX-B jelenlétében. E kísérletek egyértelműen arra utaltak, hogy a trastuzumab antiproliferatív hatását a CTX-B előkezelés növelte (29B. ábra). Ugyanezen sejteken megvizsgáltuk az ErbB2 expressziós szintjét, és azt tapasztaltuk, hogy a trastuzumab önmagában jelentősen csökkentette az ErbB2 szintet a sejtekben, de a CTX-B-vel történő együttkezelés során ez tovább nem csökkent (29B. ábra). A fenti eredmények azért voltak rendkívül érdekesek, mert akkoriban a trastuzumab hatásmechanizmusának az ErbB2 leszabályozódását tekintették. Eredményeink szerint azonban a CTX-B kezelés következményei a trastuzumab antiproliferatív és ErbB2 leszabályozódást indukáló hatásaira nem korreláltak egymással, hiszen a CTX-B a trastuzumab rövidtávú, ErbB2 leszabályozódást kiváltó hatását csökkentette, antiproliferatív hatását viszont növelte. Ezért levontuk azt a következtetést, hogy a trastuzumab antiproliferatív hatását valószínűleg nem az ErbB2 leszabályozódása által fejti ki.



29. ábra. A CTX-B keresztkötés hatása az ErbB2 biológiai funkcióira.

(A) SKBR-3 sejteket jelöletlen trastuzumabbal kezeltünk 37°C-on CTX-B nélkül vagy jelenlétében, és a kezelést követően a sejtfelületen maradó ErbB2-t fluoreszcens 7C2 antitesttel festettük. (B) 50000 SKBR-3 sejtet tettünk tenyésztő edényekbe, majd 5 napig tenyésztettük őket az ábrán feltüntetett körülmények között, majd Coulter számlálóval meghatároztuk a sejtszámot és a feltripszinezett sejtek 7C2 festésével az ErbB2 expressziós szintet. A *-gal jelölt oszlopok szignifikánsan eltérnek a kontrolltól (mind az ErbB2 szint, mind a sejtszám tekintetében; $p < 0,05$). (C) Kontroll és CTX-B előkezelte, szérumban éheztetett SKBR-3 sejteket heregulin (HRG) stimuláltunk 10 percig. A CTX-B előkezelés 30 percig tartott. Ezt követően sejtlizátumot készítettünk, amiből immunprecipitáltuk az ErbB2-t vagy a Shc-et, majd a Western blotot foszforizáns ellenes antitesttel jelöltük. A foszforizált ErbB2 (p-ErbB2) vagy Shc (p-Shc) mennyiségét az adott fehérje expressziós szintjére normalizáltuk.

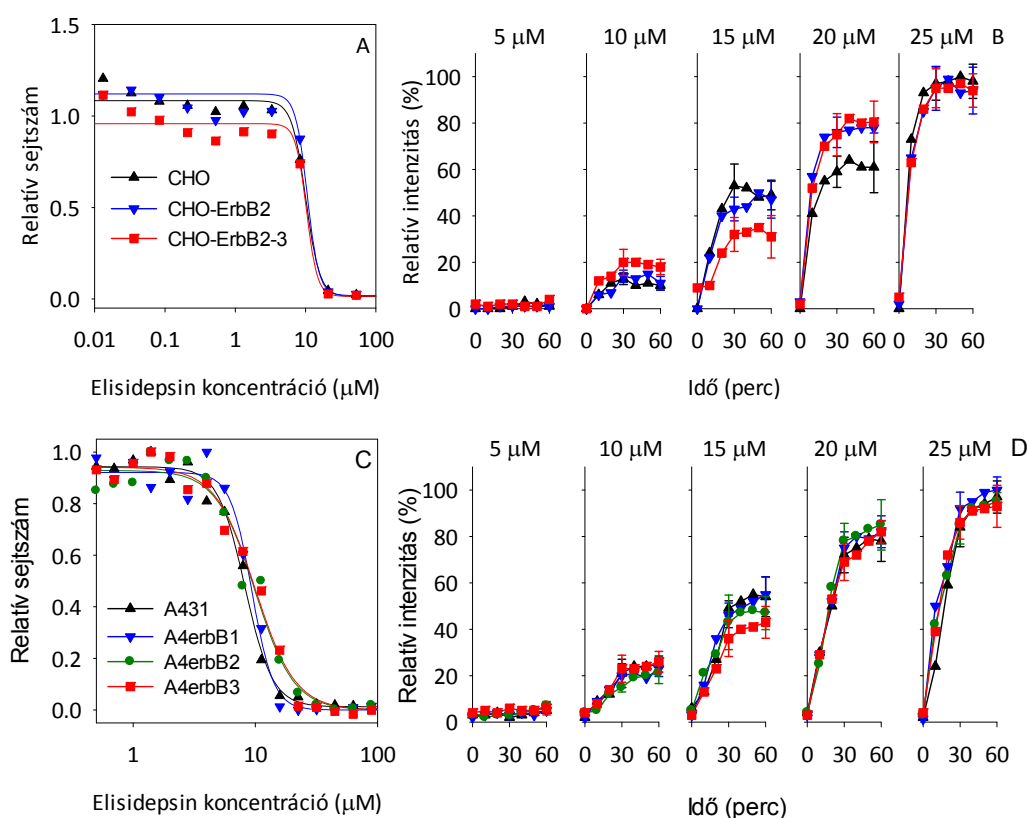
Végül megvizsgáltuk, hogy az ErbB2 heregulin általi stimulációjára milyen hatással van a CTX-B előkezelés. Eredményeink szerint a heregulin a várakozásoknak megfelelően mind az ErbB2, mind a Shc tirozin foszforilációját növelte, és ezt a hatást a CTX-B előkezelés meggátolta (29C. ábra). Ezen változás összhangban van a CTX-B kezelésnek az ErbB2-3 heteroasszociációra kifejtett hatásával, hiszen a CTX-B ezt is csökkentette (FRET határfok az ErbB2-3 heterodimerre kontroll mintákban: $19,7 \pm 0,9\%$; ugyanez CTX-B előkezelt mintákban: $7,3 \pm 2,1\%$). A fenti eredmények arra utalnak, hogy a lipid tutajok lipid környezete az ErbB2 homoasszociációt csökkenti, de az ErbB2-3 heteroklaszterizációt és a heregulin által kiváltott biológiai hatásokat valószínűleg elősegíti. Bár pontos magyarázat a homo- és heteroasszociációk eltérő lipid környezet érzékenységeire nincs, valószínű, hogy a transzmembrán domének ezen asszociációs formák fenntartásában betöltött eltérő szerepe lehet a jelenség hátterében. (A tárgyalt cikk a referencialistában: (42).)

4.12. Az ErbB fehérjék expressziója nincs hatással sejtek elisidepsin iránti érzékenységére

Az előző fejezetekben a lipid tutajok szerepe a transzmembrán jelátvitel és a fehérje asszociációk szemszögéből került tárgyalásra. A következőkben bemutatott eredmények arra utalnak, hogy a lipid tutajok gyógyszerhatás szempontjából is fontosak, hiszen az elisidepsin nevű kísérleti kemoterápiás szer támadáspontja a sejtmembrán, valószínűleg a lipid tutajok.

Az előrehaladott daganatok kemoterápiás kezelése a jelentős előrelépések ellenére sok kihívást rejt magában. Ezért kiemelt fontosságú új gyógyszerek fejlesztése, melyektől akkor várhatjuk a legjelentősebb javulást a terápiában, ha újféle hatásmóddal rendelkeznek. A racionális gyógyszertervezés mellett az új gyógyszerek fejlesztésének másik módja a természetes lelőhelyek molekuláinak szűrése és szisztematikus vizsgálatuk potenciális daganatellenes hatású vegyületek jelenlétéért (220). Az elisidepsin egy tengeri puhatestűből (*Elysia rufescens*) izolált ciklikus depsi-peptid, amely a National Cancer Institute „Compare” analízise alapján egyedi hatásspektrummal rendelkezik (221). Hatásmechanizmusa vizsgálataink elkezdésekor még nagyrészt ismeretlen volt, de voltak arra mutató kísérleti eredmények, hogy a lizoszómális membrán károsítását okozza, és hatásossága korrelációt mutatott az ErbB fehérjék, különösen az ErbB3, expressziójával (222-224). A hatásmechanizmusát érintő bizonytalanságok ellenére bevezették a klinikai vizsgálatokba (225, 226), ezért is volt sürgető, hogy támadáspontja tisztázásra kerüljön.

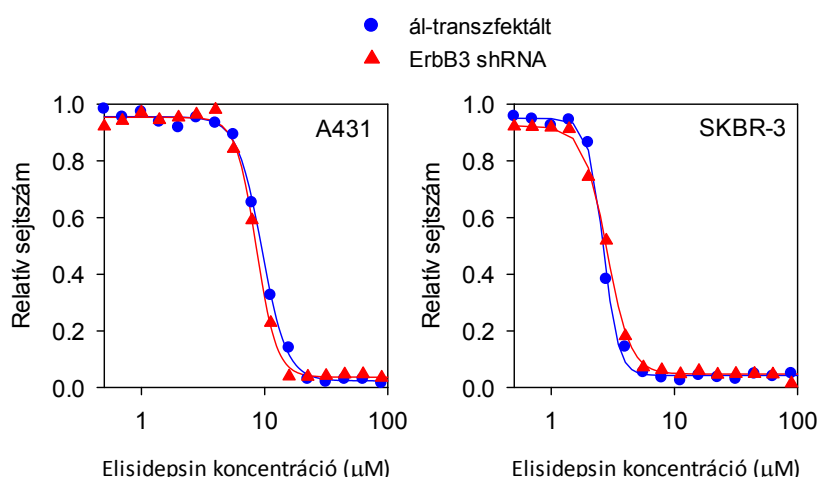
Vizsgálataink elején azt ellenőriztük, hogy az ErbB fehérjék expressziójának valóban meghatározó szerepe van-e az elisidepsin hatásában. Először CHO sejtekben, ill. ennek ErbB2-vel és ErbB3-mal stabilan transzfektált variánsaiban mértük meg az elisidepsin IC50 értékét a gyógyszer hosszútávú hatása alapján. A kísérletek meggyőzően bizonyították, hogy sem az ErbB2, sem az ErbB3 nem gyakorolt számottevő hatást a CHO sejtek elisidepsin iránti érzékenységre (IC50 CHO sejtekben (átlag $\pm$ SEM): 10 $\pm$ 0.7 μ M; IC50 CHO-ErbB2 sejtekben: 10.5 $\pm$ 0.9 μ M; IC50 CHO-ErbB2-3 sejtekben: 10.3 $\pm$ 0.8 μ M; $p > 0.1$ ANOVA felhasználásával; 30. ábra). Hasonlóan nem tapasztaltunk semmilyen változást az ErbB2 vagy ErbB3 transzfekció után a sejtek rövidtávú elisidepsin érzékenységekben (30. ábra). Mivel CHO sejtek ErbB protein kifejezése elhanyagolható (l. 3.1. fejezet), ezért az, hogy jelentős mennyiségű ErbB2 és ErbB3 expressziója sem változtatta meg az elisidepsin iránti érzékenységet, meggyőzően bizonyítja, hogy e fehérjék nem játszanak szerepet az elisidepsin hatásában.



30. ábra. Az ErbB fehérjék expressziója nem változtatja meg a CHO és A431 sejtek elisidepsin érzékenységét. Vad típusú és transzfektált CHO sejteket (A,B), ill. A431 és ebből ErbB1-3 fehérjék transzfekciójával generált sejt vonalakat (C,D) vizsgáltunk az elisidepsin iránti hosszú- (A,C) és rövidtávú (B,D) érzékenység szempontjából a 3.10. fejezetben leírt módszerek szerint. A rövidtávú érzékenység statisztikai értékelése során két ANOVA tesztet hajtottunk végre 30 és 60 percnél, de nem tapasztaltunk szignifikáns eltérést a transzfektált sejt vonalakat és az eredeti sejtek között ($p > 0,1$). Az A és C ábrákon a szimbólumok a mérési pontokat, a vonalak a (21)-es egyenlet pontokra való illesztését mutatják.

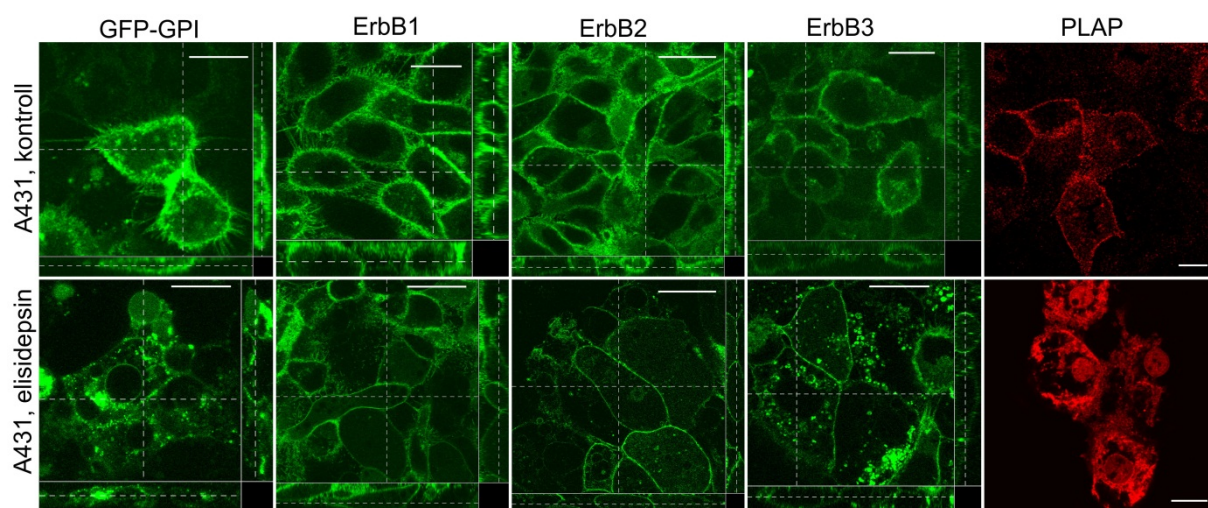
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Annak eldöntésére, hogy az ErbB fehérjék hatástalansága esetleg a CHO sejtek sajátossága lenne, ErbB1-3 fehérjék fluoreszcens proteinnel fuzionált variánsaival transzfektált A431 sejtek elisidepsin iránti rövid- és hosszútávú érzékenységet hasonlítottuk össze az eredeti sejtvonalával. A CHO sejtekhez hasonlóan az ErbB fehérje expressziójának itt sem volt semmilyen hatása (IC₅₀ A431-ben: 8.2 ± 0.8 μM ; IC₅₀ A4erbB1-ben: 9.4 ± 0.5 μM ; IC₅₀ A4erbB2-ben: 9.9 ± 1.0 μM ; IC₅₀ A4erbB3-ben: 9.9 ± 0.9 μM ; $p > 0.1$ ANOVA felhasználásával; 30. ábra). Végül ellenőriztük, hogy az elisidepsin hatásmechanizmusával legtöbbször kapcsolatba hozott ErbB3 expressziójának RNS interferencia segítségével történő gátlása befolyásolja-e az elisidepsin IC₅₀ értékét. Az ErbB3 ellenes shRNS-t generáló plazmid transzfekciója után 2 nappal az ErbB3 kifejeződése a kontroll $\sim 15\%$ -ra esett vissza, amely ~ 500 és ~ 2500 ErbB3 fehérjének felel meg A431, ill. SKBR-3 sejtek esetében. A 31. ábra tanúsága szerint az ErbB3 expresszió gátlása sem gyakorolt semmilyen hatást az IC₅₀ értékekre (IC₅₀ ál-transzfektált SKBR-3 sejtekben: 2.64 ± 0.8 μM ; IC₅₀ ErbB3 shRNS-sel transzfektált SKBR-3-ban: 2.82 ± 1.1 μM ; IC₅₀ ál-transzfektált A431-ben: 9.48 ± 1.2 μM ; IC₅₀ ErbB3 shRNS-sel transzfektált A431-ben: 8.55 ± 1.4 μM). A fenti eredmények rendkívül meggyőzően bizonyították, hogy az ErbB1-3 fehérjéknek nincsen jelentős hatásuk az elisidepsin iránti érzékenység meghatározásában, és cáfolták a korábbi, erre utaló kísérleti eredményeket. Feltételezésünk szerint a korábbi kísérletekben az ErbB expresszió és az elisidepsin érzékenység közötti gyenge korreláció alapján vonhattak le téves következtetéseket. (A tárgyalt cikk a referencialistában: (227).)

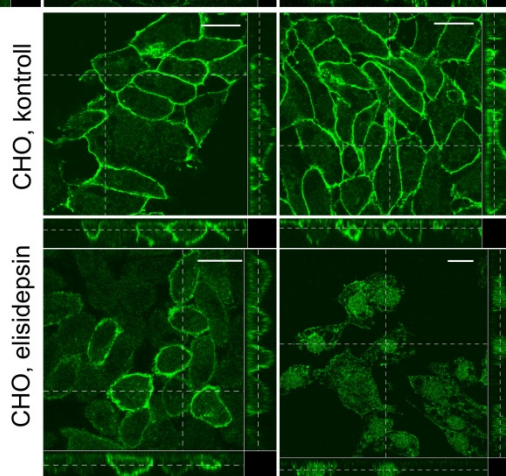


31. ábra. Az ErbB3 expresszió RNS interferenciával történő gátlása nem hat az elisidepsin iránti érzékenységre.

Ál-transzfektált és ErbB3 ellenes shRNS-t generáló plazmiddal transzfektált A431 és SKBR-3 sejtek elisidepsinre vonatkozó IC₅₀ értékét határoztuk meg a transzfekciót követő második napon történt elisidepsin kezeléssel. A szimbólumok a mérési pontokat, a folytonos vonal a (21)-es egyenlet pontokra történt illesztését mutatják.



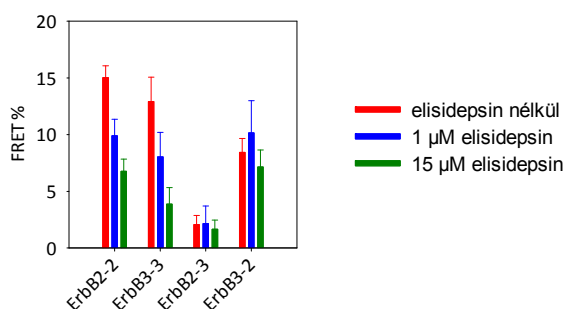
32. ábra. Lipid tutaj asszociált és ErbB fehérjék eloszlása kontroll és elisidepsin kezelt CHO és A431 sejtekben. A zöld tónusú képek konfokális mikroszkópos képsorozatok ortogonális nézeteit, a pirosan színezett képpár egyetlen konfokális szeletet mutat. A PLAP-t, ill. az ErbB2-t és ErbB3-t CHO sejtekben immunfluoreszcensen festettük, míg A431 sejtekben az ErbB1-3 fehérjéket fluoreszcens proteinek segítségével tettük láthatóvá. A sejtek kezelése 10 μ M elisidepsinnel történt 30 percig. A méret indikátorok 10 μ m-nek felelnek meg.



4.13. Az elisidepsin elsődleges támadáspontja a sejtmembrán

Bár az előző fejezetben tárgyalt eredmények alapján bebizonyosodott, hogy az ErbB fehérjék minden valószínűség szerint semmilyen elsődleges szerepet nem játszanak az elisidepsin iránti érzékenység meghatározásában, másodlagos hatásoknak azonban mégis ki lehetnek téve. Ezért megvizsgáltuk, hogy elisidepsin kezelést követően az ErbB fehérjék mikroszkópos és szubmikroszkópos eloszlásában milyen változások következnek be. ErbB1-eGFP-t kifejező A4erbB1 és ErbB2-mYFP-t kifejező A4erbB2 sejtek elisidepsin kezelése a két fehérje eloszlásában nem okozott számottevő változásokat konfokális mikroszkópos vizsgálatok alapján (32. ábra). Hasonló következtetésre jutottunk az ErbB2-vel kapcsolatban, amikor CHO-ErbB2-3 sejteken az ErbB2 receptort immunfluoreszcensen tettük láthatóvá (32. ábra). Ezzel ellentétben az elisidepsin az ErbB3 fehérje (CHO-ErbB2-3 sejteken) és az ErbB3-citrine fúziós fehérje (A4erbB3 sejteken) eloszlásában jelentős változást indukált már ~30

perc alatt: a kezelés előtti tipikus plazmamembránban való elhelyezkedés diffúz intracelluláris és vezikuláris, néhol nukleáris lokalizációra változott (32. ábra).



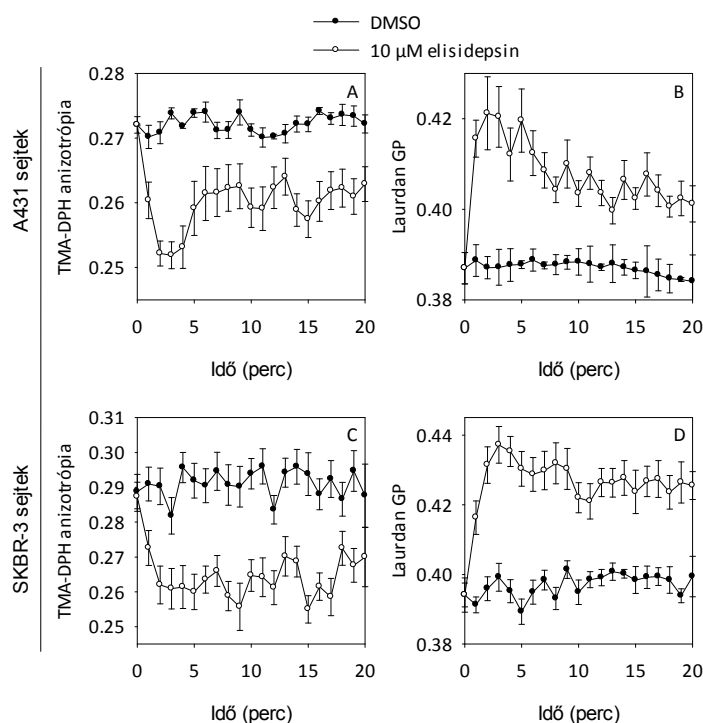
33. ábra. Elisidepsin által indukált változások az ErbB2 és ErbB3 homo- és heteroasszociációiban.

Áramlási citometriás FRET mérésekkel meghatároztuk az ErbB2 és ErbB3 homoasszociációját (ErbB2-2 és ErbB3-3) és heteroasszociációikat kontroll, ill. 1 μM és 15 μM elisidepsinnel kezelt CHO-ErbB2-3 sejteken. Az ErbB2-3 jelölés arra utal, hogy az ErbB2 ellenes antitest volt donorral, míg az ErbB3 ellenes akceptorral jelölt, az ErbB3-2 jelölés esetében a fluoreszcens jelek sorrendjét felcseréltük.

A fénymikroszkópos vizsgálatok nem adnak felvilágosítást a fehérje interakciók molekuláris szintjéről. Ezért áramlási citometriás FRET mérésekkel meghatároztuk, hogy az ErbB2 és ErbB3 homo- és heteroasszociációja milyen átalakulásokon megy keresztül elisidepsin hatására. Eredményeink szerint mindkét fehérje homoasszociációját az elisidepsin dózisfüggő módon csökkentette, míg a két protein heteroasszociációjára nem volt hatással (33. ábra). A heteroasszociáció vizsgálatok a kapott FRET hatásfok jelentősen magasabb volt akkor, amikor az ErbB2-t jelöltük akceptorral konjugált antitesttel. Mivel a használt CHO-ErbB2-3 sejteken az ErbB2 magasabb expressziót mutat, ez a különbség összhangban van a FRET hatásfok akceptor mennyiségtől való függésével (228).

Következőekben megvizsgáltuk, hogy milyen hatása van az elisidepsinnek lipid tutaj markerek eloszlására. Először a GPI-kötött GFP-re (GFP-GPI) gyakorolt hatását analizáltuk a fehérjével tranziensen transzfektált A431 sejteken. A konfokális mikroszkópos mérések szerint az elisidepsin az ErbB3-hoz hasonló átrendeződést okozott a GFP-GPI eloszlásában (32. ábra). Bár a GFP-GPI gyakran alkalmazott lipid tutaj marker, felmerült bennünk, hogy nem biztos, hogy hűen reprezentálja az endogén lipid tutaj asszociált fehérjék eloszlását és elisidepsinre adott válaszát. Ezért A431 sejteken elvégeztük egy endogén lipid tutaj asszociált fehérje, a PLAP (placentáris alkalikus foszfatáz), immunfluoreszcens festését kontroll és

elisidepsin kezelt sejteken. Eredményeink szerint ez a lipid tutaj asszociált fehérje is a már korábban látott jellegzetes redisztribúciót mutatta elisidepsin hatására: diffúz intracelluláris, néhol vezikuláris és nukleáris lokalizáció volt felismerhető (32. ábra).



34. ábra. Elisidepsin által indukált változások a plazmamembrán struktúrájában.

A TMA-DPH anizotrópiájában és a Laurdan generalizált polarizációjában 10 μ M elisidepsin által kiváltott változásokat követtük fluoriméteren SKBR-3 és A431 sejteken. Kontrollként az elisidepsin oldószerét, DMSO-t használtuk. Az ábrán három független mérés eredményének átlaga ($\pm$ SEM) látható.

Mivel a mérések alapján úgy tűnt, hogy az elisidepsin több membránfehérje mikroszkópos szintű eloszlását és molekuláris kölcsönhatásait átalakítja, felmerült annak a lehetősége, hogy mindezek a változások másodlagosan következnek be valamilyen elsődleges effektus után. Feltételeztük, hogy az elisidepsin támadáspontja a plazmamembrán, hiszen ekkor elképzelhető, hogy a fehérjék lipid környezetének átalakulása sokrétű változásokat okoz a membránfehérjék eloszlásában. Az olyan gyógyszerek, amelyek támadáspontja a sejtmembrán, gyakran okoznak átalakulást a membrán fluiditásában vagy rendezettségében (229-231). Két fluoreszcens indikátort használtunk a membrán struktúrájának vizsgálatához. A TMA-DPH fluoreszcencia anizotrópiája a membrán fluiditásáról, míg a Laurdan generalizált polarizációja (GP) a membrán hidratáltságáról, ill. rendezettségéről ad információt (125, 194, 196, 197, 232). Az A431 és az SKBR-3 sejteken az

elisidepsin 1-2 perc alatt jelentős csökkenést indukált a TMA-DPH fluoreszcencia anizotrópiájában, míg a Laurdan GP-jában ezzel ellentétes irányú változást okozott (34. ábra). Általában a membrán fluiditásának növekedését (amelyre a TMA-DPH fluoreszcencia anizotrópia emelkedése utal) a rendezettség csökkenésének kellene követnie, amely esetben a Laurdan GP-jának csökkenni kellene. A két paraméter ellentétes irányú változása arra utal, hogy olyan strukturális átalakulás történt, amely a rendezettséget és a fluiditást is növelte. Ez több közlemény alapján a folyékony rendezetlen doménekre, azaz a lipid tutajokra jellemző (125, 233). Az a tény, hogy a membrán szerkezetében feltárt fenti változásokat az elisidepsin 1-2 perc alatt kiváltja, arra utal, hogy ez ténylegesen a gyógyszer elsődleges hatása, és a többi változás ennek a következménye lehet.

Kísérleteinkkel többé-kevésbé egy időben két közlemény is megjelent, amelyek alátámasztják a fentebb körvonalazott elképzeléseket. Molina-Guijarro és mtsai kimutatták, hogy az elisidepsin valóban a plazmamembránhoz kötődik (234). Továbbá Herrero és mtsai kísérletei feltárták, hogy az elisidepsin hatásossága korrelál a zsírsav 2-hidroxiláz (fatty acid 2-hydroxylase, FA2H) szintjével (235). A hidroxilált zsírsavak és más lipid tutajokra jellemző lipidek (elsősorban koleszterin és szfingolipidek) hidrogénkötések hálózatával kompakt struktúrát hoznak létre, amely csökkenti a membrán hidratáltságát (124, 236). Az elisidepsin valószínűleg a hidroxilált zsírsavakkal alkot hidrogénkötéseket (235), és ezáltal a koleszterinhez hasonlóan hidrogénkötések kialakítása révén segíti elő a lipid tutajok képződését, és végül a membrán permeabilizálódását, amelyet propidium jodid felvételi kísérleteink támasztanak alá (30. ábra).

Felmerül a kérdés, hogy az elsődleges hatásokat követő másodlagos effektusok miért mutatnak bizonyos szelektivitást egyes fehérjékre. Az magától értetődő a fentiek alapján, hogy a lipid tutaj asszociált fehérjék (GFP-GPI és PLAP) miért rendeződnek át elisidepsin hatására, de hogy az ErbB fehérjék közül miért csak az ErbB3 sejtszintű eloszlása változott meg, és hogy a molekuláris kölcsönhatások közül miért csak az ErbB2 és ErbB3 homoasszociációi változtak, jelenleg nyitott kérdések. Feltételezéseink szerint mindazon fehérje komplexek, melyek fenntartásában a lipid környezet szerepet játszik, szenzitívebbek az elisidepsin hatására, mind a közvetlen protein-protein interakciókon alapuló asszociátumok. Saját méréseink szerint az ErbB2 (35), Landgraf és Eisenberg kísérletei szerint pedig az ErbB3 is nagyméretű klasztereket alkot (41). Mivel a 4.9. fejezetben javasolt modell alapján ezek összetartásában a lipid környezet fontos szerepet játszik, nem meglepő, hogy a

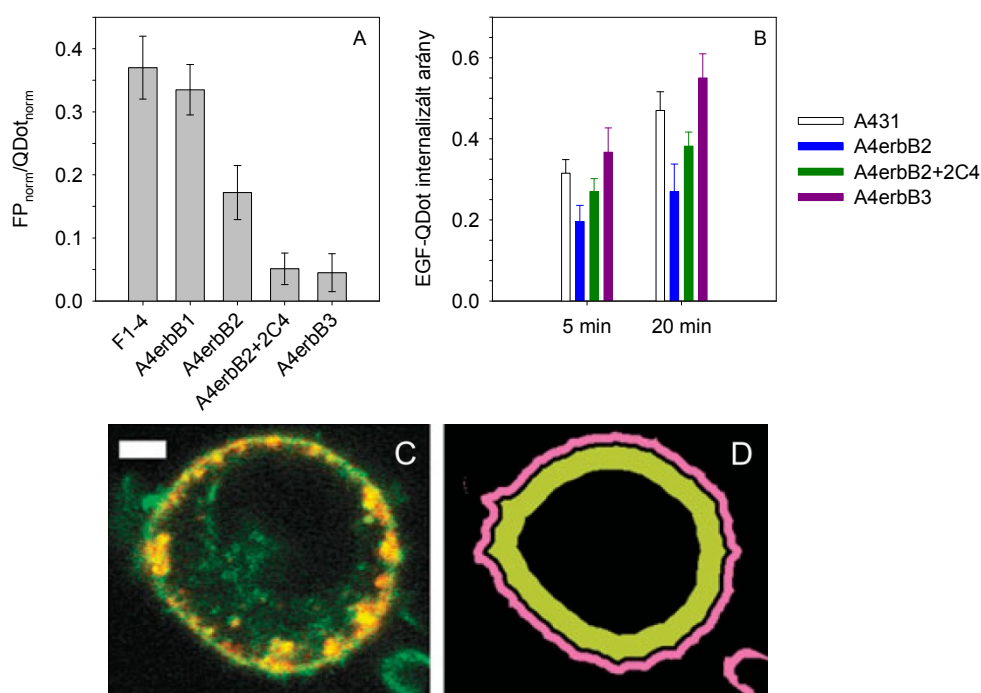
két fehérje homoasszociációit jellemző FRET értékek csökkentek elisidepsin hatására. Ezzel szemben a heteroasszociátumok fenntartásában feltételezhetően elsősorban közvetlen fehérje-fehérje kölcsönhatások vesznek részt, amelyeket az elisidepsin nem borít fel.

Nagyon fontos, de szintén nyitott kérdés az, hogy ilyen általános támadásponttal rendelkező gyógyszer, mint az elisidepsin, hogyan mutathat specificitást daganatsejtekre. Elképzeléseink szerint ezen jelenség mögött a daganatsejtekre jellemző lipidösszetételbeli módosulások állhatnak, amelyek a telített/telítetlen zsírsavak arányának növekedését, a kevésbé komplex gangliozidok felhalmozódását és a hidroxilált zsírsavak mennyiségének növekedését is magukba foglalják (237-239). E változások, különösen az utolsó, állhat az elisidepsin daganat specificitásának hátterében. *(A tárgyalt cikk a referencialistában: (227).)*

4.14. Az ErbB2 és ErbB3 koexpresszió hatása ErbB1 ligand indukált internalizációjára

A „Bevezetés” 1.2. fejezetében ismertetettek szerint az ErbB1 ligand indukált leszabályozódását az ErbB2 koexpresszió gátolja, és ezáltal fokozza az EGF által kiváltott jelátviteli folyamatokat. Egyes vélemények szerint ez a gátlás az internalizáció blokkolásával, míg mások szerint a recirkuláció fokozásával valósul meg (48-50). Vannak olyan eredmények is, amelyek szerint az ErbB3 szintén gátolja az ErbB1 EGF indukált internalizációját (240). Mivel a fenti eredmények legtöbbjét hagyományos biokémiai módszerekkel érték el, célunk az volt, hogy kvantitatív fluoreszcens analízis segítségével ellenőrizzük a fenti állításokat. Ebben a projektben készítettük el az ErbB1-eGFP-vel, ErbB2-mYFP-vel és ErbB3-citrine-nel stabilan transzfektált, A431 sejtéből származó sejtvonalakat (A4erbB1, A4erbB2 és A4erbB3). Bár az áramlási citometriás analízishez a hagyományos fluoreszcens festékek is megfelelnek, a hosszadalmas fluoreszcens mikroszkópos megfigyelés során fellépő fotoelhalványodás problémás lehet. Ezért az A431-ből származó sejtvonalak generálása mellett e projekt úttörő eredménye volt a fluoreszcens kvantum dot-ok (QDot) alkalmazása kvantitatív analízisre.

Először fluoreszcens mikroszkóppal vizsgáltuk az ErbB1 EGF által kiváltott internalizációját. A sejtek stimulálásához streptavidin-QDot-hoz kapcsolt biotinált EGF-et használtunk (EGF-QDot). A komplexben az EGF:QDot molarány 6:1 volt. A sejteket intenzitás alapú küszöböléssel jelöltük ki a fluoreszcens proteineknek megfelelő képeken, és az így azonosított egyedi sejtek határát tekintettük a sejtmembránnak. Ezt a 3.9. fejezetben leírtaknak megfelelően befelé megvastagítottuk 3-10 pixellel, és az így előálló területet használtuk membrán maszkként. Az ettől a területtől további 15-35 pixellel befelé



35. ábra. EGF-QDot internalizáció kvantitatív analízise.

A. A fluoreszcens protein-ErbB konstrukciók internalizációjának meghatározása a QDot-EGF internalizációhoz képest. B. Az EGF-QDot internalizált arányának áramlási citometriás analízise. C-D. Az ábra A részében bemutatott eredmények kiértékelésekor használt maszkok. A C képen a zöld és a vörös szín rendre az ErbB1-eGFP és az EGF-QDot fluoreszcenciáját jelöli. A D ábrán a lila keret a membrán maszkot, a világoszöld terület az intracelluláris maszkot jelöli.

elhelyezkedő területet pedig intracelluláris maszkként használtuk. Azért tartózkodtunk a teljes intracelluláris tér kiértékelésétől, mert a kísérlet időtartama alatt az EGF-QDot nem távolodott el a membrántól ennél messzebbre, és így az autofluoreszcencia zavaró hatását csökkenteni lehetett (35. ábra). Az analízist mindkét maszkon belül az EGF-QD jeleknek megfelelő foltokban végeztük el. Kiértékeljük a fluoreszcens proteinek (ErbB1-eGFP, ErbB2-mYFP vagy ErbB3-citrine) jelének intracelluláris (FP_{ic}) és membrán (FP_{mem}) maszkban mért hányadosát, és ezt osztottuk az EGF-QD jel intracelluláris (QD_{ic}) és membrán (QD_{mem}) maszkokban található hányadosával:

$$\frac{FP_{norm}}{QDot_{norm}} = \frac{FP_{ic}/FP_{mem}}{QDot_{ic}/QDot_{mem}} \quad (56)$$

Az így kapott hányados ($FP_{norm}/QDot_{norm}$) a normalizáció miatt független a fluoreszcens proteinek kiégésétől, és azt jellemezte, hogy a QDot-hoz kapcsolt EGF milyen mértékben

indukálja a fluoreszcens proteinekhez kapcsolt ErbB fehérjék internalizációját. Eredményeink szerint az EGF-QDot jelentős mértékű ErbB1 internalizációt váltott ki mind F1-4, mind A4erbB1 sejteken. Az EGF-QDot által aktivált endogén ErbB1 kisebb mértékben kiváltotta az ErbB2-mYFP internalizációját is. Azt, hogy ez az ErbB1-ErbB2 heterodimerizáció következménye volt, 2C4 előkezeléssel bizonyítottuk, amely az ErbB2 heterodimerizációjának gátlásával teljesen blokkolta az EGF-QDot indukálta ErbB2 internalizációt. Az endogén ErbB1-gyel koexpresszált ErbB3-citrine, valószínűleg az ErbB1-3 heterodimerek gyenge képződésének köszönhetően, nem mutatott internalizációt EGF-QDot stimulációt követően (35. ábra).

A fenti eredmények arra utaltak, hogy az ErbB2 az ErbB1 ligand indukált internalizációját gátolja, míg az ErbB3 koexpresszió, az ErbB1-3 heterodimer kialakulásának elmaradása miatt, nincsen ilyen hatása. Ezen konklúziókat az EGF-QDot internalizáció áramlási citometriás analízisével is megerősítettük. Az internalizációt a 3.9. fejezetben leírt savas mosásos protokoll alapján határoztuk meg. A mikroszkópos eredményekkel összhangban az EGF-QDot jelentős mértékű ErbB1 internalizációt váltott ki, amelyet az ErbB2 koexpresszió gátolt. Az ErbB2 hatását 2C4 előkezeléssel gátolni lehetett, hiszen a 2C4 antitest blokkolja az ErbB2-t magába foglaló heterodimerek létrejöttét. Az ErbB3 koexpresszió nem volt jelentős hatása az EGF-QDot internalizáció sebességére (35. ábra). A fenti eredmények arra utaltak, hogy az ErbB2 koexpresszió az ErbB1-2 heterodimerek képződése révén gátolja az ErbB1 EGF által indukált internalizációját, de még jelentős mennyiségű ErbB3 kifejeződésének sincs számottevő hatása erre. *(A tárgyalt cikk a referencialistában: (185).)*

4.15. A trastuzumab rezisztens JIMT-1 sejteken a MUC4 transzmembrán mucin maszkírozza az ErbB2-t

A receptor klaszterizáció, internalizáció és transzmembrán jelátvitel után a dolgozat hátralevő részében az ErbB fehérjékkel, mint potenciális terápiás célpontokkal fogok foglalkozni. Az 1.3-1.4. fejezetekben leírtak szerint a trastuzumab a legáltalánosabb monoklonális antitest, amelyet az ErbB receptorok ellen használnak. A trastuzumabot elsősorban ErbB2-t fokozottan kifejező emlőtumzorok ellen használják, és bár jelentős terápiás előnyt jelent a konvencionális kemoterápiákkal szemben, a rezisztencia jelentős kihívást jelent. A jelen fejezetben ismertetendő munka előtt a trastuzumab rezisztencia

mechanizmusát lényegében homály fedte. Az experimentális sejtbiológiai gyakorlatban alkalmazott emlőtumor sejtvonalak trastuzumab szenzitívek voltak, ami megnehezítette a rezisztencia jelenségének tanulmányozását. Kollaborációs partnerünk egy trastuzumab rezisztens, ErbB2-t overexpresszáló tumorral rendelkező beteg pleurális folyadékgyüleméből előállította a JIMT-1 sejtvonalat, amely *in vitro* is megőrizte rezisztens fenotípusát (184), melyet munkánk során próbáltunk karakterizálni.

A vizsgálatainkat megelőző években körvonalazott elképzelések szerint a trastuzumab hatásmechanizmusa mögött az antitest indukálta ErbB2 leszabályozódás, a rezisztencia mögött pedig az ErbB2 expresszió elvesztése áll. (Érdekes, hogy azóta, az 1.4. fejezetben ismertetettek szerint, mindkét feltételezés nagyrészt megdőlt.) Ezért ellenőriztük, hogy az ErbB és IGF1R expressziók, trastuzumab indukálta ErbB2 internalizáció és leszabályozódás tekintetében van-e különbség a JIMT-1 sejtvonal és más, trastuzumab szenzitív sejtvonalak között. Eredményeink szerint a paraméterek között nem volt olyan mértékű különbség, amely a biológiai válaszok eltérését magyarázhatta volna (7. táblázat).

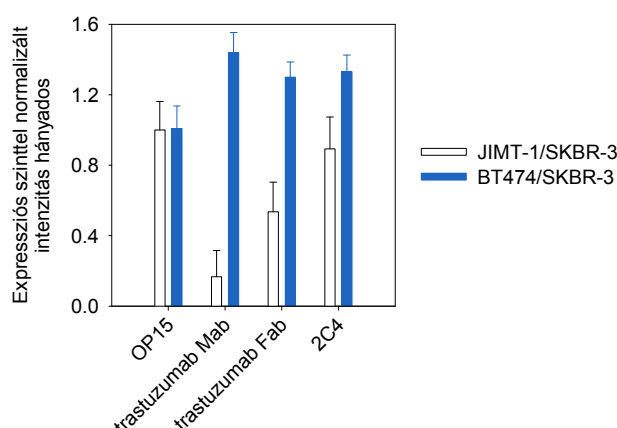
	JIMT-1	SKBR-3	BT-474	MDA-453
Expressziós szint ($\cdot 10^3$)				
ErbB1	160	190	16	60
ErbB2	620	1100	1450	620
ErbB3	10	42	20	18
ErbB4	0	0	0	2
IGF1R	5	4	9	5
trastuzumab indukálta ErbB2 internalizáció	33 $\pm$ 4%	25 $\pm$ 1%	22 $\pm$ 2%	16 $\pm$ 2%
trastuzumab indukálta ErbB2 leszabályozódás	40 $\pm$ 5%	31 $\pm$ 2%	27 $\pm$ 2%	38 $\pm$ 3%

7. táblázat. IGF1R és ErbB fehérjék expressziója, internalizációja és leszabályozódása JIMT-1 és trastuzumab szenzitív sejtvonalakon.

Az expressziós szinteket Qifikit segítségével áramlási citometriával határoztuk meg. A következő antitesteket használtuk: Mab528 (ErbB1), OP15 (ErbB2), H3.90.6 (ErbB3), H4.77.16 (ErbB4), Ab1-Clone 24-31 (IGF1R). A trastuzumab indukálta válaszok mérésekor a sejteket 10 μ g/ml trastuzumabbal kezeltük, és az ErbB2 internalizáció és leszabályozódás mértékét szintén áramlási citometriával mértük. Az internalizációt 3 órás trastuzumab kezelés után vizsgáltuk, és a kezdeti kötött trastuzumab százalékos arányában adtuk meg. A leszabályozódást 48 órás trastuzumab kezelést követően határoztuk meg, és a kezdeti ErbB2 expressziós szint százalékában adtuk meg.

Felmerült bennünk az, hogy bár az ErbB2 intracelluláris részéhez kötődő antitest (OP15) segítségével meghatározott expressziós szint tekintetében a JIMT-1 sejtek nem különböztek jelentősen a vizsgált szenzitív sejtvonalaktól, a trastuzumab kötődés mégis

gátolt lehet. Ezért megvizsgáltuk a különböző ErbB2 epitópok elérhetőségét különböző sejtvonalakon. Eredményeink szerint a membránhoz proximálisan kötődő (241) trastuzumab antitest jelentősen kisebb mértékben kötődött a JIMT-1 sejtekhez, mint a szenzitív SKBR-3 vagy BT474 sejtvonalakhoz (36. ábra). Az epitóp maszkírozása jelentős mértékben csökkent, ha teljes antitest helyett a trastuzumab Fab fragmentumával jelöltük a sejteket, és jelentősen kisebb mértékű volt abban az esetben is, ha az ErbB2-t a membrántól disztálisan kötődő 2C4 antitesttel jelöltük ((241), 36. ábra).



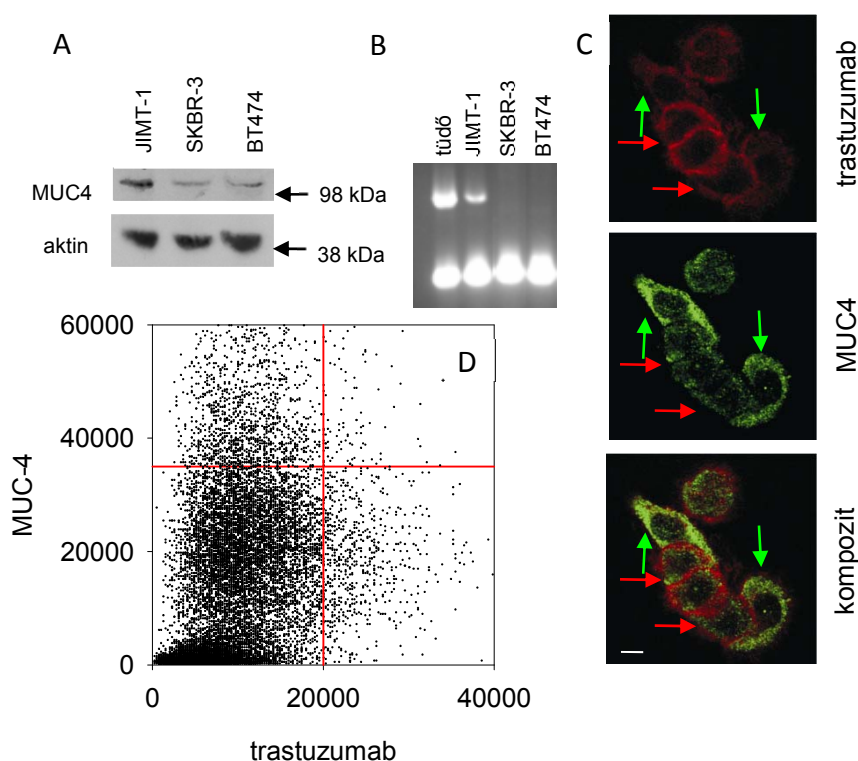
36. ábra. A trastuzumab kötő epitóp maszkírozása JIMT-1 sejteken.

Az ábrán feltüntetett antitestek kötődését elsődleges vagy másodlagos immunfluoreszcens módszerrel mértük meg áramlási citometria segítségével. A JIMT-1/SKBR-3 és JIMT-1/BT474 hányadosokat az ErbB2 törtben szereplő két sejten mérhető expressziós hányadosával normalizáltuk, így a kapott hányados az antitest vagy Fab két sejthez való relatív kötődét fejezi ki.

Az eredmények alapján nyilvánvaló volt, hogy valamilyen faktor gátolja a trastuzumab sejtekhez kötődését. A tény, hogy az antitest molekulatömegének harmadával rendelkező Fab és a membrántól távolabb kötődő 2C4 antitest kötődését az ismeretlen faktor nem vagy csekélyebb mértékben gátolta, egyértelműen a membránban jelen lévő sztérikus gátló hatásra utalt. A patkány MUC4 molekula egy nagy molekulatömegű, extenzív mértékben glikozilált membránhoz kötött mucin, amely EGF-szerű doménje segítségével az ErbB2-t aktiválni képes, és méretéből fakadóan gátolja antitestek ErbB2-höz való kötődését (242-244). A jelenséget azonban humán tumorok vonatkozásában a humán MUC4-gyel kapcsolatban még nem vizsgálták.

Igazoltuk, hogy a MUC4 mind mRNS, mind fehérje szinten magasabb szinten expresszálódik a JIMT-1 sejteken, mint trastuzumab szenzitív sejtvonalakon (37. ábra). Ezt

követően a JIMT-1 sejteken immunfluoreszcensen megfestettük a MUC4-et és az ErbB2-t trastuzumabbal jelöltük. A MUC4 expresszió és a trastuzumab kötődése között határozott negatív korrelációt találtunk, ami arra utalt, hogy a MUC4 ténylegesen gátolja a trastuzumab ErbB2-höz való kötődését. Mind az immunfluoreszcens képeken, mind a kétdimenziós hisztogramon a magas MUC4 intenzitás és a magas trastuzumab intenzitás kizárták egymást (37. ábra).

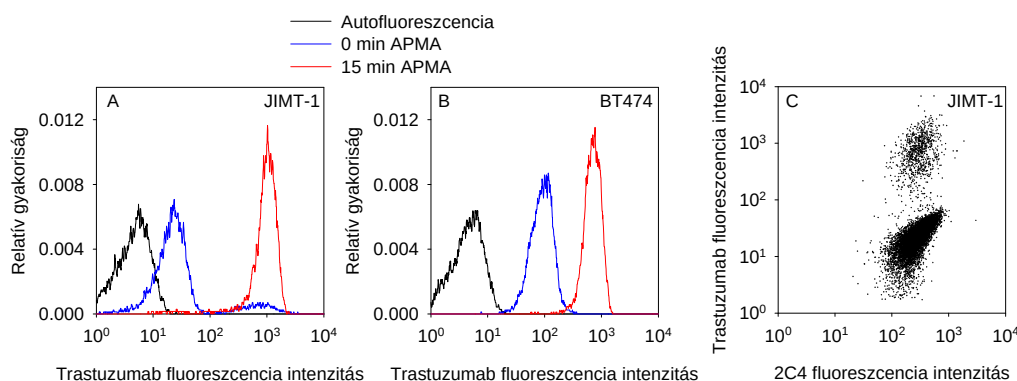


37. ábra. A MUC4 fokozott kifejeződést mutat JIMT-1 sejtekben és lokális denzitása negatívan korrelál a trastuzumab kötődésével.

(A) A feltüntetett sejtvonalakból teljes sejt lizátumot készítettünk, majd a MUC4-et felismerő 1G8 antitesttel blottoltuk. A membránt, megtisztítása („stripping”) után az aktint felismerő A4700 antitesttel jelöltük. (B) Reverz transzkripció PCR-rel a MUC4 mRNS-t (felső csíkok, 414 bp) és kontrollként GAPDH mRNS-t (glicerinaldehid-foszfát-dehidrogenáz, alsó csíkok, 204 bp) detektáltunk a feltüntetett sejtvonalakból és tüdő szövetből (pozitív kontroll). (C) Fixált és permeabilizált JIMT-1 sejteket Cy5-trastuzumabbal (piros csatorna) és a MUC4-et felismerő 1G8 antitesttel, majd Cy2 GAMIG-gal jelöltünk (zöld csatorna). A zöld nyilak olyan területeket jelölnek, ahol magas a MUC4 lokális denzitás, és alacsony a trastuzumab kötődése. A piros nyilak magas trastuzumab kötődésű és alacsony MUC4 denzitású pixeleket jelölnek. A képek kétdimenziós hisztogramja az ábra D részén látható.

Az epitóp maszkírozás egy újabb bizonyítékával szolgált a metalloproteázokat aktiváló APMA felhasználása. A maszkírozást nem mutató, trastuzumab szenzitív BT474 sejtvonalon az APMA előkezelés a trastuzumab kötődést ~10-szeresére, míg a maszkírozást mutató JIMT-1 sejtvonalon ~40-szeresére növelte (38AB. ábra). Ez arra utal, hogy az APMA által

aktivált metalloproteázok azon sejtfelszíni fehérjéket is hasítják, amelyek az ErbB2 maszkírozásáért felelősek. JIMT-1 sejteken a trastuzumab kötődés szempontjából két populáció volt jelen (38C. ábra). Az APMA kezelés az alacsony szintű trastuzumab kötődéssel rendelkező populációban a trastuzumab kötődést a magas szintű kötődést mutató populáció szintjére emelte, ami amellet szól, hogy a két populációban az ErbB2 expressziója azonos, csak a maszkírozás tekintetében különböznek (38A. ábra).



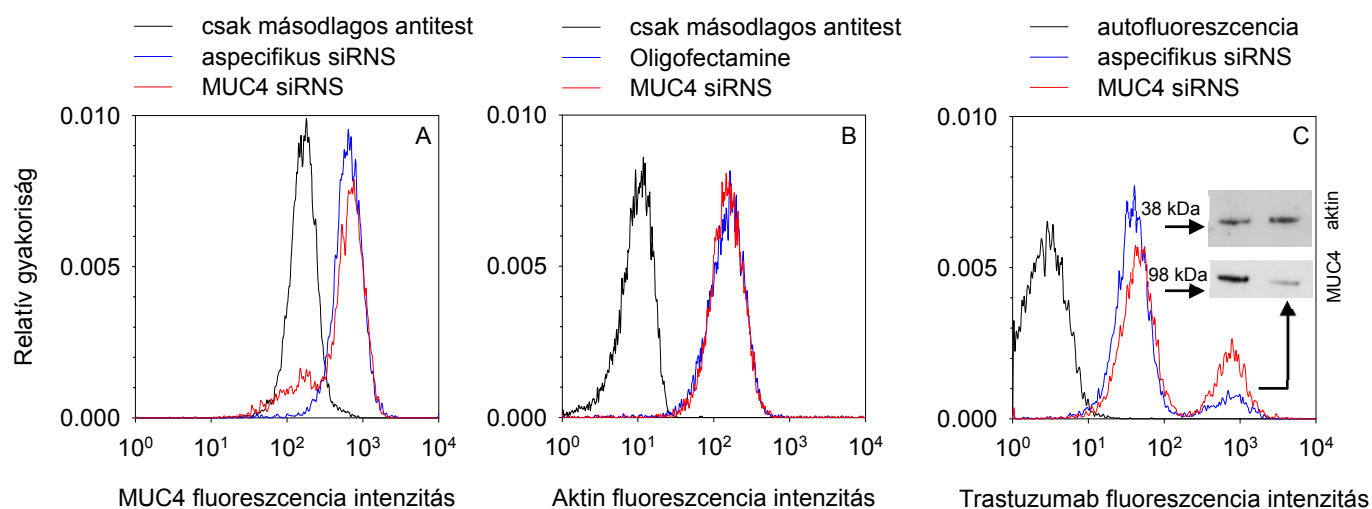
38. ábra. Az ErbB2 APMA indukálta feltárása.

A-B. JIMT-1 és BT474 sejteket 1 mM APMA-val kezeltünk, majd fluoreszcens trastuzumabbal jelöltük és a kezeletlen, ill. a festetlen kontrollokkal párhuzamosan áramlási citometriával analizáltuk. (C) JIMT-1 sejteket kettősen jelöltünk AlexaFluor488-2C4-gyel és AlexaFluor633-trastuzumabbal, majd áramlási citometriával vizsgáltuk a két fluoreszcens antitest kötődése közötti korrelációt.

A MUC4 maszkírozásban betöltött szerepét RNS interferenciával bizonyítottuk be. Bár a JIMT-1 sejteknek csak kb. 15%-át sikerült transzfektálni, a transzfektált szubpopulációban a MUC4 ellenes siRNS-sel hatékonyan gátoltuk a MUC4 expressziót. A hatás specificitását úgy bizonyítottuk, hogy egy irreleváns, GFP ellenes siRNS a MUC4 expressziót nem befolyásolta, ill. a MUC4 ellenes siRNS-nek semmilyen hatása nem volt egy irreleváns fehérje, az aktin kifejeződésére (39AB. ábra). A MUC4 siRNS-sel kezelt JIMT-1 sejteket a transzfekciót követően két nappal fluoreszcens trastuzumabbal festettünk meg, és a magas trastuzumab kötődést mutató szubpopuláció mérete jelentősen megnőtt. A növekmény nagysága megfelelt a MUC4 ellenes siRNS-sel transzfektált populáció arányának (~15%). A transzfektált mintát a trastuzumab festődés alapján áramlási citometriás szeparálással két populációra választottuk, és mindkét frakcióból húszezer sejtet izoláltunk. A két populációban megvizsgáltuk a MUC4 expresszióját Western blottal, és a magas szintű

trastuzumab kötődéssel jellemezhető szubpopuláció jelentősen alacsonyabb MUC4 expressziót mutatott (39C. ábra).

Összefoglalásul elmondható, hogy a kísérletek meggyőzően bizonyították, hogy a MUC4 JIMT-1 sejtekben maszkírozza az ErbB2 trastuzumab kötő epitópját. Eredményeink hozzájárultak a trastuzumab rezisztencia egy valószínűleg klinikai szempontból is releváns mechanizmusának feltárásához. (A tárgyalt cikk a referencialistában: (113).)



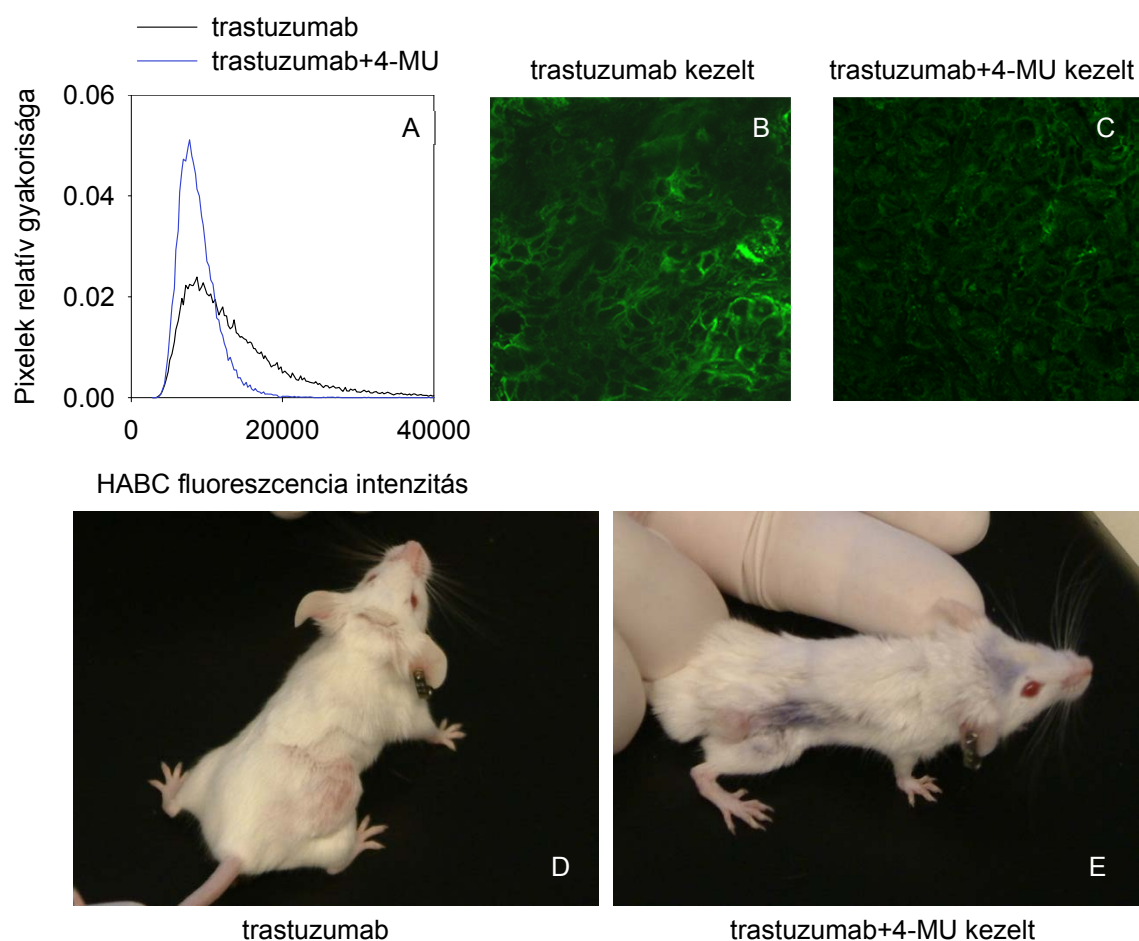
39. ábra. A MUC4 expresszió RNS interferenciával történő gátlása fokozza a trastuzumab kötődését.

JIMT-1 sejteket MUC4 ellenes és egy irreleváns, GFP ellenes siRNS-sel transzfektáltunk Oligofectamine transzekciós ágens felhasználásával, majd a transzekciót követően 48 óra múlva immunfluoreszcens festéssel áramlási citometria segítségével vizsgáltuk a MUC4 (A) és az aktin expresszió szintjét (B), ill. a trastuzumab kötődését (C). A MUC4 ellenes siRNS-sel transzfektált mintában a magas és alacsony trastuzumab kötődést mutató sejtpopulációkból 20-20 ezer sejtet izoláltunk áramlási citometriás szeparálás segítségével, majd Western blottal megmértük a MUC4 expressziót mindkét frakcióban (C).

4.16. A CD44 ligand hialuronsav szintén maszkírozza az ErbB2 trastuzumab kötő epitópját

A JIMT-1 sejtek további karakterizálása és a JIMT-1 xenograftok vizsgálata során került előtérbe, hogy a sejtvonal mind *in vitro*, mind *in vivo* nagyon magas CD44 expressziót mutat, amely a hialuronsav legfontosabb celluláris receptora. Ezenkívül a munkacsoport azt a jelentős megfigyelést tette, hogy a trastuzumab rezisztencia csak ~5 hét alatt alakul ki *in vivo* (98). Ez felvetette annak a lehetőségét, hogy az epitóp maszkírozásban a CD44 is részt vesz az *in vivo* lassan kiépülő, hialuronsavat is tartalmazó kötőszövetes mátrix által. Ezért

részletes vizsgálatnak vetettük alá a hialuronsav szintézis hatását a trastuzumab kötődés mértékére.

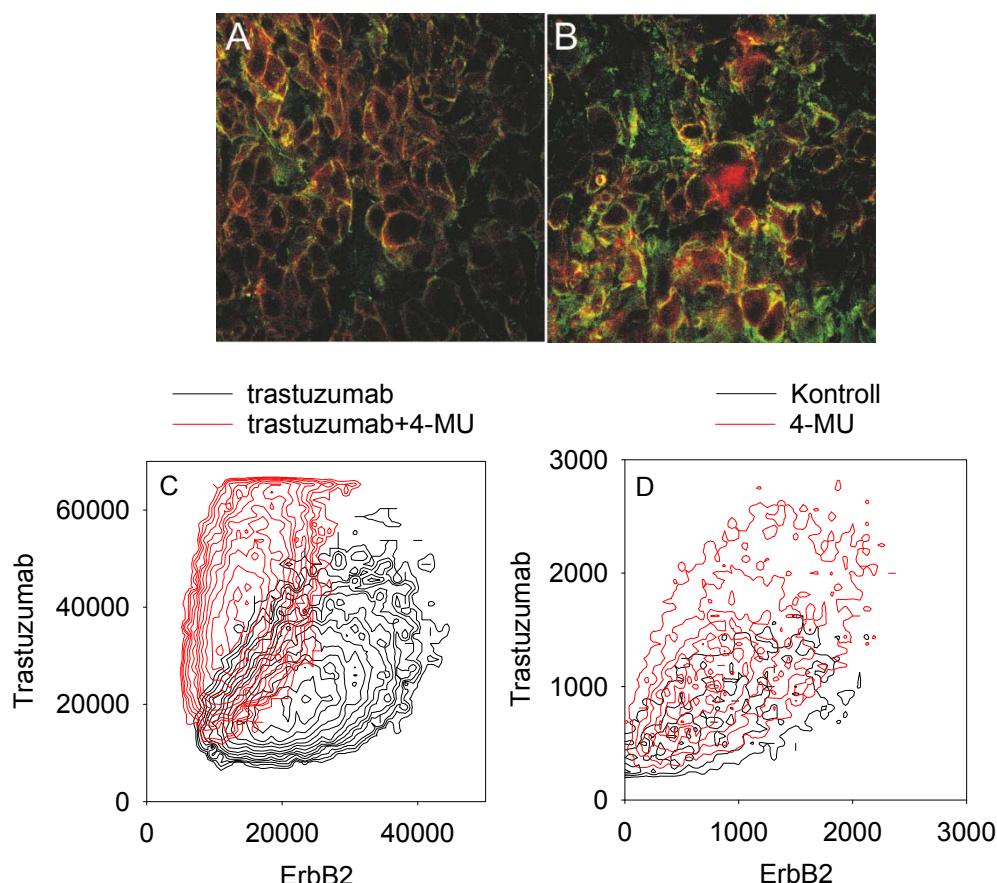


40. ábra. Egerek *in vivo* 4-MU kezelésének hatása a hialuronsav szintézisre.

(A-C) JIMT-1 xenograftokkal oltott egereket trastuzumabbal vagy trastuzumab+4-MU kombinációjával kezeltünk 8 héten keresztül, majd a xenograftokból készített metszeteken HABC festés segítségével megmértük a hialuronsav szintet. Reprezentatív mikroszkópos felvételek láthatóak a csak trastuzumabbal kezelt (A) és a trastuzumab+4-MU kombinációjával (B) kezelt állatok metszeteiről. A képek kvantitatív analízisének eredményét az ábra A része demonstrálja.

(D-E) A kétféle módon kezelt állatok szőrzetének megjelenése is különbözött.

A hialuronsav hatásának felméréséhez a szintézisét gátló 4-metilumbelliferont (4-MU) használtuk (245). Először igazoltuk, hogy a 4-MU kezelés hatásosan csökkenti a hialuronsav szintézist *in vivo*. A JIMT-1 tumor xenograftokban a hialuronsav mennyiségét HABC festéssel kvantitáltuk (246), mely egyértelműen bizonyította, hogy a hialuronsav mennyisége jelentősen alacsonyabb volt a 4-MU-val kezelt egerek mintáiban. A 4-MU kezelés sikerét támasztotta alá az egerek megjelenése is, hiszen a 4-MU-val kezelt állatok szőrzete ritkásabbnak és gyengébbnek tűnt (40. ábra).



41. ábra. A hialuronsav gátolja a trastuzumab kötődését *in vivo* és *in vitro*.

(A-C) A JIMT-1 xenograftokat hordozó egereket trastuzumabbal (A) vagy trastuzumab+4-MU kombinációjával (B) kezeltük, majd a xenograft szövetmintákat ErbB2-76.5 antitesttel (vörös csatorna) és a trastuzumabot felismerő anti-humán IgG-vel (zöld csatorna) festettük. A két képen a trastuzumab kötődés és az ErbB2 expresszió szintjének összefüggését az ábra C részén látható kétdimenziós hisztogram ábrázolja, amely pixelenként mutatja a két paraméter eloszlását. (D) JIMT-1 sejtkultúrát két napon keresztül 4-MU-val kezeltünk, majd AlexaFluor488-trastuzumabbal és AlexaFluor647-2C4-gyel festettük a sejteket. Konfokális mikroszkópos vizsgálattal elemeztük a trastuzumab kötődés és az ErbB2 expresszió (2C4 antitest) összefüggését. A C és D kontúrbrák készítésekor csak a manuálisan indított vízfeltöltéses algoritmus által azonosított membránpixelek intenzitását vettük figyelembe.

A JIMT-1 xenograftokkal oltott immundeficiens egereket trastuzumabbal vagy trastuzumab és 4-MU kombinációjával kezeltük 8 héten keresztül. Az állatok feláldozását követően a metszeteken az ErbB2 expressziós szintjét ErbB2-76.5 antitesttel történő festéssel, az *in vivo* kötődött trastuzumab mennyiségét anti-humán IgG-vel történő jelöléssel jellemeztük. A mikroszkópos képek megtekintése és kvantitatív analízise egyaránt arra utalt, hogy a 4-MU-val kezelt mintában jelentősen nőtt a trastuzumab relatív kötődése (41A-C. ábra). Hasonló megközelítést alkalmazva bizonyítottuk, hogy a JIMT-1 sejteken a hialuronsav

in vitro is gátolja a trastuzumab kötődését. JIMT-1 sejteket tenyésztőedényben két napon keresztül 4-MU-val kezeltünk, majd az ErbB2 mennyiségét 2C4 immunfluoreszcens festéssel, a trastuzumab kötő epitóp elérhetőségét fluoreszcens trastuzumabbal történő jelöléssel karakterizáltuk. A kísérlet egyértelműen bizonyította, hogy a 4-MU kezelés *in vitro* is növelte a trastuzumab relatív kötődését.

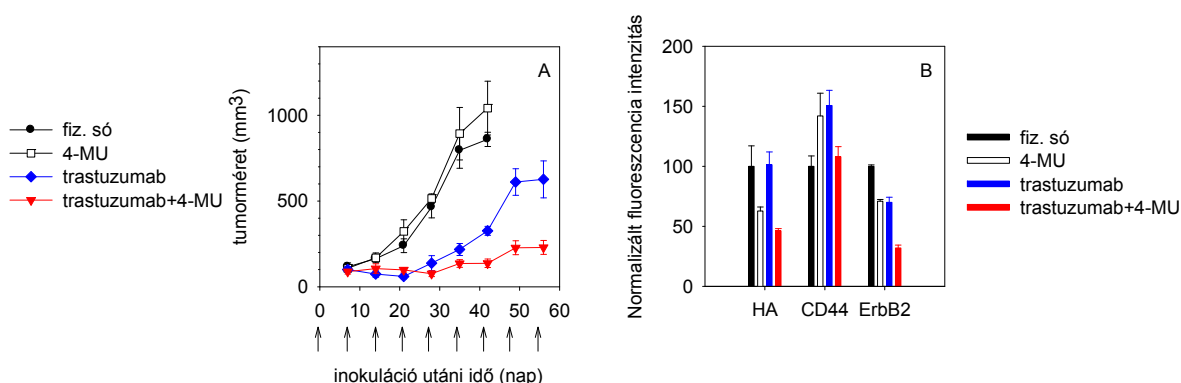
Kísérleteink bizonyították, hogy a hialuronsav mind *in vitro*, mind *in vivo* maszkírozza az ErbB2 trastuzumab kötő epitópját. Ezáltal a maszkírozásnak, mint a trastuzumab rezisztencia egyik lehetséges mechanizmusának, egy újabb manifesztációját írtuk le. (A tárgyalt cikk a referencialistában: (114).)

4.17. A hialuronsav szintézis gátlása növeli a trastuzumab hatását *in vivo*

Az ErbB2 hialuronsav általi maszkírozásának demonstrálása mellett fontosnak éreztük a hialuronsav szintézis gátlásának a tumorok növekedésére kifejtett hatásának vizsgálatát. Ezért JIMT-1 sejtekből xenograftokat hoztunk létre immundeficiens egerekben, és vizsgáltuk a trastuzumab, a 4-MU és kombinációjuk hatását a tumorok növekedésére. A trastuzumabot heti intraperitoneális injekció formájában 5 µg/g dózisban adtuk, míg a 4-MU-t szájon át 3 mg/g dózisban naponta kétszer. A hialuronsav szintézis gátlása önmagában nem volt hatással a daganatok növekedésére, míg a trastuzumab a várakozásoknak megfelelően (98) csökkentette a JIMT-1 xenograftok növekedését. A 4-MU a trastuzumab hatását jelentős mértékben fokozta, és a kombinációs terápiával kezelt egerekben ~6 hétig alig volt észlelhető növekmény a daganatok méretében (42. ábra).

Az állatok feláldozását követően a tumorokból metszeteket készítettünk, és megmértük a sejtek CD44 és ErbB2 expresszióját, ill. a pericelluláris hialuronsav mennyiségét. A hialuronsav denzitás a 4-MU-val és a trastuzumab+4-MU kombinációval kezelt állatok mintáiban alacsonyabb volt, mint a kontrollban. A 4-MU kezelés növelte a CD44 expresszió mértékét, valószínűleg a csökkent hialuronsav mediálta leszabályozódás miatt (l. 43. ábra, (247)). A trastuzumab kezelt sejtek magasabb CD44 szintje valószínűleg a CD44 shedding trastuzumab általi gátlásának a következménye (l. 4.18. fejezet, (68)). A trastuzumab a korábbi eredményekkel összhangban (l. 7. táblázat) csökkentette az ErbB2 kifejeződését. A 4-MU ugyanolyan mértékben csökkentette az ErbB2 expressziós szintjét, mint a trastuzumab. Ezt annak tulajdonítjuk, hogy a 4-MU növelte a sejtekben a CD44 mennyiségét, ami viszont saját eredményeink szerint az emelkedett ErbB2 endocitózissal

korrelál (114). A 4-MU kezelés nemcsak a tumor növekedés gátlása tekintetében fokozta a trastuzumab hatását, hanem az ErbB2 leszabályozódás vonatkozásában is (42. ábra).



42. ábra. A 4-MU szinergikusan hat a trastuzumabbal JIMT-1 xenograftok növekedésének gátlására. A. Immundeficiens egereket JIMT-1 sejtekkel oltottunk, majd a 4-MU kezelést naponta, a trastuzumab kezelést hetente a nyíllal jelölt napokon végeztük. A heti fiziológiás só (fiz. só) vagy trastuzumab kezeléssel egy időben a tumorok méretét (átlag $\pm$ SEM) meghatároztuk, és az idő függvényében ábrázoltuk. B. Az egerek feláldozását követően a tumorokból metszeteket készítettünk, és azokat hármass fluoreszcens jelzéssel festettük. A CD44-t és az ErbB2-t elsődlegesen jelölt antitesttel, a hialuronsavat (HA) biotinált HABC kezelést követő fluoreszcens avidin festéssel tettük láthatóvá. A fluoreszcencia intenzitásokat a manuálisan indított vízfeltöltéses algoritmussal azonosított sejtmembrán maszkon belül analizáltuk, és az átlag $\pm$ SEM értékeket tüntettük fel.

Összefoglalásképpen elmondható, hogy állatkísérletekkel bizonyítottuk, hogy a hialuronsav által mediált ErbB2 maszkírozás *in vivo* is jelentős, és a hialuronsav mátrix kiépülésének gátlásával a trastuzumab hatékonysága jelentősen fokozható. (A tárgyalt cikk a referencialistában: (114).)

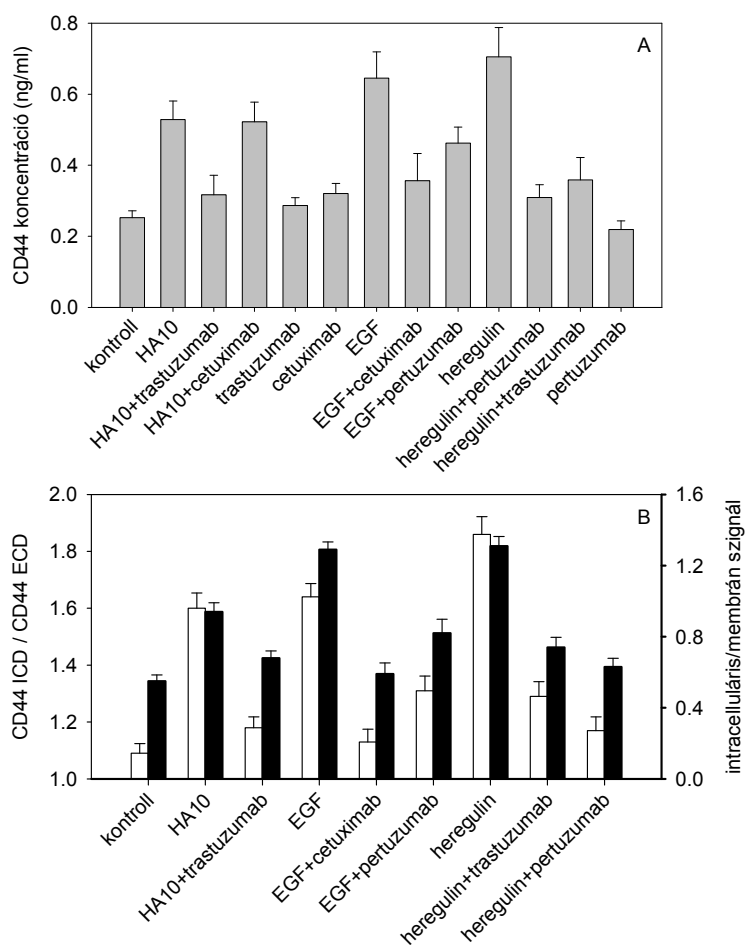
4.18. Az ErbB receptorok ligandjai által indukált CD44 shedding

A „Bevezetés” 1.2. fejezetében ismertetettek szerint az ErbB fehérjék számtalan szignalizációs útvonal csomópontjában helyezkednek el. Ezek egyike a CD44, amely a JIMT-1 sejtvonalon jelentős mértékű expressziót mutat. Ezért tűnt érdekesnek meghatározni, hogy milyen kommunikáció van a CD44 és az ErbB receptorok között ezen a sejten, és ennek mi a biológiai következménye.

A tenyésztőedényben levő sejteket tíz monoszacharid egységből álló hialuronsav fragmentummal (HA10), EGF-fel vagy heregulinnal kezeltük 60 percen keresztül, majd ELISA segítségével meghatároztuk a CD44 intramembrán proteolízise révén a felülúszóba került

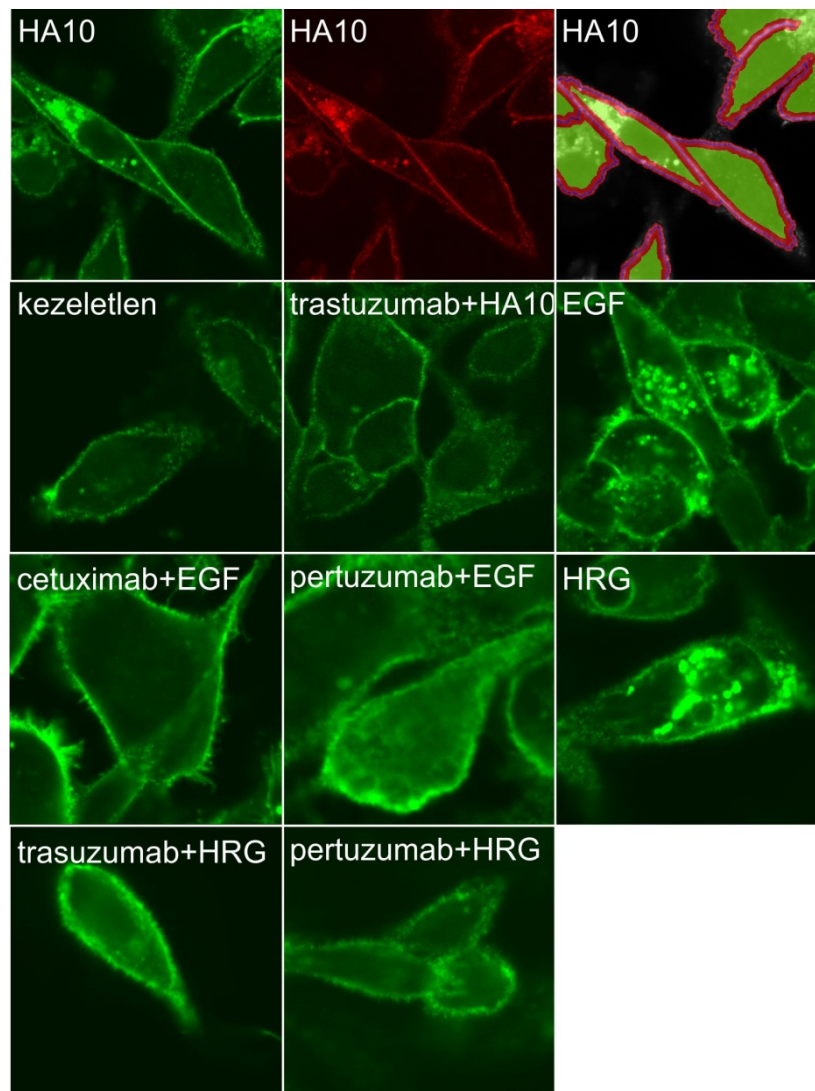
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CD44 ektodomén koncentrációját. Mindkét növekedési faktor a HA10-hez hasonló mértékű CD44 sheddinget váltott ki (43A. ábra).



43. ábra. Növekedési faktorok és ErbB ellenes monoklonális antitestek hatása a CD44 sheddingre és intramembrán proteolízisre. A. JIMT-1 sejteket 30 percig jégen kezeltünk a monoklonális antitestek telítő koncentrációjával, majd 100 µg/ml hialuronsav oligoszachariddal (HA10), 10 nM EGF-fel vagy 10 nM heregulinval történő inkubáció következett 60 percig 37°C-on. A felülúszóból a sejtörmelék és a levált sejtek eltávolítása után ELISA-val meghatároztuk a CD44 ektodomén koncentrációját. Három mérés átlaga ($\pm$ SD) látható az ábrán. B. Fedőlemezekre növesztett JIMT-1 sejteket az ábrán feltüntetett ágensekkel kezeltünk, majd a CD44 intracelluláris doménjét (anti-CD44cyto) és a CD44 extracelluláris doménjét (Hermes-3) felismerő antitesttel jelöltük őket. Ezután meghatároztuk az anti-CD44cyto antitest teljes, háttér korrigált fluoreszcencia intenzitását a sejtekben (CD44 ICD), és a Hermes-3 antitest hasonló paraméterét (CD44 ECD). A jobb oldali tengelyen feltüntetett paraméter kiszámításához meghatároztuk az anti-CD44cyto antitest teljes, háttér korrigált fluoreszcencia intenzitását az intracelluláris és membrán maszkokon belül (l. 44. ábra), és a kettő hányadosát ábrázoltuk. A bal oldali tengelyen ábrázolt paraméter a CD44 proteolízisével, a jobb oldali tengelyen ábrázolt pedig a CD44 internalizáció mértékével arányos.

A CD44 proteolízise mellett internalizációját is követtük konfokális mikroszkópia segítségével. HA10-zel, EGF-fel és heregulinnal kezelt JIMT-1 sejteket a CD44 intracelluláris (anti-CD44cyto) és extracelluláris (Hermes-3) doménje elleni antitesttel kettősen festettünk. A mikroszkópos képek alapján mindhárom anyag jelentős mértékű CD44 internalizációt váltott ki, amit a CD44-pozitív intracelluláris vezikulumok megjelenése bizonyított (44. ábra).

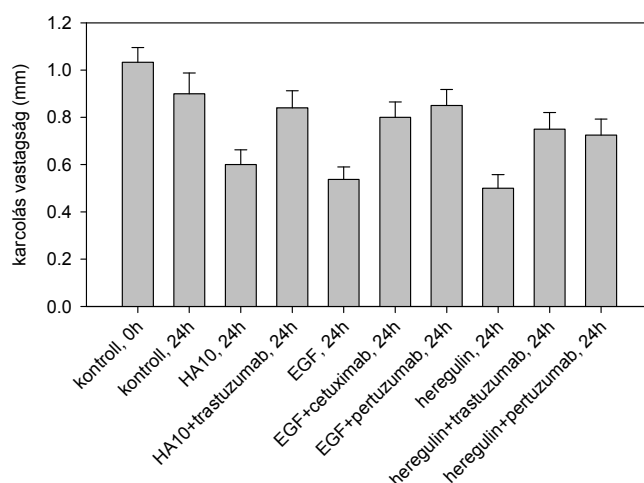


44. ábra. CD44 shedding és internalizáció analízise konfokális mikroszkópiával.

Fedőlemezekre növesztett JIMT-1 sejteket az ábrán feltüntetett ágensekkel kezeltünk egy éjszakán át 37°C-on (HRG=heregulin), majd fixálást és permeabilizációt követően vagy Hermes-3 antitesttel (piros kép) vagy a CD44 intracelluláris doménje ellenes antitesttel (anti-CD44cyto, zöld képek) jelöltük őket. A 43. ábra elkészítéséhez használt maszkok a jobb felső képen láthatók. A manuálisan indított vízfeltöltéses algoritmus által azonosított membránt (lila terület) megvastagítottuk a piros területtel, és a kettő együtt alkotta a membrán maszkot, amelyen belül elhelyezkedő, zölddel jelölt területet tekintettük az intracelluláris térnek.

A CD44 internalizáció mértékét kvantitáltuk az intracelluláris térben és a plazma membránban levő CD44 mennyiségének meghatározásával. Az analízis megerősítette, hogy mindhárom ágens szignifikánsan növelte a CD44 endocitózist (43B. ábra, $p < 0.05$). A CD44 shedding jelenségét a konfokális mikroszkópos képek elemzésével is bizonyítottuk. A CD44 proteolízise egy olyan trunkált fehérjét generál, amelyet csak az anti-CD44cyto antitest ismer fel. A tény, hogy az anti-CD44cyto antitest fluoreszcencia intenzitása a CD44 extracelluláris doménjét felismerő Hermes-3 antitest intenzitásához képest jelentősen nőtt, alátámasztotta a CD44 intramembrán proteolízis bekövetkeztét (43B. ábra).

Mivel a hialuronsav által kiváltott CD44 shedding és intramembrán proteolízis a sejtek motilitását fokozza, meg kívántuk vizsgálni, hogy az ErbB receptorokat stimuláló növekedési faktorok is kiváltanak-e ilyen hatást. *In vitro* karcolásos vizsgálat („scratch assay”) segítségével bizonyítottuk, hogy mindkét növekedési faktor a HA10-hez hasonló mértékű migráció fokozódást váltott ki (45. ábra).



45. ábra. EGF, heregulin és ErbB ellenes antitestek hatása JIMT-1 sejtek motilitására.

JIMT-1 sejtek konfluens kultúrájába egy ~1 mm széles sejtmentes területet karcoltunk, majd képeket vettünk fel azonnal és egy nappal a karcolás után. A sejtmentes területek szélességének átlagát ($\pm$ SD) tüntettük fel az ábrán. Mivel a 0 h-nál felvett képeken a karcolás vastagsága nem különbözött egymástól szignifikánsan, ezért ehhez az időponthoz csak a kezeletlen minta értékét tüntettük fel.

A CD44-ErbB kommunikációról eddig bemutatott eredmények meggyőzően bizonyítják, hogy az ErbB receptorcsalád és a hialuronsav legfontosabb celluláris receptora között együttműködés valósul meg. Mivel a CD44 intracelluláris fragmentjének keletkezése a

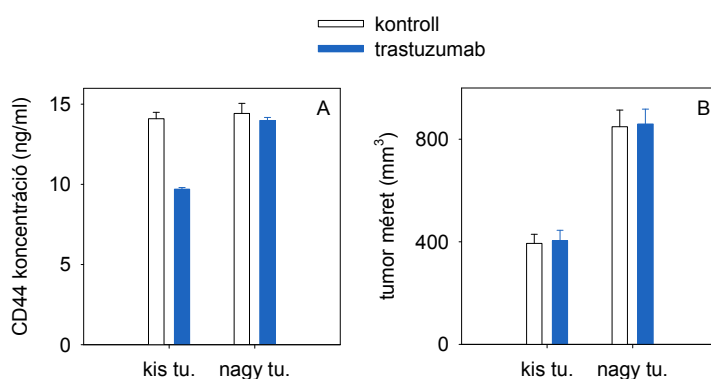
motilitás fokozódásához vezet, ez egy külön utat biztosít az ErbB fehérjéket aktiváló növekedési faktoroknak a metasztázis fokozására. (A tárgyalt cikk a referencialistában: (68).)

4.19. ErbB1 és ErbB2 ellenes antitestek hatása a CD44 proteolízisére és az általa indukált migrációra

Az előző fejezetben leírt ErbB-CD44 együttműködés felveti azt a lehetőséget, hogy a CD44 intramembrán proteolízisét és a motilitás következményes fokozódását befolyásolni lehet ErbB protein ellenes monoklonális antitestekkel. A kísérletekben három ErbB ellenes antitest hatását vizsgáltuk: a trastuzumabét, amely *in vitro* körülmények között az ErbB2 szignalizáció modulálásával fejt ki hatását; a pertuzumabét, amely az ErbB2 dimerizációját gátolja; és a cetuximabét, amely az ErbB1 EGF kötésének kompetitív inhibitora. Először az EGF és heregulin által kiváltott effektusokra kifejtett hatásukat vizsgáltuk meg. ELISA kísérletek azt mutatták, hogy a pertuzumab szignifikánsan gátolta a heregulin és az EGF által kiváltott CD44 sheddinget (43A. ábra), intramembrán proteolízist (43B. ábra) és internalizációt (43B. és 44. ábra, $p < 0,05$). A cetuximab esetében csak az EGF által kiváltott válaszokra kifejtett hatásokat vizsgáltuk, és eredményeink szerint az antitest teljesen blokkolta az EGF hatását mindhárom paraméterre (43-44. ábra, $p < 0,05$). A trastuzumab nemcsak a heregulin által kiváltott hatásokat, de a hialuronsav oligoszacharid által okozott CD44 sheddinget, internalizációt és intramembrán proteolízist is gátolta (43-44. ábra, $p < 0,05$).

Mivel a trastuzumabot széleskörűen alkalmazzák ErbB2-t fokozottan expresszáló emlőtumorsejtek kezelésére, ezért érdekesnek tűnt annak megvizsgálása, hogy az antitest milyen hatást fejt ki a CD44 sheddingre *in vivo*. A mérésekhez a 4.17. fejezetben leírt kísérleti állatokat használtuk. Mivel az egerek CD44 szérumszintje korrelációt mutatott a tumorok nagyságával, és a trastuzumab a tumorok méretét csökkentette (l. 42. ábra), ezért a CD44 szérumszintben a trastuzumab által okozott hatások egy részéért a tumornövekedés gátlása is felelős lehet. Hogy ezt a hatást kiküszöböljük, közel azonos méretű tumorok esetében hasonlítottuk össze a CD44 szérumszinteket. A kisméretű tumorok térfogata $\sim 400 \text{ mm}^3$ volt. Ilyen méretű daganatokkal a kontroll egerek a kísérlet ötödik hetében, míg a trastuzumabbal kezelt állatok a 9-11. héten rendelkeztek. Az ekkor vett vérmintákból meghatározott szérumszint CD44 koncentráció szignifikánsan alacsonyabb volt a trastuzumabbal kezelt állatokban (46. ábra, $p < 0,05$). Mivel a használt ELISA a humán CD44-

re specifikus, a detektált CD44 csak a JIMT-1 xenograftokból származhatott. Ezért az eredmények arra utaltak, hogy a trastuzumab hatásosan gátolja a CD44 sheddinget *in vivo* is. A nagyméretű daganatokkal rendelkező állatokból a vérvételt a 7-9. héten végeztük a kontroll, és a 13-14. héten a trastuzumabbal kezelt állatok esetében. Az átlagos tumorméret $\sim 850 \text{ mm}^3$ volt. Ebben az esetben nem volt szignifikáns eltérés a kontroll és a kezelt csoportok értékei között (46. ábra, $p > 0,05$). Ennek az lehet a magyarázata, hogy a trastuzumab nem képes a nagyobb méretű daganatokba kellő mértékben penetrálni vagy a daganatok nagy mérete, vagy az addigra kiépülő kötőszövetes, hialuronsavas mátrix miatt (l. 4.17. fejezet).



46. ábra. A trastuzumab gátolja a CD44 sheddinget kis méretű tumorokban *in vivo*.

JIMT-1 tumorsejtekkel injektált immundeficiens egerekből vért vettünk. A vérmintákat két csoportra osztottuk a vérvételkor tumorméret alapján. A vizsgált tumorok térfogata között nem volt lényeges eltérés a kontroll és trastuzumab kezelt állatok között (B). A CD44 szérumkoncentrációját ELISA segítségével meghatároztuk, és a minimum négy állat mérési adataiból meghatározott átlag $\pm$ SD értékeket tüntettük fel az ábrán (A).

A terápiás hatás megítélése szempontjából fontos volt az ErbB ellenes antitestek motilitásra kifejtett hatásának analízise. *In vitro* karcolásos vizsgálattal bizonyítottuk, hogy a hialuronsav oligoszacharid és a heregulin által kiváltott motogén hatást a trastuzumab szignifikánsan gátolta (45. ábra, $p < 0,05$). Az EGF által kiváltott motilitás fokozódás szenzitív volt cetuximabra, és a pertuzumab mind az EGF, mind a heregulin által indukált motogén választ blokkolta (45. ábra, $p < 0,05$). Az antitestek motilitás vizsgálatokkal feltárt inhibíciós profilja megegyezett a CD44 sheddingre, internalizációra és proteolízisre kifejtett hatásspektrumukkal.

Az ebben a fejezetben ismertetett eredmények meggyőzően alátámasztják, hogy az ErbB fehérjék fontos reguláló szerepet töltenek be a CD44 által mediált folyamatokban. Ez

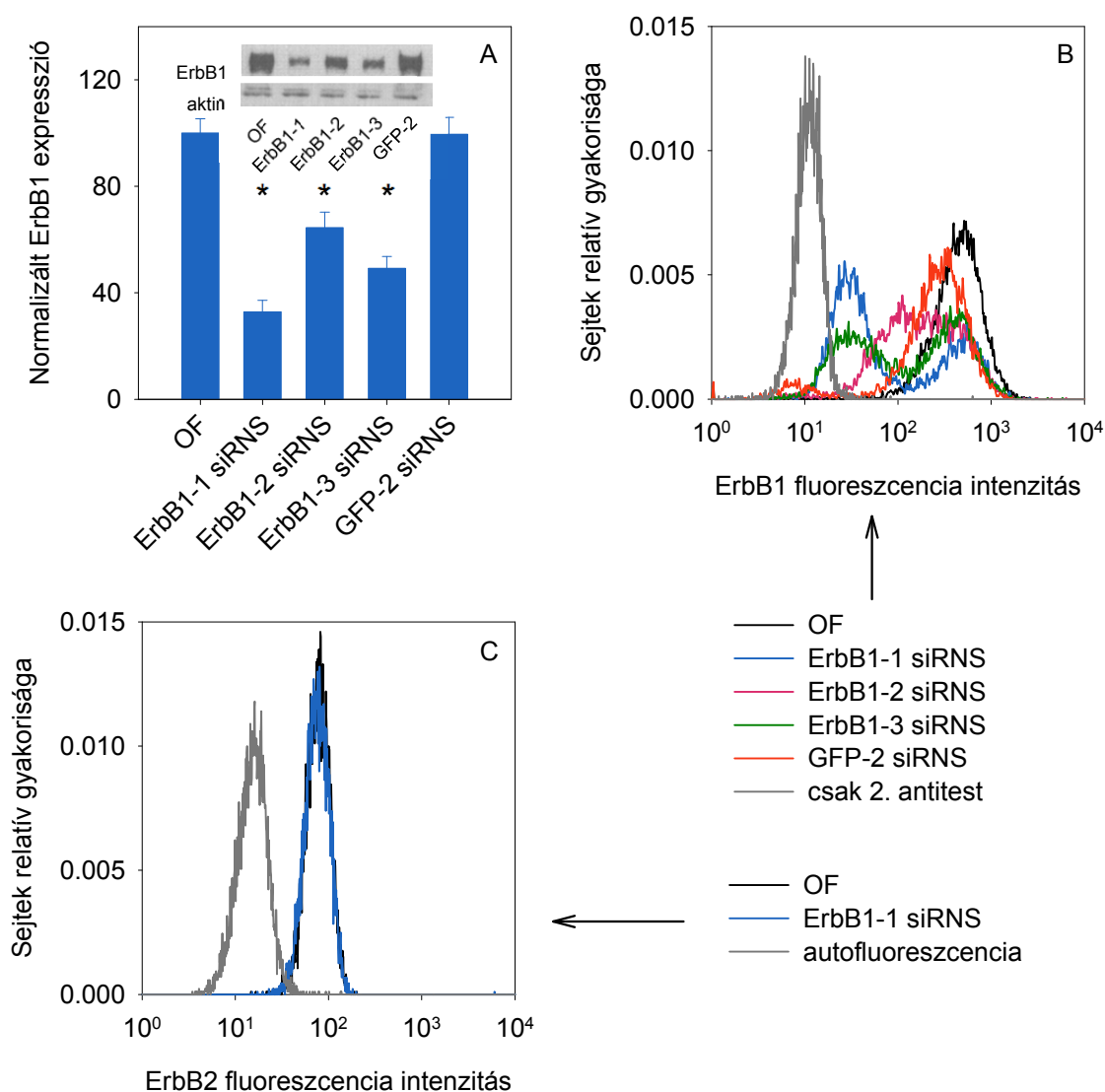
megnyilvánul abban, hogy az ErbB fehérjéket aktiváló növekedési faktorok hatásainak egy részéért (pl. motilitás fokozódás) a CD44 intracelluláris doménjének felszabadulása és az általa indukált válaszok felelősek. Ez egyrészt fokozza az ErbB receptorokat és a CD44-et együttesen kifejező daganatsejtek malignitását, hiszen a motilitás a metasztázis képződés fontos alapeleme. Ugyanakkor az a tény, hogy az ErbB ellenes antitestek ezen válaszokat gátolják, terápiás beavatkozási lehetőséget is kínál. Különösen jelentős, hogy a trastuzumab nemcsak a heregulin, hanem a CD44 saját liganduma, a hialuronsav által kiváltott válaszokat is gátolta, ami az antitest hatásmechanizmusának egy eddig nem ismert vetületét képviseli. *(A tárgyalt cikk a referencialistában: (68).)*

4.20. Az ErbB1 expresszió RNS interferenciával történő gátlása apoptózist vált ki és csökkenti az EGF indukálta hatásokat

Az ErbB1-et fokozottan expresszáló és tőle függő daganatok terápiájában a tirozin kináz inhibitorok és monoklonális antitestek mellett szóba jön a fehérje expresszió gátlása. A 2000-es évek elején ígéretes új eljárásként került a köztudatba az RNS interferencia, amelyről bebizonyosodott, hogy eukarióta sejtekben is specifikusan és nagy hatékonysággal képes gének expresszióját gátolni (248). A jelen fejezetben ismertetendő vizsgálatokkal úttörő módon bizonyítottuk, hogy az ErbB1 expresszió RNS interferenciával történő gátlásának az ErbB1-et fokozottan kifejező daganatokra biológiai hatása van.

A Thomas Tuschl által kialakított elveknek megfelelően (249) három különböző siRNS-t terveztünk ErbB1 ellen (ErbB1-1, ErbB1-2 és ErbB1-3 siRNS), és egyet GFP ellen (GFP-2 siRNS), amit általában negatív kontrollként használtunk (l. 2. táblázat). A431 sejtekben mindhárom ErbB1 ellenes siRNS hatásosnak bizonyult az ErbB1 expresszió gátlására, amit Western blottal és áramlási citométerrel is igazoltunk (47A-B. ábra). A jelenség specifikus voltát többféleképpen bizonyítottuk. Egyrészt az irreleváns siRNS szekvencia (GFP-2 siRNS) nem befolyásolta az ErbB1 expresszióját. Másrészt az ErbB1 siRNS-ek nem gátolták a molekulacsalád másik tagjának, az ErbB2 receptornak az expresszióját annak ellenére, hogy a két gén jelentős homológiát mutat (47C. ábra, csak az ErbB1-1 siRNS-re vonatkozó adatok láthatók). Az RNS interferencia jelenségének áramlási citometriás tanulmányozása rámutatott, hogy a Western blottal történő ellenőrzés a transzfekció heterogenitását figyelmen kívül hagyja. Mindhárom ErbB1 ellenes siRNS esetében voltak nem transzfektált sejtek, de a transzfektált és nem transzfektált populációk csak az ErbB1-1 és ErbB1-3 siRNS-

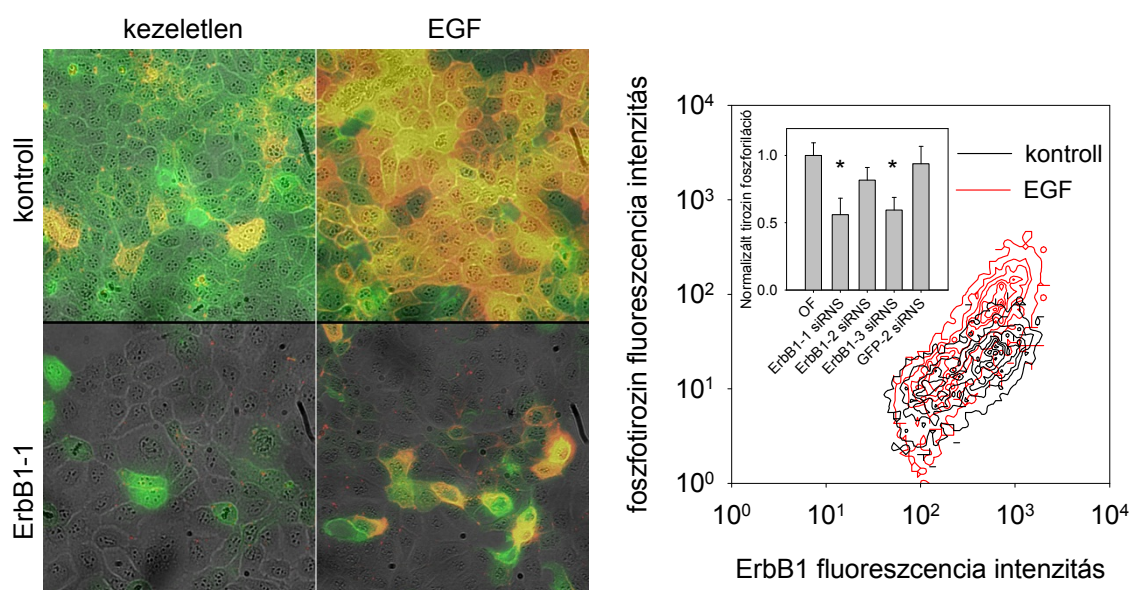
ek esetében különültek el egymástól annyira, hogy két Gauss görbét illesszünk a hisztogramokra. Ezek segítségével meghatároztuk, hogy az ErbB1-1 és ErbB1-3 siRNS-ek esetében a sejtek rendre $69\pm 5\%$ -a és $48\pm 4\%$ -a transzfektálódott, és a transzfektálódott szubpopulációkban az ErbB1 expressziós szintje $1,6\times 10^5 \pm 3\times 10^4$ (a kontroll A431 sejtek $8\pm 2\%$ -a) és $1,8\times 10^5 \pm 4\times 10^4$ (a kontroll A431 sejtek $9\pm 2\%$ -a) volt a két siRNS esetében (47B. ábra). A tény, hogy a sikeresen transzfektált sejtekben az expresszió a kontroll kevesebb, mint 10%-ra esett, a Western blot analízis nem fedte fel.



47. ábra. ErbB1 ellenes RNS interferencia kvantitatív kiértékelése A431 sejtekben.

A-B. A431 sejteket a transzekciós ágenssel (Oligofectamine, OF) kezeltünk vagy a feltüntetett siRNS-ekkel transzfektáltunk, és a transzekció után 48 órával Western blottal (A) vagy áramlási citometriával (B) megmértük az ErbB1 expressziós szintjét. Az A ábrán az inzertben bemutatott Western bloton az ErbB1 expressziós szintjét az aktinéra normalizáltuk az oszlopdiaagramon történő ábrázoláshoz (* - $p < 0,05$). (C) A431 sejteket ErbB1-1 siRNS-sel transzfektáltunk és a transzekció után 48 órával áramlási citometriával vizsgáltuk az ErbB2 expressziós szintjét.

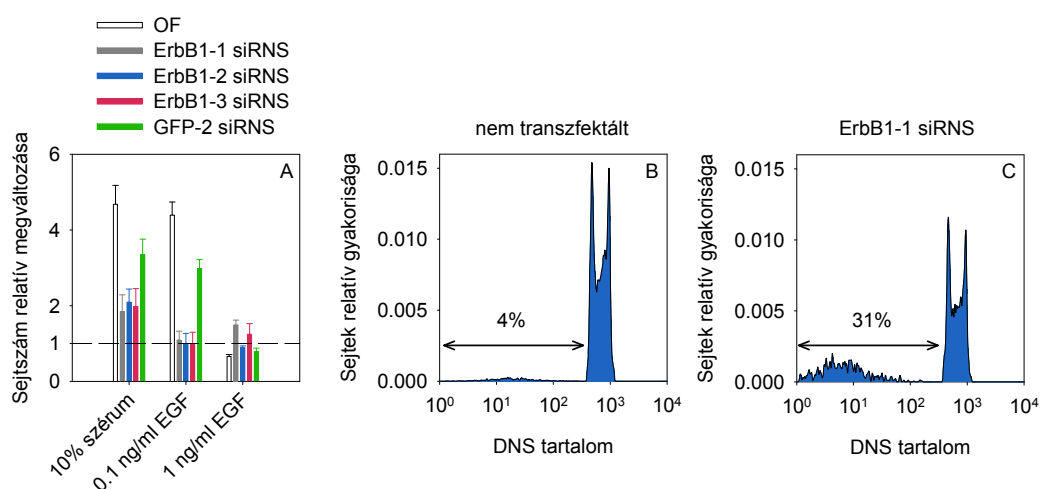
Az EGF indukálta válaszok RNS interferencia általi gátlásának demonstrálására kontroll és ErbB1 ellenes siRNS-sel transzfektált A431 sejteket stimuláltunk 0.1 ng/ml EGF-fel 5 percig 37°C-on, majd a sejteket fixálást és permeabilizálást követően ErbB1 és foszfortirozin ellenes antitestekkel festettük. A mikroszkópos képek meggyőzően bizonyították, hogy a kontroll sejtekben az EGF a várakozásoknak megfelelően jelentős mértékű tirozin foszforilációt okozott, míg az ErbB1 ellenes siRNS-sel transzfektált sejtekben ez gátolt volt (48. ábra). Az áramlási citometria jobb statisztikai megbízhatósága miatt a tirozin foszforiláció kvantitálását ezzel az eljárással végeztük el. Eredményeink szerint az EGF indukálta tirozin foszforilációt csak az ErbB1 expressziót erősen gátló ErbB1-1 és ErbB1-3 siRNS-ek gátolták szignifikánsan ($p < 0,05$). A jelenség specificitását alátámasztja az ErbB1 és foszfortirozin ellen kettősen festett, ErbB1-1 siRNS-sel transzfektált sejtek kontúrábrája, amelyen a transzfektálódott, alacsony ErbB1 expresszióval rendelkező sejtek egyáltalán nem válaszoltak az EGF-re, míg a nem transzfektálódott szubpopuláció foszfortirozin szintje jelentősen megemelkedett EGF hatására (48. ábra).



48. ábra. Az EGF indukálta tirozin foszforiláció gátlása RNS interferenciával.

A431 sejteket transzfektáltunk háromféle ErbB1 ellenes siRNS-sel, majd a transzfektáció után 48 órával a sejteket 0.1 ng/ml EGF-fel stimuláltuk 5 percig 37°C-on. Ezután a sejteket fixálást és permeabilizálást követően ErbB1 (zöld csatorna) és foszfortirozin (vörös csatorna) ellenes antitesttel jelöltük fluoreszcens mikroszkópiás vizsgálathoz (bal oldali panel). A vizsgálatokat áramlási citometriával is elvégeztük. A jobb oldali panelen az inzer a foszfortirozin ellenes antitest fluoreszcencia intenzitásának átlagát ($\pm$ SD) tünteti fel. A csillagok szignifikáns eltérést jelölnek az Oligofectamine-nal (OF) kezelt mintához képest ($p < 0,05$). A kontúrábra a kezeletlen és EGF-fel stimulált, ErbB1-1 siRNS-sel transzfektált sejtek egy reprezentatív mérését mutatja.

Az ErbB1 expresszió RNS interferenciával történő gátlása csökkentette az A431 sejtek szérum és 0,1 ng/ml EGF által kiváltott proliferációját. A hatás mindhárom ErbB1 ellenes siRNS esetében szignifikáns volt (49. ábra, $p < 0,05$). Megjegyzendő, hogy az irreleváns GFP-2 siRNS is csökkentette a sejtek osztódásának mértékét, ami aspecifikus hatás jelenlétére utal. Az ErbB1 ellenes siRNS a transzfektálódott sejtekben apoptózist váltott ki, amit a szub-G1 csúcs megjelenése bizonyított a DNS hisztogramon (49B-C. ábra). A431 sejteken az EGF 1 ng/ml-es koncentrációban a proliferációt gátolja. Az ErbB1 ellenes siRNS-sel történő transzfekció ezt a hatást is blokkolta (49A. ábra).



49. ábra. ErbB1 specifikus siRNS-ek gátolják az EGF indukálta sejtciklus válaszokat.

A. A431 sejteket az ábrán feltüntetett módon kezeltünk, és a transzfekció után 12 órával egy napig éhezettük őket 0.1% FCS tartalmú médiumban. Ezt követően két napig a vízszintes tengelyen feltüntetett körülmények között tartottuk őket, és a kísérlet végén a sejtszámot Coulter számláló segítségével határoztuk meg. A végső, két napos inkubáció alatti relatív sejtszám változást (átlag $\pm$ SD) mutatja be az ábra (OF=Oligofectamine). B-C. Az A részben leírt módon ErbB1-1 siRNS-sel transzfektált, majd 10% szérum jelenlétében tenyésztett A431 sejteket ErbB1 ellenes antitesttel jelöltünk és propidium jodiddal festettünk. A magas ErbB1 expressziót mutató, nem transzfektálódott sejtpopuláció DNS eloszlása az ábra B részén, míg az alacsony ErbB1 expressziót mutató, transzfektálódott szubpopuláció hisztogramja az ábra C részén látható.

A fenti kísérletekkel bizonyítottuk, hogy RNS interferenciával az ErbB1 expresszió és az EGF indukálta válaszok hatékonyan gátolhatók. Az ErbB1-től függő, azt fokozott mértékben expresszáló sejtekben az ErbB1 kifejeződés RNS interferenciával történő gátlása apoptózist okoz. Ez, az *in vivo* transzfekció technikai problémáinak megoldása után, elvileg lehetőséget ad ilyen jellegű tumorok kezelésére. (A tárgyalt cikk a referencialistában: (250).)

5. Következtetések, hasznosíthatóság

Az ErbB receptorok klaszterizációjával kapcsolatos eredményeink új megvilágításba helyezik az EGF receptorcsalád tagjainak aktivációs mechanizmusait, és több olyan módszertani fejlesztést is magukba foglalnak, melyek jelentős előrelépést jelentenek a problémakör biofizikai karakterizálása szempontjából.

- Kifejlesztettünk két áramlási citometriás módszert, melyek a fluoreszcens proteinekkel végzett kvantitatív FRET méréseket, ill. a fluoreszcens antitesttel jelölt fehérjék homoklaszterizációjának szintén kvantitatív jellemzését teszik lehetővé.
- A heteroklaszterizáció kvantitatív jellemzésére kidolgozott FSAB módszer hagyományos konfokális mikroszkóp felhasználásával teszi lehetővé az egymással kölcsönható receptorok arányának meghatározását.
- Mások által kifejlesztett és a fentebb említett módszerek alkalmazásával leírtuk a receptor asszociáció dimernél magasabb hierarchikus szintjeit. Ennek több módszerrel történt megerősítése bizonyítja a háromszintű receptor klaszterizáció modell létjogosultságát. Rámutattunk arra, hogy az ErbB1 és ErbB2 jelentősen különböznek klaszterizációs tulajdonságaik szempontjából. Bár a közepes méretű asszociátumok biológiai szerepe fehérjénként más és más lehet, az ErbB2 esetében az auto-aktivációban gátolt, de heteroasszociáció által aktiválható fehérjék rezervoárjaként funkcionálnak.
- Bebizonyítottuk, hogy az ErbB2 a lipid tutajokban helyezkedik el, és a tutajok lipid környezete gátolja az ErbB2 homoasszociációját. Az ErbB3 magas lokális expressziós szintje szintén csökkenti az ErbB2 homoasszociáció mértékét.
- Az ErbB1 ligand indukálta internalizációját az ErbB2 gátolja, míg az ErbB3-nak nincs erre jelentős hatása.
- A fehérje asszociációk magasabb szintjeinek biológiai szerepe arra utal, hogy a receptorok aktivációja ezek strukturális megbolygatásával is elérhető és a jövőben akár daganatok terápiájában is kihasználható.

A dolgozatban leírt másik jelentős kísérletsorozatban az ErbB receptorok és lipid tutajok terápiás célpontként való felhasználhatóságával, ill. a trastuzumab rezisztencia kialakulásának molekuláris mechanizmusával kapcsolatban tettünk fontos megállapításokat.

- Leírtuk, hogy a MUC4 transzmembrán mucin fokozott expressziója gátolja a trastuzumab kötődését ErbB2-t fokozottan kifejező emlőtumor sejtekhez az antitestkötő epitóp maszkírozása által.
- A pericellulárisan elhelyezkedő hialuronsav szintén maszkírozza az ErbB2-t. A hialuronsav hatását mind *in vitro*, mind *in vivo* igazoltuk.
- A maszkírozás a trastuzumab rezisztencia kialakulásának egy általunk leírt új mechanizmusa. Ezen eredményeinknek nyilvánvalóan fontos klinikai következményei vannak, hiszen rámutatnak arra, hogy az ErbB2 maszkírozása trastuzumab rezisztenciához vezet, és az ezért felelős molekulák termelésének gátlása szinergikusan növelheti a trastuzumab terápiás hatékonyságát.
- A hialuronsav receptor CD44 funkcionális molekuláris komplexet képez az ErbB receptorokkal, és mind ligandumjaik, mint a hozzájuk kötődő antitestek az egész komplex működését befolyásolják. Így az ErbB ellenes antitestek (trastuzumab, pertuzumab, cetuximab) gátolják a motilitást fokozó CD44 shedding és internalizáció folyamatát.
- Megállapítottuk, hogy az ErbB1 expresszió RNS interferencia segítségével történő gátlása apoptózishoz vezet a receptort fokozottan kifejező sejtekben.
- Bebizonyítottuk, hogy az elisidepsin hatását a sejtek ErbB expressziója nem befolyásolja, és hogy a gyógyszer a lipid tutajokon keresztül, folyékony rendezett domének indukálása révén fejti ki citotoxikus hatását.

6. Köszönetnyilvánítás

Egy nagydoktori disszertáció hosszú évtizedek munkájának gyümölcse, hiszen nemcsak azt a mintegy egy évtizedet kell tekintetbe venni, amely alatt a benne tárgyalt cikkek publikációra kerültek, hanem az azt megelőző hosszú évek tanulmányait és kutató munkáját is. Ezért, talán kicsit formabontóan, először családomnak szeretnék köszönetet mondani, hogy a munkájába elmerülő kutatót támogatták. Szüleim példamutató kitartása, szerénysége és odaadása, mellyel a család, a gyermek és a munka felé fordultak, túl nem értékelhető háttérrel és lendülettel adott nekem, és példaként áll még ma is előttem. Édesapám sajnos nem élhette meg ezt a napot, ezért Neki szeretném külön kifejezni hálámat, amiért csendesen a háttérből mindig utat mutatott nekem. Édesanyám kifogyhatatlan szeretete és féltése olyan útravalóval látott el, amit friss apaként értek meg csak igazán. Feleségem és kislányom édesanyja, Ági, nemcsak a meleg családi fészek megteremtésével, de közlemények társszerzőjeként is segítő kezet nyújtott. Kislányunk, Anna, még talán nem sokat ért az előző oldalakon leírtakból (bár ebben nem vagyok mindig biztos), tengerkék szemének igéző tekintete és ragaszkodó szeretete mindig meggyőz arról, hogy a kihívásokkal szembe kell nézni.

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Természetesen köszönöm a cikkekben társszerzőként megjelenő, továbbá név szerint meg nem említett munkatársaimnak is a támogatást és a kooperációt.

7. Támogató pályázatok

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10. A dolgozatban tárgyalt cikkek másolata

Lipid rafts and the local density of ErbB proteins influence the biological role of homo- and heteroassociations of ErbB2

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Summary

The ErbB family of transmembrane receptor tyrosine kinases plays an important role in the pathogenesis of many cancers. The four members of the family, ErbB1-4, form various homo- and heterodimers during the course of signal transduction. A second hierarchical level of molecular associations involving 10^2 - 10^3 molecules, termed large-scale clustering, has also been identified, but the regulatory factors and biological consequences of such structures have not been systematically evaluated. In this report, we describe the states of association of ErbB2 and their relationship to local ErbB3 density and lipid rafts based on quantitative fluorescence microscopy of SKBR-3 breast cancer cells. Clusters of ErbB2 colocalized with lipid rafts identified by the GM1-binding B subunit of cholera toxin. Pixel-by-pixel analysis of fluorescence resonance energy transfer between labeled antibodies indicated that the homoassociation (homodimerization) of ErbB2 was proportional to the local density of ErbB2 and inversely proportional to that of ErbB3 and of the raft-specific lipid

GM1. Crosslinking lipid rafts with the B subunit of cholera toxin caused dissociation of the rafts and ErbB2 clusters, an effect that was independent of the cytoskeletal anchoring of ErbB2. Crosslinking also decreased ErbB2-ErbB3 heteroassociation and the EGF- and heregulin-induced tyrosine phosphorylation of Shc. When cells were treated with the anti-ErbB2 monoclonal antibody 4D5 (parent murine version of Trastuzumab used in the immunotherapy of breast cancer), internalization of the antibody was inhibited by crosslinking of lipid rafts, but the antiproliferative activity of 4D5 was retained and even enhanced. We conclude that local densities of ErbB2 and ErbB3, as well as the lipid environment profoundly influence the association properties and biological function of ErbB2.

Key words: ErbB proteins, Lipid rafts, Breast cancer, Fluorescence resonance energy transfer

Introduction

The type I family of transmembrane receptor tyrosine kinases comprises four members: epidermal growth factor receptor (EGFR or ErbB1), ErbB2 (HER2 or Neu), ErbB3 and ErbB4 (Alroy and Yarden, 1997; Yarden and Sliwkowski, 2001). ErbB proteins engage in an extensive network of homo- and heteroassociations resulting in signal diversification (Tzahar et al., 1996). The relative expression levels of the various receptors and the concentrations of their respective ligands determine the composition of homo- and heterodimers (Pinkas Kramarski et al., 1996; Graus Porta et al., 1997). ErbB2 is the preferred heteroassociation partner of all other ErbB proteins, enhancing ligand binding affinity and signaling potency by virtue of its potent latent kinase activity (Karunagaran et al., 1996). Activation-induced endocytosis and downregulation are important in limiting the duration of receptor activation, but their efficiency is low for all members of the ErbB family with the exception of ErbB1 (Baulida et al., 1996). This is probably

due to impaired coupling of cbl, a protein thought to be involved in activation-induced degradation, to ErbB2, ErbB3 and ErbB4 (Levkowitz et al., 1996). The significance of ErbB2 is underscored by its overexpression in about 30% of breast carcinomas, a phenomenon associated with poor prognosis (Slamon et al., 1987).

The signaling network of ErbB proteins is based on receptor homo- and heterodimers. In addition to these small-scale associations, higher order associations involving large-scale clusters of ErbB2 have also been identified (Nagy et al., 1999b). These clusters contain hundreds of ErbB2, are $\sim 0.5 \mu\text{m}$ in diameter, a size that increases upon ErbB2 activation. The significance of such large-scale protein clusters probably results from the high local concentration of ErbB and other signaling proteins facilitating the formation of receptor complexes (Nagy et al., 1999a).

ErbB2 plays an important role in the development and progression of many breast cancers and other malignancies

(Park et al., 2000). An anti-ErbB2 monoclonal antibody, 4D5, inhibits the proliferation of tumor cells that overexpress ErbB2 (Sarup et al., 1991). The humanized version of 4D5 (Trastuzumab, Herceptin™) has significant anticancer activity (Cobleigh et al., 1999; Slamon et al., 2001). Its exact mechanism of action is as yet unclear. The antibody induces partial tyrosine phosphorylation of ErbB2 but does not activate the MAP kinase pathway, although it does activate the cyclin-dependent kinase inhibitor p27KIP (Sliwkowski et al., 1999). It has been proposed that the antiproliferative activity of Trastuzumab is due largely to the downmodulation of surface ErbB2 by receptor-mediated endocytosis (Sarup et al., 1991; Sliwkowski et al., 1999). Cbl-mediated ubiquitination is thought to play a key role in this process (Klapper et al., 2000; Levkowitz et al., 2000). Antibodies that efficiently internalize ErbB2 but fail to inhibit tumor cell proliferation have also been identified (Neve et al., 2001), suggesting that antibody-mediated internalization may not be a good marker of anti-tumor activity.

Higher order associations (i.e. the organization of individual and oligomeric membrane proteins into several hundred-nanometer-scale complexes) have attracted increased attention in studies of receptor biology and signal transduction. Membrane domains are often thought of as organizing forces beyond such associations. For example, lipid rafts, sphingolipid- and cholesterol-rich membrane microdomains, participate in diverse cellular functions such as membrane trafficking and receptor signaling (Simons and Ikonen, 1997; Janes et al., 1999). There is intense debate about the size and nature of lipid rafts, but recent evidence supports the existence of small (<0.1 µm in diameter) lipid rafts that can dynamically associate with each other to form larger signal transducing platforms (Harder and Simons, 1997; Kurzchalia and Parton, 1999; Pralle et al., 2000; Edidin, 2001; Anderson and Jacobson, 2002). Lipid rafts and a special form thereof, caveolae, are thought to be involved in EGFR-mediated signaling (Furuchi and Anderson, 1998; Mineo et al., 1999) presumably by altering the activity of protein kinases (Ilangumaran et al., 1999) and changing the association state of membrane proteins (Bodnár et al., 1996; Vereb et al., 2000; Matkó et al., 2002).

We have previously reported the existence of plasma membrane domains in which the homoassociation of ErbB2 is unusually high (Nagy et al., 1998). The significance of this finding is underlined by the fact that overexpression-driven constitutive homoassociation of ErbB2 induces ligand-independent activation of ErbB2 and other ErbB proteins (Worthylake et al., 1999). Although the overall effect of changes in the relative expression levels of ErbB proteins has already been studied (Tzahar et al., 1996), the role of local differences in the relative densities of receptors, which may explain differences in the association of proteins within a single cell (Chamberlin and Davies, 1998), has not been previously addressed. In our current experiments we show that membrane domains with high degree of ErbB2 homoassociation have high ErbB2 and low ErbB3 densities. The association state of ErbB2 is also influenced by lipid rafts. ErbB2 is dislodged from the latter after crosslinking GM1, a raft-associated ganglioside (Harder et al., 1998), by the B subunit of cholera toxin (CTX-B), which is associated with decreased ErbB2-ErbB3 heterodimerization and reduced tyrosine phosphorylation of

ErbB2 upon heregulin stimulation. It is of interest to point out that 4D5-mediated internalization of ErbB2 is blocked in CTX-B-pretreated cells, whereas the antiproliferative effect of 4D5 is not. Our results emphasize that alterations in the local environment of ErbB2 strongly influence its association properties with significant consequences for its biological activity.

Materials and Methods

Cells lines

The SKBR-3 breast tumor cell line was obtained from the American Type Culture Collection (Rockville, MD) and grown according to its specifications to confluency. For microscopic experiments cells were cultured in slide chambers (Nunc, Naperville, IL), and labeled and measured without trypsinisation. For flow cytometric measurements cells were harvested with trypsinization.

Antibodies, growth factors

4D5 and 7C2 antibodies against the ErbB2 protein were a kind gift from Genentech (South San Francisco, CA), and were labeled with fluorescent dyes for FRET and for colocalization measurements. The dye/protein ratio was 1-3 as determined by spectrophotometric measurements. The H3.90.6 anti-ErbB3 antibody was purchased from NeoMarkers (Fremont, CA), and the sc-894 polyclonal rabbit antibody against caveolin was from Santa Cruz Biotechnology (Heidelberg, Germany). Cy2-, Cy3- and Cy5-labeled polyclonal rabbit Fab fragments against mouse IgG (anti H+L) were from Jackson ImmunoResearch (West Grove, PA). The anti-phosphotyrosine antibody PY99 (sc-7020) and the anti-Shc antibody (sc-967) were from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-ErbB2 antibody c-neu/Ab-3 (OP15) was from Calbiochem (Darmstadt, Germany). Protein G-coated Sepharose 'Fast flow' beads were purchased from Sigma-Aldrich (Schnelldorf, Germany).

Epidermal growth factor and the EGF-like domain of heregulin-β1 were from R&D Systems (Minneapolis, MN). Cells were starved in 0.1% FCS-containing medium for 48 hours before stimulation with EGF or heregulin.

Labeling of cells with antibodies and subunit B of cholera toxin

ErbB proteins were labeled with saturating concentration of antibodies for 30 minutes on ice in PBS. Unbound antibodies were removed by washing twice with PBS. When two primary labeled antibodies were used to label cells, they were added simultaneously. When one of the labelings was indirect, cells were first incubated with the unlabeled primary antibody followed by the dye-conjugated secondary Fab. Subsequently, cells were labeled with a labeled primary antibody against the other antigen.

Prior to labeling caveolin, cells were fixed in 3.7% formaldehyde (30 minutes on ice) and incubated with the sc-894 anti-caveolin antibody in the presence of 0.1% BSA and 0.1% TritonX-100 for 30 minutes on ice followed by secondary labeling with Cy3-conjugated F(ab')₂ fragment of goat anti-rabbit immunoglobulin (Jackson ImmunoResearch, West Grove, PA).

In order to label lipid rafts cells were incubated in the presence of 8 µg/ml FITC-labeled subunit B of cholera toxin (Sigma-Aldrich, Schnelldorf, Germany) for 30 minutes on ice. For crosslinking lipid rafts incubation with subunit B of cholera toxin was carried out at 37°C for 30 minutes.

Confocal microscopy

A Zeiss LSM410 confocal laser scanning microscope (Carl Zeiss, Thornwood, New York) was used to image cells. Alexa488, Cy2 and

FITC were excited with the 488 nm line of an argon/krypton ion laser and emission from these fluorophores was detected through an FT 488/568 nm dual-band dichroic mirror and a 515-565 nm band-pass filter. Cy3 was excited with the 568 nm line of an argon/krypton laser and emission was detected through an FT 488/568 nm dual-band dichroic mirror and a 575-640 nm band-pass filter. Cy5 was excited at 647 nm and emission was measured through a FT 665 nm long-pass dichroic mirror.

During the experiments with membrane clusters of ErbB proteins, the focal plane of the confocal microscope was adjusted such that either the top or the bottom flat layer of the cell membrane could be imaged in a single confocal slice.

Donor photobleaching FRET measurements

In some of the experiments, the association of membrane proteins was measured using the donor photobleaching FRET method (Jovin and Arndt Jovin, 1989). Briefly, one of the proteins was labeled with a fluorescein (donor)-labeled antibody and the other protein was labeled with a Cy3 (acceptor)-labeled antibody (double-labeled sample). Another sample was also prepared in which only the donor-labeled antibody was applied (donor only sample). The kinetics of donor photobleaching was measured in both samples. The fluorescence intensity curves were fitted to a double exponential equation with a custom-written program in LabView (National Instruments, Austin, TX). An effective rate constant was calculated by taking the amplitude-weighted time constants of the exponential terms. Any process (e.g. FRET) competing with photobleaching slows the rate of the process. Therefore it is possible to calculate the FRET efficiency on a pixel-by-pixel basis by comparing the rate of donor photobleaching in the double-labeled sample to that in the donor-only-labeled sample according to the expression $\text{FRET} = 1 - \tau_{\text{eff,D}} / \tau_{\text{eff,DA}}$, where $\tau_{\text{eff,D}}$ is the average effective photobleaching time constant in the donor-only-labeled sample, and $\tau_{\text{eff,DA}}$ is the effective photobleaching time constant of a pixel in the donor-acceptor double-labeled sample.

Acceptor photobleaching FRET measurements

This technique is based on the detection of increased donor fluorescence intensity following irreversible photodestruction of the acceptor (Wouters et al., 1998). A sample was labeled with Cy3- and Cy5-tagged antibodies. First, an image was taken in the Cy3 (donor) channel (I_q) followed by an image in the Cy5 (acceptor) channel. Then, the acceptor was bleached completely, after which another Cy3 image was taken reporting the unquenched fluorescence intensity of the donor (I_{uq}). Images were corrected for spectral spillover, and the second Cy3 image was corrected for bleaching by the 647 nm laser line used to bleach Cy5. The FRET efficiency was calculated according to the equation $\text{FRET} = 1 - I_q / I_{uq}$.

Analysis of correlation between FRET efficiency and fluorescence intensity

In order to correlate the FRET efficiency (measured either with the donor or the acceptor photobleaching FRET method) with the fluorescence intensity on a pixel-by-pixel basis, images were analyzed with Scil-Image (University of Amsterdam, The Netherlands) after correction for shift and spectral spillover. The correlation between FRET efficiency and unquenched donor fluorescence intensity was calculated and plotted according to Demandolx and Davoust (Demandolx and Davoust, 1997).

Calculation of the overlap between clusters

Low frequency background was removed using either a Fourier transformation based method (Hwang et al., 1998) or top-hat

transformation (Glasbey and Horgan, 1995). Low frequency background is a constant or a smoothly changing intensity component that may be caused by inhomogeneous illumination, autofluorescence or non-specific staining. Removal of high frequency background (smoothing) was carried out either by low-pass filtering or a morphology operation (opening) (Glasbey and Horgan, 1995). Cluster and background pixels were discriminated with the entropy threshold segmentation algorithm of Scil-Image, and the distribution of particle sizes in this binary mask was determined with ScionImage (Scion Corp., Frederick, MD). Cluster diameter was determined by judiciously positioning the cursor on the screen on opposing edges of the bright regions (Nagy et al., 1999b). In order to calculate the area of overlap between clusters, segmented binary images (in which digit 1 and 0 correspond to cluster and background pixels, respectively) recorded in the different fluorescence channels were multiplied with each other. In the resulting image foreground pixels (value 1) reflect the overlap of clusters in the two input images. The fractional overlap was calculated by dividing the area of foreground pixels in the resulting image by the area of foreground pixels in one of the original images. Since this area was approximately the same in both original images, we report only one of the obtained ratio values.

Flow cytometric energy transfer measurements

A modified Becton Dickinson FACStar Plus flow cytometer equipped with dual argon ion laser excitation was used to measure the FRET efficiency between fluorescently labeled membrane proteins. This parameter is a quantitative measure of protein associations and can be derived from three fluorescence intensities (Szöllősi et al., 1984). Two intensities resulted from excitation at 488 nm and detection at 540 ± 20 and above 580 nm, and the third signal was excited at 514 and detected above 580 nm. Forward angle light scattering (at 514 nm) was used to gate out debris. All data were stored in list mode format. FRET efficiency was calculated on a cell-by-cell basis from the three fluorescence intensities of 10,000 cells.

Flow cytometric measurement of antibody internalization

Three methods were used to detect antibody internalization. (1) SKBR3 cells were incubated with unlabeled 4D5 (10 $\mu\text{g/ml}$) at 37°C. Samples were taken every 10 minutes, and non-internalized ErbB2 was labeled with Alexa488-7C2 for 30 minutes on ice. Fluorescence was detected with a FacsCalibur Flow Cytometer. (2) SKBR3 cells were incubated in the presence of 10 $\mu\text{g/ml}$ 4D5 antibody at 37°C. The non-internalized fraction of 4D5 was labeled with Cy2-tagged Fab fragments of anti-mouse IgG, and the fluorescence intensity of Cy2 was measured on the flow cytometer. (3) SKBR3 cells were treated with fluorescein labeled 4D5 antibody at 37°C. Samples taken every 10 minutes were divided into two parts. Part 1 was subjected to acid-wash (pH 2.4, 3 min) in order to remove 4D5 from the cell surface (Baulida and Carpenter, 1997). Part 2 was not acid-treated. The difference between the fluorescence intensities of these two samples represented the amount of 4D5 on the cell surface.

Measurement of cell proliferation

50,000 cells were seeded in a culture dish and kept in the presence of 10% FCS for 1 day. They were subsequently incubated in medium containing 0.1% FCS for 1 day. This was followed by a 5-day culture in the presence of 10% FCS. In order to study the antiproliferative effect of 4D5, 10 $\mu\text{g/ml}$ of the antibody was added to the medium with or without 8 $\mu\text{g/ml}$ cholera toxin (subunit B). The culture medium supplemented with 4D5 (and cholera toxin) was replenished every second day. On day 5 the cells were counted and the cell surface expression of ErbB2 was determined by labeling cells with Alexa488-7C2.

Measurement of tyrosine phosphorylation of ErbB2 and Shc

Cells were scraped off the culture substrate. Whole cell lysates of control or stimulated cells were prepared by incubating the cells in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 10% (w/v) glycerol, 1 mM EGTA, 1% (w/v) Triton X-100, 1 mM Na_3VO_4 , 1 mM PMSF, Complete Mini protease inhibitor cocktail from Roche, pH 7.5) for 10 minutes on ice. The lysate was centrifuged at 12,000 *g*, and ErbB2 or Shc was immunoprecipitated from the supernatant using OP15 and sc-967 antibodies, respectively, and protein G beads. The immunoprecipitates were run on SDS-polyacrylamide gels, and blotted to nitrocellulose membranes. The membranes were probed with anti-phosphotyrosine antibody PY99, then stripped and reprobed with either anti-ErbB2 or anti-Shc antibodies to check whether equivalent amounts of proteins were immunoprecipitated in each sample. Bands on the blots were quantitated using enhanced chemiluminescence (Amersham, Freiburg, Germany).

Results

ErbB2 homoassociation is not significantly different inside and outside large-scale clusters

Previously we have found that the homoassociation of ErbB2 is uneven on the cell surface, i.e. there are membrane patches with a diameter of ~ 0.5 – 1 μm in which ErbB2 homoassociation is significantly higher than in the surrounding area (Nagy et al., 1998). Later we have identified large-scale clusters of ErbB2 containing ~ 1000 ErbB2 proteins in which the density of ErbB2 is 1–2 orders of magnitude higher than outside of them. Their characteristic diameter is ~ 0.5 μm (Fig. 2A) (Nagy et al., 1999b). The similar size of large-scale ErbB2 clusters and membrane patches showing a high degree of ErbB2 homoassociation raised the possibility that the two might be related. Therefore we measured the homoassociation of ErbB2 on a pixel-by-pixel basis using the donor pbFRET technique in confocal microscopy (Jovin and Arndt Jovin, 1989) in order to find out whether ErbB2 homoassociation was the same inside and outside of large-scale clusters of ErbB2. The fluorescence intensity of pixels outside large-scale clusters was not high enough for reliable FRET measurements prompting two approaches for increasing the signal-to-noise ratio. First, large polygon-shaped regions of interest (ROI) were placed separately on areas outside and inside large-scale clusters, and the fluorescence intensity was summed inside these ROIs. The FRET efficiency was calculated on the summed fluorescence intensities. There was no significant difference between the FRET efficiencies evaluated over pixels inside and outside the large-scale clusters. In FRET experiments the FRET efficiency is a measure of protein associations: the higher the FRET efficiency is, the tighter the labeled proteins associate with each other. Therefore, the lack of any significant difference in the measured FRET efficiencies indicated that the homoassociation of ErbB2 was the same inside and outside ErbB2 clusters: FRET efficiencies for ErbB2 homoassociation were $16 \pm 3\%$ and $18 \pm 3\%$ (mean $\pm$ s.e.m.) for pixels inside and outside ErbB2 clusters, respectively. According to the second approach, the size of images was reduced in a way that square-shaped areas (superpixels) consisting of 3×3 , 5×5 or 7×7 pixels were collapsed to a single pixel, the fluorescence intensity of which was the summed intensity of the area it replaced. Evaluation of the FRET efficiency was carried out on a pixel-by-pixel basis on these compressed images, separately for superpixels outside and inside large-scale clusters. These

calculations corroborated that there was no significant difference between the homoassociation of ErbB2 inside and outside large-scale clusters of this protein (FRET efficiencies for ErbB2 homodimerization were $17 \pm 2\%$ and $16 \pm 2\%$ for superpixels inside and outside large-scale ErbB2 clusters, respectively, with all three sizes of superpixels).

ErbB2 homoassociation is influenced by the local density of ErbB2 and ErbB3

Since the homoassociation of ErbB2 did not differ significantly inside and outside large-scale clusters, we turned our attention to the local densities of ErbB2 and ErbB3 as possible determinants of ErbB2 homoassociation. SKBR-3 breast tumor cells were labeled with FITC-4D5 and Cy3-4D5 as donor-acceptor pair against ErbB2, and ErbB3 was labeled indirectly using a Cy5-tagged secondary antibody. The homoassociation of ErbB2 was calculated on a pixel-by-pixel basis using the donor pbFRET technique and plotted as a function of the local ErbB2 and ErbB3 densities. The analysis, carried out on pixels inside ErbB2 clusters, showed that ErbB2 homoassociation was positively correlated with the local density of the protein, but negatively correlated with the local density of ErbB3 (Fig. 1). A very similar dependence of ErbB2 homoassociation on the local densities of ErbB2 and ErbB3 was found outside clusters; i.e. there were areas with high ErbB2 homoassociation outside clusters as well (data not shown). However, the signal-to-noise ratio was much lower in pixels outside clusters, therefore we restricted our detailed analysis to the clusters. Membrane patches with unusually high ErbB2 homoassociation were located in areas where the local density of ErbB2 was high and that of ErbB3 was comparatively low (Fig. 2A–C), as shown in Fig. 2D–F. In addition, for areas with high ErbB2 intensity and very high ErbB3 density (as those

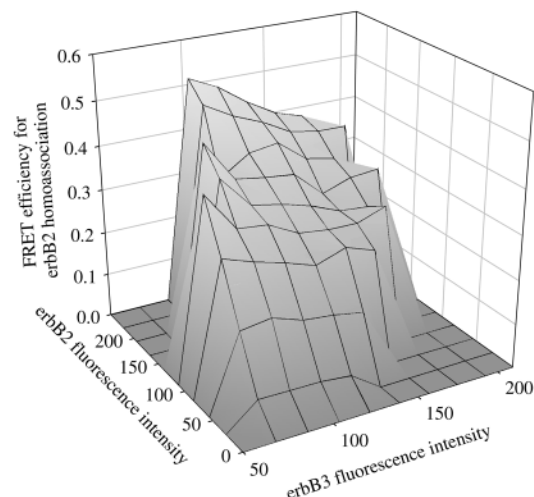


Fig. 1. Dependence of ErbB2 homoassociation on local parameters. SKBR-3 breast tumor cells were labeled with FITC-4D5 and Cy3-4D5 against ErbB2 to measure the homoassociation of ErbB2; ErbB3 was labeled with Cy5-tagged secondary Fab following labeling with H3.90.6. The homoassociation of ErbB2 was measured with the donor pbFRET method, and it was correlated with the local density of ErbB2 and ErbB3 on a pixel-by-pixel basis. The average FRET efficiency for ErbB2 homoassociation is plotted as a function of the local density of ErbB2 and ErbB3.

marked with arrows in images A-C, Fig. 2), the homoassociation of ErbB2 is lower than would be expected from the high ErbB2 density alone.

ErbB2 clusters colocalize with lipid rafts

Lipid rafts and lipid raft clusters are envisioned to be in the range of ~10-1000 nm in size (Harder et al., 1998; Jacobson and Dietrich, 1999; Anderson and Jacobson, 2002). Since lipid rafts can serve as signal transduction platforms (Kurzchalia and Parton, 1999), determining the colocalization between ErbB2 and lipid rafts is important and relevant. Lipid rafts were visualized by labeling cells with FITC-labeled subunit B of cholera toxin (CTX-B), which binds GM1 ganglioside, a lipid component highly abundant in lipid rafts (Harder et al., 1998). ErbB2 was labeled with Cy3-4D5. These experiments revealed that the overlap between lipid rafts and ErbB2 clusters was $77\pm6\%$ (Fig. 3A).

ErbB2 homoassociation is negatively correlated with the density of GM1

We next examined the spatial relationship between lipid rafts and the homoassociation of ErbB2. Rafts were labeled with FITC-CTX-B, and ErbB2 was labeled with a 1:1 mixture of Cy3- and Cy5-tagged 4D5. ErbB2 homoassociation was measured with the acceptor photobleaching technique. We found a negative correlation between ErbB2 homoassociation and the fluorescence intensity of FITC-CTX-B (Fig. 3A,B, Fig. 4). We detected no FRET between FITC-CTX-B and Cy3-4D5 (data not shown) implying that the fluorescence intensity of FITC-CTX-B reflected its local density. As CTX-B labels GM1 ganglioside, an abundant lipid component of lipid rafts, we concluded that there was a negative correlation between ErbB2 homoassociation and the local density of the raft marker.

Cells were stimulated with EGF (50 ng/ml), heregulin (50 ng/ml) or MAB 4D5 (10 μ g/ml) to determine whether the colocalization between rafts and large-scale clusters of ErbB2 was altered upon activation. The colocalization of ErbB2 clusters and lipid rafts was not significantly influenced by any of the above treatments, as evidenced by similar cluster overlap values ($77\pm6\%$ on control cells; $71\pm6\%$, $76\pm9\%$ and $73\pm9\%$ after stimulation with EGF, heregulin and 4D5, respectively). We have previously reported that the size of large-scale ErbB2 clusters increases upon stimulation of cells with EGF, heregulin or 4D5 (Nagy et al., 1999b). Thus, the fact that the overlap between large-scale ErbB2 clusters and lipid rafts did not change upon any of these

stimuli, suggested that the size of lipid rafts also increased after stimulation with EGF, heregulin or 4D5. This conclusion was confirmed by calculation of lipid raft diameter, which increased from 0.43 ± 0.02 μ m in control cells to 0.53 ± 0.02 μ m, 0.54 ± 0.03 μ m and 0.51 ± 0.03 μ m upon stimulation with EGF, heregulin and 4D5, respectively ($P<0.05$ for the increase; Fig. 3C). Since both the control and the treated samples were labeled with FITC-CTX-B and labeling was carried out on ice, we believe that the observed increase in raft size was not due to pentavalent binding of FITC-CTX-B.

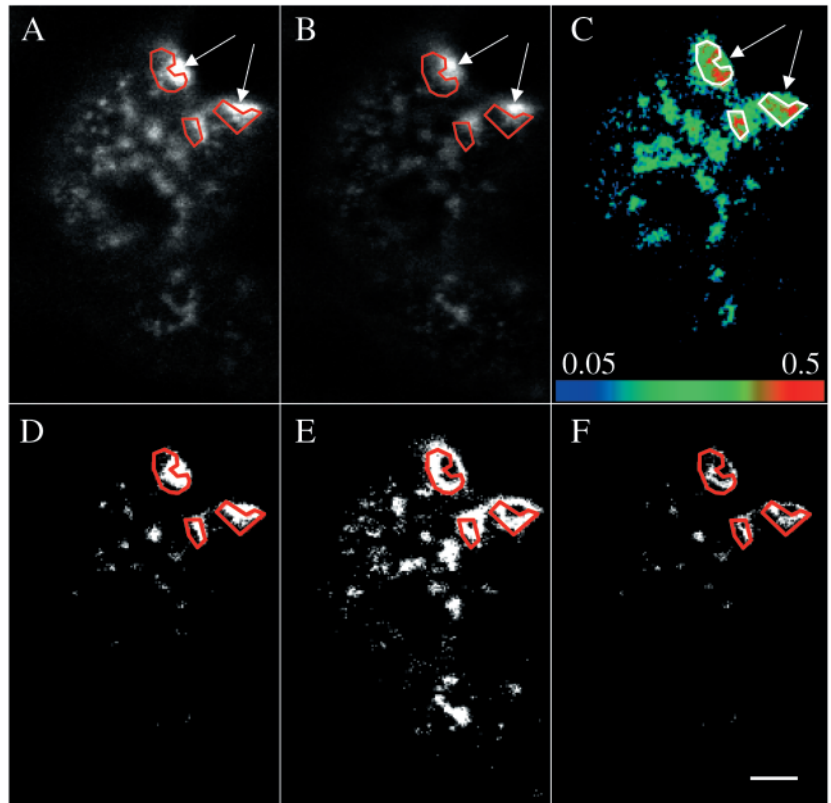


Fig. 2. Membrane areas with anomalously high ErbB2 homoassociation colocalize with membrane domains with high ErbB2 and comparatively low ErbB3 densities. SKBR-3 breast tumor cells were labeled with FITC-4D5 and Cy3-4D5 against ErbB2, and with unlabeled H3.90.6 anti-ErbB3 antibody followed by secondary labeling with a Cy5-tagged Fab. Images A and B show the fluorescence intensity distribution of Cy3-4D5 (ErbB2) and the Cy5-tagged secondary Fab (ErbB3), respectively. The FRET efficiency for ErbB2 homoassociation is displayed in image C. The areas inside the white polygons in image C have the highest FRET values. The same areas are marked with red polygons in images A, B, D-F. These areas have very high ErbB2 density and comparatively low ErbB3 density. The areas at the head of the arrows have the highest ErbB2 and ErbB3 densities, but ErbB2 homoassociation in these pixels is lower than inside the marked areas. Image A was thresholded, and pixels with high ErbB2 fluorescence intensity are white in image D, whereas pixels with intensity below the threshold are black. Image B was 'bi-thresholded': two intensity values were determined, and only pixels whose intensity is between the two threshold values are white in image E, all other pixels are displayed in black. Threshold values were adjusted so that white pixels in image E have low ErbB3 intensities, but still above a certain level. Pixels with intensity values lower than the lower threshold are background pixels, and they have to be excluded from the analysis. In image F a pixel is displayed in white if the pixel is white in both image D and E. The distributions of white areas in image F correlate with pixels with high ErbB2 homoassociation in image C. Bar, 1 μ m.

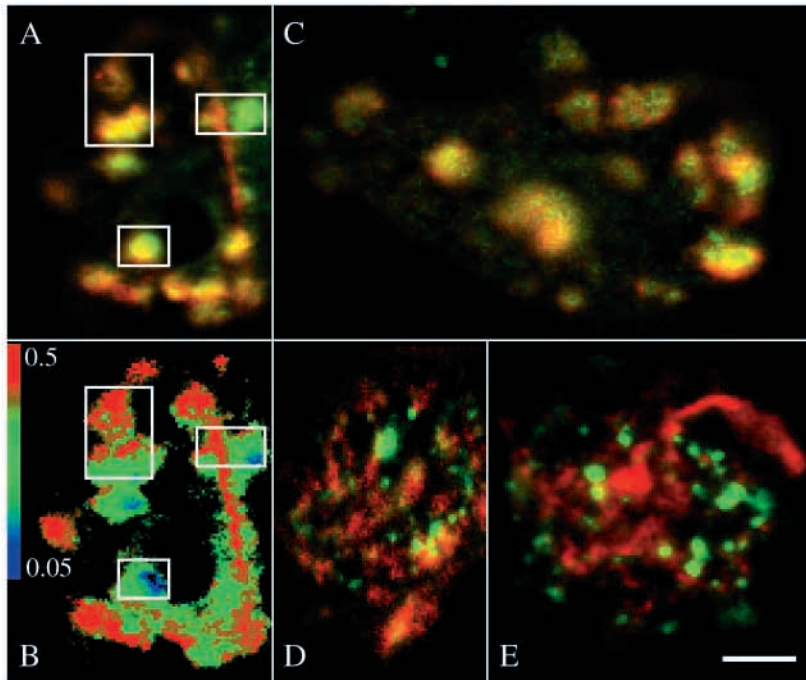


Fig. 3. The relationship between lipid rafts and the small- and large-scale clustering of ErbB2. (A,B) SKBR-3 cells were labeled with FITC-CTX-B, Cy3-4D5 and Cy5-4D5 Fab. The FITC (green) and Cy3 (red) channels are overlaid in image A. Yellow areas correspond to colocalization between CTX-B-labeled lipid rafts and ErbB2 clusters. The homoassociation of ErbB2 was calculated using the acceptor photobleaching FRET method, and the FRET efficiency is displayed in panel B. FRET efficiency is color-coded according to the color-scale. The white boxes mark areas with inverse correlation between the local CTX-B-labeling density and ErbB2 homoassociation; the greener the area is in image A, the lower the FRET efficiency is in the corresponding part of the cell in image B. (C) Heregulin-stimulated SKBR-3 cells were labeled with FITC-CTX-B and Cy3-4D5 Fab. (D) SKBR-3 cells were treated with FITC-CTX-B at 37°C for 30 minutes, and they were subsequently labeled with Cy3-4D5 Fab. (E) SKBR-3 cells were pretreated with FITC-CTX-B and then incubated with Cy3-4D5 antibody at 37°C for 30 minutes. In part A, C and E the FITC image (green) is overlaid on the Cy3 (red) image. Yellow areas correspond to significant colocalization between lipid rafts and ErbB2 clusters. Bar, 1 μ m.

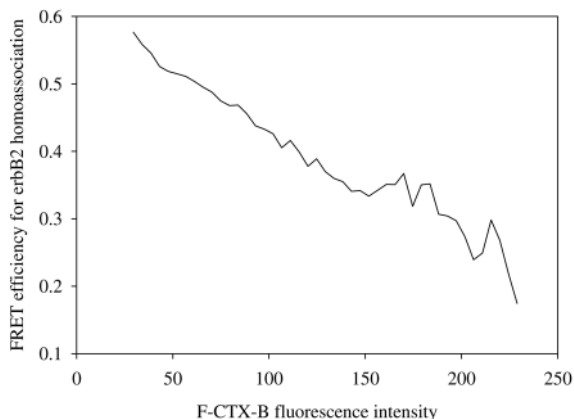


Fig. 4. Lipid rafts influence the homoassociation of ErbB2. Lipid rafts were visualized with FITC-CTX-B, and ErbB2 was labeled with Cy3-4D5 and Cy5-4D5 antibodies in order to measure its homoassociation with the acceptor photobleaching FRET method. FRET efficiency was averaged in pixels with the same FITC-CTX fluorescence intensity, and the average FRET efficiency is plotted as a function of lipid raft labeling.

CTX-B induces significant changes in the large-scale association of ErbB2

In view of the inverse correlation between the density of GM1 ganglioside and ErbB2 homoassociation, we investigated whether lipid raft crosslinking by CTX-B had any effect on the association of ErbB2. CTX-B not only binds GM1, but prolonged exposure of cells to CTX-B at 37°C can crosslink lipid rafts and induce characteristic changes in the distribution of membrane proteins (Harder et al., 1998). Cells were first treated with 8 μ g/ml FITC-CTX-B at 37°C for 30 minutes, and then labeled with Cy3-4D5 Fab against ErbB2 on ice. Analysis by confocal microscopy showed that the overlap

between lipid rafts and large-scale clusters of ErbB2 significantly ($P < 0.05$) decreased to $29 \pm 9\%$ (Fig. 3D), implying that crosslinking of GM1 by CTX-B sequestered these domains from ErbB2. By crosslinking GM1 CTX-B induces translational diffusion of lipid rafts and the accumulation of GM1 at sites of endocytosis (Nichols, 2002). One possibility is that cytoskeletal anchoring of ErbB2 might impede its movement with lipid rafts, resulting in ErbB2 being left behind. However, disrupting microfilaments with cytochalasin D treatment (10 μ g/ml, 2.5 hours, 37°C) did not perceptibly modify the CTX-B-induced separation between lipid rafts and large-scale clusters of ErbB2; the overlap between lipid rafts and large-scale clusters of ErbB2 was $28 \pm 5\%$ in CTX- and cytochalasin D-treated cells. Cytochalasin D by itself did not change the colocalization between ErbB2 clusters and lipid rafts, from which we conclude that cytoskeletal anchoring was not responsible for the inability of ErbB2 to move with lipid rafts.

Relationship between ErbB2 and caveolin clusters to GM1-enriched domains

Caveolae are considered to be specialized lipid rafts, that can be visualized with confocal microscopy (Kurzchalia and Parton, 1999). In order to determine whether FITC-CTX-B-labeled domains correspond to caveolae, we labeled SKBR-3 cells with an anti-caveolin antibody and FITC-CTX-B (Fig. 5A). There was an almost complete separation between caveolae and the domains labeled by CTX-B as evidenced by the low cluster overlap value ($13 \pm 4\%$). This result suggested that ErbB2 protein clusters, which colocalize with CTX-B, should not overlap with caveolae. Indeed, we found only a negligible colocalization between ErbB2 clusters and caveolae ($17 \pm 6\%$, Fig. 5B). However, CTX-B induced crosslinking of GM1 induced its migration into caveolae, leaving ErbB2 clusters behind (Fig. 5C).

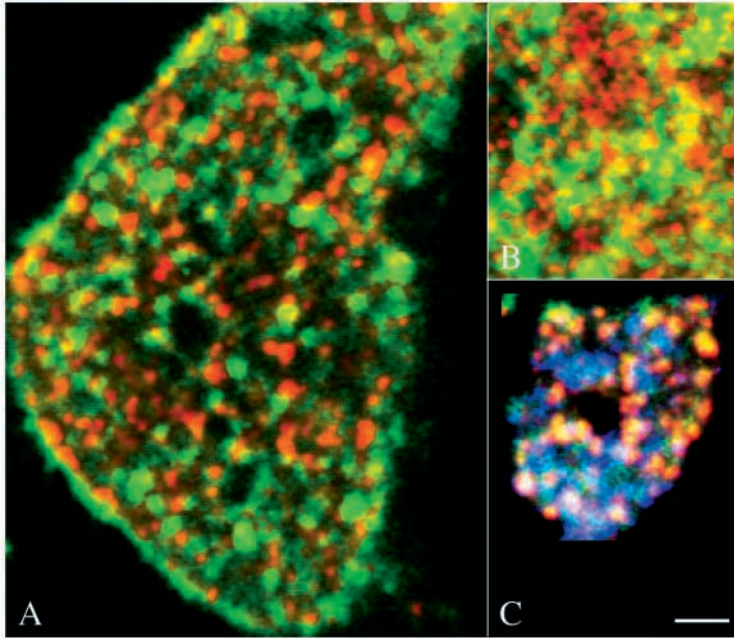


Fig. 5. Relationship between caveolin, GM1-enriched domains and ErbB2 clusters. (A) Quiescent SKBR-3 cells were labeled with FITC-CTX-B (green channel) and with anti-caveolin antibody sc-894 followed by secondary staining with Cy3-conjugated goat anti-rabbit immunoglobulin to visualize sc-894 labeling (red channel). (B) Quiescent SKBR-3 cells were stained against ErbB2 (with Alexa488-4D5, green channel) and against caveolin (as in part A, red channel). (C) SKBR-3 cells were treated with FITC-CTX-B for 30 minutes at 37°C (green channel), and then stained against ErbB2 (Cy5-4D5, blue channel) and against caveolin (as in part A, red channel). Bar, 1 μ m.

CTX-B-induced changes in the small-scale association of ErbB2

We investigated the small-scale association of ErbB2 in control and CTX-B-treated cells using flow cytometric FRET measurements. In these experiments, cells were treated with unlabeled CTX-B, and then labeled with donor- and acceptor-labeled antibodies against the ErbB proteins. The heteroassociation of ErbB2 with ErbB3 significantly decreased, but the homoassociation of ErbB2, and its heteroassociation with ErbB1 was not affected by CTX-B treatment (Fig. 6A). In control experiments, CTX-B treatment did not change the expression level of ErbB1, ErbB2 and ErbB3 implying that the measured changes in the FRET efficiencies reflect alterations in protein association. One possible origin for the decreased heteroassociation between ErbB2 and ErbB3 is that CTX treatment separates ErbB2 but not ErbB3 from lipid rafts. This would result in the separation of large-scale clusters of ErbB2 and ErbB3, which would make their heteroassociation impossible. However, confocal microscopy showed that both ErbB2 and ErbB3 remained in the same large-scale clusters after CTX treatment (data not shown).

ErbB2 and Shc tyrosine phosphorylation induced by EGF and heregulin in cells pretreated with CTX-B

Since a heterodimer of ErbB2 and ErbB3 forms a functional

heregulin receptor, we expected a decreased effect of this growth factor on CTX-B-pretreated SKBR-3 cells. Control and CTX-B-pretreated samples were incubated in the presence of heregulin or EGF. The tyrosine phosphorylation of ErbB2 was high even in non-stimulated cells, and neither EGF, nor heregulin was able to elicit a significant increase in ErbB2 tyrosine phosphorylation ($0.05 < P < 0.1$, Fig. 6B). In CTX-B-pretreated cells, heregulin induced a marked decrease in ErbB2 tyrosine phosphorylation, indicating a shift in the balance between phosphorylation and dephosphorylation towards the latter ($P < 0.01$ for the comparison between the heregulin-induced ErbB2 tyrosine phosphorylation of CTX-B-pretreated and non-treated samples). The lack of EGF-induced response was unchanged in CTX-B-pretreated cells (Fig. 6B). In order to observe a growth factor-elicited response in SKBR-3 cells, we searched for a target downstream of ErbB2 in the signal transduction cascade. The tyrosine phosphorylation of Shc was detectable even in unstimulated cells, and treatment with either of EGF, heregulin or CTX-B induced an increase in Shc tyrosine phosphorylation. Interestingly, both EGF and heregulin induced a decrease in Shc tyrosine phosphorylation when applied to CTX-B-pretreated cells (Fig. 6B).

CTX-B significantly alters the internalization and antiproliferative effect of 4D5

The 4D5 antibody is a partial agonist of ErbB2, which after inducing tyrosine phosphorylation of ErbB2, efficiently downregulates the protein and blocks cell proliferation (Sarup et al., 1991). We investigated whether treatment of SKBR-3 cells with CTX-B alters 4D5-induced ErbB2 downregulation and the cytostatic effect of the antibody. According to flow cytometric measurements, ErbB2 was efficiently downregulated by 4D5 (Fig. 6C), a finding consistent with previous reports (Sarup et al., 1991). Furthermore, the downregulation was significantly blocked by treatment of cells with CTX-B. Three flow cytometric methods were used to monitor the downregulation of ErbB2 (described in Materials and Methods), but only one of them is shown in Fig. 6C, since results from the other approaches were the same. Flow cytometry yields statistically reliable data but requires cells in suspension. Therefore, we also used confocal microscopy to detect the internalization of fluorescently labeled 4D5 antibody and confirmed that treatment of SKBR-3 cells with CTX-B blocks the accumulation of 4D5 in the cytoplasm (Fig. 6D). We found previously that CTX-B treatment segregates lipid rafts from clusters of ErbB2 (Fig. 3D). We wanted to determine how colocalization between lipid rafts and ErbB2 clusters is influenced by co-treatment with CTX-B and 4D5. The overlap between ErbB2 clusters and lipid rafts was as low as in the case of cells treated with CTX-B alone (Fig. 3E).

The 4D5 antibody blocks the proliferation of breast tumor cells overexpressing ErbB2, and this effect is often attributed to its ability to downregulate ErbB2. Therefore, we checked whether treatment of cells with CTX-B influences the cytostatic effect of 4D5. MAb 4D5 significantly blocked the

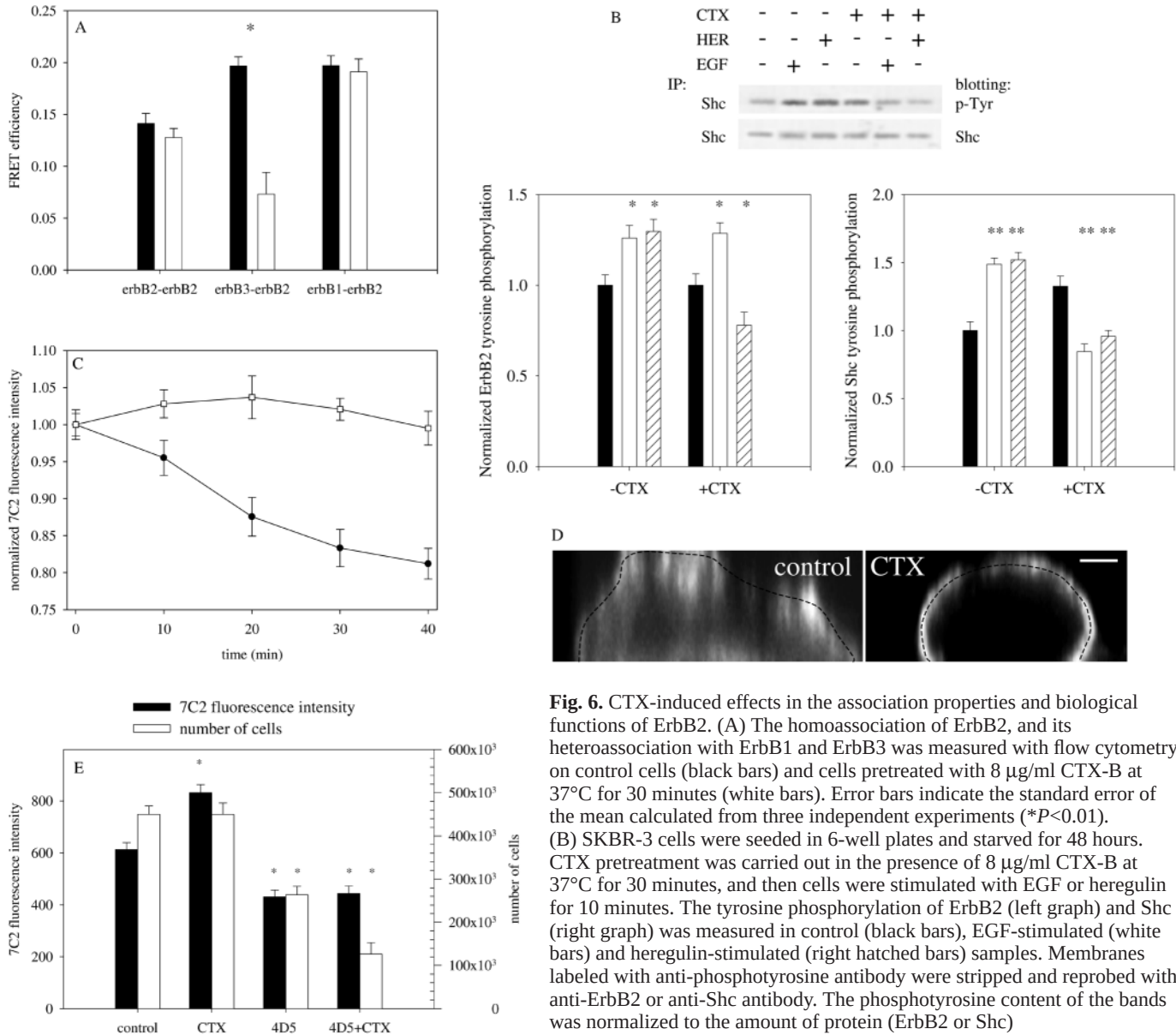


Fig. 6. CTX-induced effects in the association properties and biological functions of ErbB2. (A) The homoassociation of ErbB2, and its heteroassociation with ErbB1 and ErbB3 was measured with flow cytometry on control cells (black bars) and cells pretreated with 8 μ g/ml CTX-B at 37°C for 30 minutes (white bars). Error bars indicate the standard error of the mean calculated from three independent experiments (* P <0.01). (B) SKBR-3 cells were seeded in 6-well plates and starved for 48 hours. CTX pretreatment was carried out in the presence of 8 μ g/ml CTX-B at 37°C for 30 minutes, and then cells were stimulated with EGF or heregulin for 10 minutes. The tyrosine phosphorylation of ErbB2 (left graph) and Shc (right graph) was measured in control (black bars), EGF-stimulated (white bars) and heregulin-stimulated (right hatched bars) samples. Membranes labeled with anti-phosphotyrosine antibody were stripped and reprobed with anti-ErbB2 or anti-Shc antibody. The phosphotyrosine content of the bands was normalized to the amount of protein (ErbB2 or Shc) immunoprecipitated and to the control samples. Asterisks indicate statistical significance of Student's t -test performed on non-stimulated and growth

factor-stimulated samples (* P <0.1, ** P <0.05). Error bars indicate the standard error of the mean calculated from three independent measurements. (C) Control (●) and CTX-pretreated (□) cells were incubated with unlabeled 4D5 antibody for 40 minutes. Samples were taken every 10 minutes, and cell surface ErbB2 was labeled with Alexa488-7C2. The fluorescence intensity of Alexa488-7C2-labeled cells was measured with flow cytometry. Error bars indicate the standard error of the mean (n =3). (D) Control and CTX-pretreated SKBR-3 cells were incubated with Alexa488-4D5 antibody at 37°C for 30 minutes. Cells were imaged using confocal microscopy, and vertical slices of the cells are shown. The approximate position of the cell membrane is marked with a black dashed line (bar, 1 μ m). (E) 50,000 cells were seeded in culture dishes. Control SKBR-3 cells and cells treated with 4D5, CTX-B and both with 4D5 and CTX-B were cultured for 5 days. Treatment with the antibody and CTX-B was carried out as described in Materials and Methods. At day 5 the cell surface expression of ErbB2 was measured with flow cytometry by labeling cells with Alexa488-7C2. The black bars show the mean fluorescence intensity after subtraction of autofluorescence. The number of cells is shown by the white bars. Error bars indicate the standard error of the mean (* P <0.05 compared with the corresponding control, n =3).

proliferation of SKBR-3 cells, and it reduced the amount of ErbB2 on the cell surface (Fig. 6E). We expected that CTX-B would hinder the antiproliferative effect of 4D5 since it prevents the internalization of ErbB2 induced by 4D5. However, 4D5 was even more efficient in blocking the proliferation of SKBR-3 cells, if they were also treated with CTX-B. CTX-B did not significantly influence the proliferation

of cells on its own but increased the amount of ErbB2 on the cell surface (Fig. 6E).

Discussion

It has been known for years that overexpression of ErbB2 significantly alters the proliferation and motility of cells

(Spencer et al., 2000), and that it is associated with poor prognosis (Slamon et al., 1987). Although many of these effects are mediated by heterodimers between ErbB2 and other ErbB proteins, ErbB2 homodimers are also present on the cell surface, particularly in cells with *c-ErbB2* gene amplification and overexpression, and these homodimers confer phenotypic effects that are likely different from that of various ErbB heterodimers (Worthylake et al., 1999; Baekstrom et al., 2000). Although much has been learned about the association properties of ErbB2, these studies failed to address the question as to how local factors influence ErbB2 association. Considering that focal activation of receptors is often observed (Brock and Jovin, 2001) we investigated the distribution of ErbB2 homoassociation in single cells.

Correlation between the association of ErbB2 and ErbB3 and their expression densities

The homoassociation of ErbB2 correlated positively with the local concentration of ErbB2, but negatively with ErbB3 local density. Consequently, membrane areas with the highest ErbB2 homoassociation displayed high and low concentrations of ErbB2 and ErbB3, respectively. The positive correlation between the local density of ErbB2 and its homoassociation probably resulted from a shift in the equilibrium between monomers and dimers of ErbB2. It has already been suggested that overexpression of ErbB2 results in constitutive homodimerization of the protein, most probably induced by a shift in the monomer-dimer equilibrium (Chamberlin and Davies, 1998; Harris et al., 1999; Worthylake et al., 1999). Our findings conform with and reinforce these previous results and point to the possible existence of a monomer-dimer equilibrium within single cells. The negative correlation between ErbB2 homoassociation and ErbB3 local density suggests that ErbB2-ErbB3 heterodimers compete with ErbB2 homodimers and therefore a high number of ErbB3 proteins can disassemble ErbB2 homodimers. A similar conclusion has already been reached based on previous work carried out on whole cell populations (Tzahar et al., 1996), but our results directly support such a competition in single cells.

Role of large-scale clustering in ErbB2 homoassociation

We have previously identified a second hierarchical level of ErbB2 association termed large-scale clustering (Nagy et al., 1999b). These large-scale clusters of ErbB2 contain $\sim 10^3$ ErbB2 proteins and their diameter is $\sim 0.5 \mu\text{m}$ in unstimulated SKBR-3 cells. We found that the extent of ErbB2 homoassociation inside clusters is not significantly different from that outside clusters. The fact that ErbB2 homoassociates even outside clusters, where the local density of the protein is low, suggests that it very avidly forms homodimers. Based on the positive correlation between the local density of ErbB2 and its homoassociation one would expect the extent of ErbB2 homoassociation to be higher inside clusters than outside. We can offer two alternative explanations. (1) The local concentration of ErbB3 is also higher inside clusters (Fig. 2B). Since ErbB3-ErbB2 dimers are preferentially formed (Tzahar et al., 1996), the higher number of ErbB3 proteins can efficiently counterbalance the effect of the increased density of ErbB2. (2) Large-scale clusters of ErbB2 colocalize with lipid

rafts, where the lipid environment decreases ErbB2 homoassociation (Figs 3, 4). Lipid rafts contain a high amount of lipids with saturated fatty acids, cholesterol and sphingolipids, and present an environment for ErbB2 that is markedly different from the bulk lipid phase. This environment might alter the association properties of ErbB2, as previously reported for another membrane protein (Vereb et al., 2000).

Involvement of lipid rafts in the association of ErbB2

According to our results ErbB2 is preferentially localized in lipid rafts. Fluorescent labeling of caveolin revealed that GM1-enriched membrane domains do not colocalize with caveolae, but crosslinking of GM1 by CTX-B induces migration of GM1 into caveolae (Fig. 5). It has been reported that GM1 appears in separate, non-caveolar membrane domains (Vyas et al., 2001), but accumulates in caveolae after crosslinking by CTX (Orlandi and Fishman, 1998; Nichols, 2002). Our results are in agreement with the above, and corroborate the existence of distinct classes of raft-like membrane domains.

Identification of a membrane protein as raft-associated is usually based on its presence in the detergent-insoluble phase of the membrane. However, this criterion is of uncertain value, because it necessitates disruption of the plasma membrane and is dependent on the nature of the detergent used (Jacobson and Dietrich, 1999). Labeling with CTX-B is accepted as a reliable raft marker (Harder et al., 1998), and application of microscopy-based approaches for the identification of raft-associated proteins has the advantage of dealing with intact plasma membranes (Jacobson and Dietrich, 1999; Kenworthy et al., 2000). The size of the domains identified by FITC-CTX-B labeling is larger than the currently accepted size of lipid rafts. However, it is acknowledged that the size and nature of lipid rafts depend upon the method of investigation (Edidin, 2001). We believe that FITC-CTX-B labeling reliably identifies lipid rafts or clusters of them.

In contrast to GPI-anchored proteins, which co-patch with CTX-B (Harder et al., 1998), lipid rafts are sequestered from ErbB2 after crosslinking with CTX-B implying a weaker interaction between raft lipids and ErbB2. Segregation of ErbB2 from lipid rafts after CTX-B treatment is not inhibited by disruption of microfilaments suggesting that cytoskeletal anchoring does not influence the raft-association of the protein. We propose that crosslinking of rafts initiates their translational diffusion in the plasma membrane, and that GPI-anchored proteins follow these motions. However, ErbB2, which has a lower lateral diffusion constant due to its transmembrane and intracellular domains, may fail to 'keep up' with the more dynamic rafts, also suggesting a less stable localization of ErbB2 in lipid rafts compared to GPI-anchored proteins.

The finding that ErbB2 is dislodged from lipid rafts and gets into the bulk lipid phase upon CTX-B treatment offered a possibility for investigating the effects of lipid environment changes on the association properties and biological function of ErbB2. Treatment of cells with CTX-B decreased the heteroassociation of ErbB2 with ErbB3, leading to inhibited tyrosine phosphorylation of Shc upon treatment with heregulin, the ligand of the ErbB2-ErbB3 heterodimer. Although the anti-Shc antibody we used recognizes both the 46 and 52 kDa forms of Shc, present in SKBR-3 cells (Le et al., 2000), we could only detect a single band. We cannot offer a rationale for this

result or for the lack of growth factor-induced tyrosine phosphorylation of ErbB2. It has already been observed by other authors that heregulin induces a hardly detectable increase in the tyrosine phosphorylation of ErbB2 (Le et al., 2000). This may be caused by the very high level of overexpression of ErbB2 in this cell line leading to self-activation of the protein (Worthylake et al., 1999). Our cell line may also be slightly different from the one used by Le and co-workers (Le et al., 2000). Neither ErbB2 homoassociation nor its heteroassociation with ErbB1 was affected by treatment with CTX-B. The fact that the EGF-induced tyrosine phosphorylation of ErbB2 was the same in control and CTX-B-pretreated cells accords nicely with this finding. However, the EGF-induced tyrosine phosphorylation of Shc was inhibited in CTX-B-pretreated cells. Previous reports suggested that Shc activation takes place inside rafts (Plyte et al., 2000), leading to the logical conclusion that ErbB2 located outside lipid rafts may not be able to activate Shc.

Internalization of ErbB2 in CTX-B-pretreated cells

Internalization of ErbB2 induced by 4D5 was significantly inhibited in cells pretreated with CTX-B. The antiproliferative effect of 4D5 is thought to be related to ErbB2 internalization followed by intracellular degradation (Sarup et al., 1991; Klapper et al., 2000; Levkowitz et al., 2000). Therefore, we expected that inhibition of ErbB2 internalization by CTX-B would be associated with decreased downregulation of ErbB2, and consequently with a decrease in the antiproliferative effect of 4D5. However, the antiproliferative effect of 4D5 was even higher in cells treated with CTX-B, suggesting that antibody-induced short-term internalization may not be a good marker for the selection of antibodies with antiproliferative effect. This proposal is in agreement with data obtained by Neve and co-workers (Neve et al., 2001), who used a single chain antibody that downregulates ErbB2 very efficiently without exerting any effect on either MAPK activity or cell proliferation. The increased cell surface expression of ErbB2 in cells treated with CTX-B alone may reflect decreased internalization of ErbB2 with a consequent shift in the equilibrium between transport of ErbB2 to and from the membrane. We do not know the reason for the increased cytostatic effect of 4D5 observed in cells treated with CTX-B. It is possible that although CTX-B slows down activation-induced internalization of ErbB2, small amounts of ErbB2 might still get internalized and degraded by a cbl-mediated pathway resulting in reduced cell proliferation (Klapper et al., 2000).

Model for the role of lipid rafts and the local density of ErbB proteins in their association

ErbB proteins form homo- and heterodimers at the molecular level and large-scale clusters in the membrane. The latter colocalize with GM1-enriched membrane domains, but are distinct from caveolae. GM1 leaves ErbB protein clusters behind upon crosslinking by CTX-B and migrates into caveolae (Fig. 7). A high local density of ErbB proteins in clusters favors their spontaneous activation, which is kept under control by the special lipid environment of rafts, that decreases ErbB2 homoassociation. A role of lipid rafts in limiting the autoactivation of the ErbB signaling system is

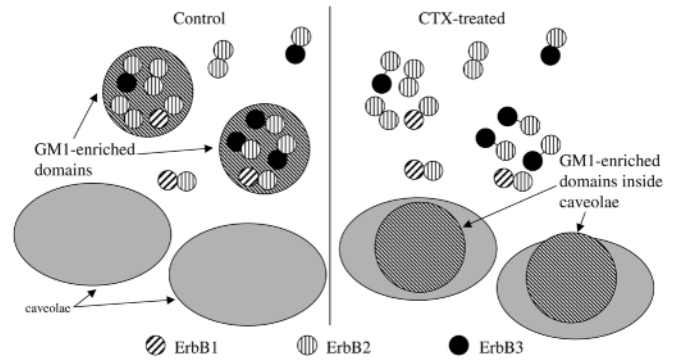


Fig. 7. Model for the association of ErbB proteins inside and outside clusters. The extent of ErbB2-ErbB2 and ErbB2-ErbB3 associations depend on the relative expression levels of the proteins: high local ErbB3 density decreases ErbB2 homoassociation. Lipid rafts, which were identified as GM1-enriched domains labeled by CTX-B in our studies, colocalize with ErbB protein clusters. Although the high local concentration of ErbB proteins inside clusters favors their spontaneous activation (e.g. the formation of highly active ErbB2 homodimers), lipid rafts keep the activation of ErbB2 under control by limiting the homodimerization of ErbB2. By contrast, ErbB proteins are maintained in a signaling competent form inside rafts (i.e. heregulin-responsive ErbB2-ErbB3 heterodimers are present), which are disassembled if ErbB proteins are removed from them. CTX-B treatment induces migration of GM1-enriched domains into caveolae and disrupts ErbB2-ErbB3, but not ErbB2-ErbB2 or ErbB2-ErbB1 dimers.

supported by the increased tyrosine phosphorylation of Shc after CTX-B treatment (Fig. 6B), which removes ErbB proteins from rafts (Fig. 3D). On the other hand, lipid rafts are also responsible for maintaining ErbB proteins in a growth factor responsive state. This is supported by the following results. (1) Neither EGF, nor heregulin is able to activate Shc, if ErbB proteins are removed from lipid rafts (Fig. 6B). (2) The formation of heregulin-responsive ErbB2-ErbB3 heterodimers and heregulin-induced ErbB2 tyrosine phosphorylation decrease, if ErbB proteins are removed from lipid rafts (Fig. 6A,B). These results parallel the suggested role of lipid rafts in T-cell-receptor-mediated signaling, where rafts are thought to be the storage sites of antigen responsive, inactive signaling components (Germain, 2001).

In summary, we have provided evidence for the role of local factors in influencing ErbB2 homoassociation. Temporal fluctuations in these factors may generate local inhomogeneities in ErbB2 homoassociation and local anomalies in signal transduction. In addition to a more thorough understanding of the complexity of the ErbB network, these results may facilitate the development of more efficient therapeutic strategies for breast cancer.

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Small interfering RNAs suppress the expression of endogenous and GFP-fused epidermal growth factor receptor (erbB1) and induce apoptosis in erbB1-overexpressing cells

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Abstract

Deregulated and excessive expression of epidermal growth factor receptor (EGFR or erbB1), a transmembrane receptor tyrosine kinase specific for the epidermal growth factor (EGF), is a feature and/or cause of a wide range of human cancers, and thus inhibition of its expression is potentially therapeutic. In RNA interference (RNAi), duplexes of 21-nucleotide RNAs (small interfering RNA, siRNA) corresponding to mRNA sequences of particular genes are used to efficiently inhibit the expression of the target proteins in mammalian cells. Here we show that by using RNAi the expression of endogenous erbB1 can be specifically and extensively (90%) suppressed in A431 human epidermoid carcinoma cells. As a consequence, EGF-induced tyrosine phosphorylation was inhibited and cell proliferation was reduced due to induction of apoptosis. We established an inverse correlation between the level of expressed erbB1 and EGF sensitivity on a cell-by-cell basis using flow cytometry. A431 cells expressing endogenous erbB1 were transfected with erbB1 fused C-terminally to enhanced green fluorescent protein (EGFP). Selective inhibition of the expression of the fusion protein was achieved with an siRNA specific for the EGFP mRNA, whereas the erbB1-specific siRNAs inhibited the expression of both molecules. siRNA-mediated inhibition of erbB1 and other erbB tyrosine kinases may constitute a useful therapeutic approach in the treatment of human cancer.

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Keywords: EGF receptor; erbB1; Inhibition of proliferation; RNA interference

Introduction

Cancer cells frequently overexpress growth factor receptors, resulting in both enhanced proliferation and dependence of tumor progression on the overexpression phenotype [1]. The erbB family of growth factor receptors are transmembrane receptor tyrosine kinases (RTKs) that are involved in the development and progression of a wide range of human cancers [2]. The four members of the family (erbB1–4) engage in a hierarchical network of interactions resulting in signal diversification [3]. Inhibition of the activity of one family member often induces profound effects by subverting the coordinated interactions within the network [4]. Thus, the erbB molecules constitute ideal targets

in cancer therapy [5]. For example, a monoclonal antibody inhibits proliferation of erbB2-overexpressing cancer cells [6], and its humanized form (trastuzumab, Herceptin) is being employed in the successful immunotherapy of breast cancer [7,8].

The epidermal growth factor receptor (EGFR or erbB1) is likewise overexpressed in a wide range of cancers [9] and thus constitutes another important therapeutic target [2,10]. A monoclonal anti-erbB1 antibody, cetuximab, is undergoing promising clinical trials in the treatment of lung and of head and neck cancers [11], as are a variety of small molecule ATP binding site inhibitors [5,12]. Although the detailed understanding of the biology and biochemistry of erbB proteins has led to the introduction of such receptor-targeted drugs in the treatment of human cancers [5], new approaches and combined therapies are required due to therapeutic failure in individual cases and the evolution of drug resistance [13,14].

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In addition to therapeutic approaches targeting the expressed proteins directly, their biosynthesis can also be inhibited, as exemplified by antisense oligonucleotides specific for erbB1 [15]. However, difficulties related to stability or specificity of the oligonucleotides or problems in target sequence selection hamper the widespread use of this approach [16]. More promising is the selective degradation of the corresponding mRNAs by RNA interference (RNAi), a process that avoids the global depression of protein synthesis induced by the double-stranded RNA-inducible interferon system present in mammalian cells [17]. In one implementation of RNAi, selective degradation of target mRNAs in mammalian cells is achieved by transfection with double-stranded, short interfering RNAs (siRNAs), leading to rapid and efficient degradation of the target [17]. Expression of siRNAs under the control of an RNA polymerase III promoter provides the means for achieving long-term suppression of protein synthesis [18,19].

RNAi of transmembrane proteins, and of RTKs in particular, has not been reported to date. Consideration of the importance of the erbB family in human cancers and the efficiency of RNAi led us to design siRNAs against erbB1 and test their effects on the proliferation and EGF-induced responses of erbB1-(over)expressing cells. We show that siRNA-induced suppression of erbB1 expression results in significant inhibition of EGF-induced tyrosine phosphorylation and in induction of apoptosis in A431 human epidermoid carcinoma cells, a cell line widely used in the study of erbB1 because it expresses this RTK to a level of $\sim 2 \times 10^6$ [20]. The inhibitory effect of high concentrations of EGF on the proliferation of A431 was suppressed in siRNA-treated cells displaying a pronounced decrease in the expression level of erbB1. In A431 cells transfected to express an erbB1-EGFP (EGFP, enhanced green fluorescent protein) fusion protein in addition to the endogenous erbB1, inhibition of both molecules was achieved with an siRNA directed against the RTK moiety whereas selective suppression of the fusion protein ensued with an siRNA specific for the EGFP segment of the mRNA. The successful application of siRNA for inhibition of proliferation in erbB1-overexpressing cells extends the list of available therapeutic modalities in the treatment of human cancer.

Materials and methods

Cell culture and transfection

Normal and stably transfected CHO, A431, and HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS and penicillin/streptomycin (Invitrogen, Karlsruhe, Germany) in 5% CO₂. Cells were reseeded at a confluency of 30–60% in the absence of antibiotics 1 day before transfection. Transfection with plasmids and siRNAs were carried out with Lipofectamine2000 and Oligofectamine (Invitrogen), respectively, according to

the manufacturer's specifications. Chemically synthesized siRNAs were kindly provided by Thomas Tuschl (Combinatorial Biochemistry Group, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany) and were used at a concentration of 100 nM in transfections.

Cells were kept in the absence of serum for 24–48 h before EGF stimulation. For flow cytometric evaluation cells were trypsinized, and trypsinization was stopped with 10% BSA to prevent possible activation of cells by serum.

Western blotting

Cells in a single well of a 24-well plate were lysed in SDS sample buffer, and proteins were separated on 7% polyacrylamide gels and transferred to nitrocellulose membranes. Anti-erbB1 monoclonal antibody F4 (E3138, Sigma, Schnelldorf, Germany) and anti-actin monoclonal antibody AC40 (A4700, Sigma) were used at 2000- and 500-fold dilutions, respectively, followed by peroxidase-conjugated goat anti-mouse immunoglobulin for immunoblotting. Enhanced chemiluminescence (Amersham, Freiburg, Germany) was used for detection.

Fluorescence microscopy

Cells in 24-well plates were fixed in 3.7% formaldehyde for 30 min on ice. Labeling with antibodies was carried out in the presence of 0.1% BSA and 0.1% Triton X-100. Anti-erbB1 mouse monoclonal antibody F4 (Sigma) anti-erbB1 rabbit polyclonal antibody sc-03 (Santa Cruz, Heidelberg, Germany), and anti-phosphotyrosine mouse monoclonal antibody sc-7020 (Santa Cruz) were used at 500-fold, 200-fold, and 1000-fold dilutions, respectively, followed by appropriate fluorescent secondary antibodies. Dual-labeling of erbB1 and phosphotyrosine was done with sc-03 and sc-7020, followed by labeling with Alexa488-goat anti-rabbit IgG and Cy3-goat anti-mouse IgG. No crossreaction of the antibodies was detected. Image processing was done with ImageJ (NIH, Bethesda, MD).

Measurement of cell cycle distribution and apoptosis

Cells were trypsinized and fixed in 70% ethanol at 4°C overnight. After rehydration cells were labeled with an anti-erbB1 antibody as described above and stained with 100 µg/ml propidium iodide (Sigma) in the presence of 100 µg/ml RNase for 60 min at 37°C followed by flow cytometric analysis. We confirmed the results obtained with this procedure with an alternative method based on staining cells in hypotonic fluorochrome solution [21]. Cell cycle analysis was carried out with the program Cylchred (Department of Hematology, College of Medicine, University of Wales, Cardiff, UK).

Table 1
siRNAs against erbB1 and EGFP

siRNA	Sense/antisense strands	Starting position of target sequence ^a
erbB1-1	5'-CUCUGGAGGAAAAGAAAGUTT-3' 3'-TTGAGACCUCCUUUCUUUCA-5'	316
erbB1-2	5'-CGUAAAGGAAUACACAGGGTT-3' 3'-TTGCAUUUCCUUUAGUGUCCC-5'	1436
erbB1-3	5'-CACAGUGGAGCGAAUCCUTT-3' 3'-TTGUGUCACCCGCUUAAGGA-5'	527
GFP-2	5'-GAACGGCAUCAAGGUGAACTT-3' 3'-TTCUUGCCGAGUCCACUUG-5'	1089 (476) ^b

^a The starting positions of target sequences corresponding to the 5' ends of the antisense strands of erbB1 and GFP siRNAs are given for the GI 12002211 and GI 1373318 sequences, respectively, relative to the first nucleotide of the published sequences.

^b The number in parentheses refers to the position of the target sequence in the GFP sequence in the GI 1373318 sequence.

Flow cytometry

Trypsinized cells were fixed in 3.7% formaldehyde (30 min on ice). Labeling of erbB2 was carried out with Mab OP15 (Calbiochem, Bad Soden, Germany) followed by staining with an appropriate secondary antibody. Labeling antibodies and conditions for erbB1 and phosphotyrosine staining were the same as those described above, except that secondary labeling of sc-7020 anti-phosphotyrosine antibody was performed with Cy5-tagged goat anti-mouse IgG. Flow cytometric analysis was with a Coulter Epics Elite flow cytometer equipped with a 488-nm argon ion laser and with a 633-nm HeNe laser. Receptor numbers were determined using Qifikit (DAKO, Hamburg, Germany). General flow cytometric data analysis was carried out using FCS Express (Denovo Software, Thornhill, Ontario, Canada). Two-parameter histograms are presented as contour plots [22].

Secondary staining of erbB1 on cells expressing erbB1–EGFP was achieved using Mab F4 and Cy5-labeled anti-mouse IgG. Since the F4 antibody labeled both endogenous erbB1 and erbB1–EGFP, corrections were made to the amount of endogenous erbB1 according to the following equation: endogenous erbB1 = $I_{Cy5} - C \cdot I_{EGFP}$, where I_{Cy5} and I_{EGFP} are the fluorescence intensities detected in the Cy5 and EGFP channels, respectively, C is a constant describing the ratio of Cy5 to EGFP signals of erbB1–EGFP, as determined on CHO cells expressing erbB1–EGFP in the absence of endogenous erbB1. Parameter calculations were made in an Excel spreadsheet. In order to discriminate cells with normal and low erbB1 expression two Gaussian peaks were fitted to the histograms with Origin (Microcal, Northampton, MA), and the percentages of cells in the two subpopulations were calculated from the areas of the two peaks.

Results

Inhibition of expression of transfected EGFP and erbB1 in CHO cells

We designed three double-stranded, 21-nucleotide-long siRNAs with TT dinucleotide 3' overhangs against the coding sequence of erbB1 (GI 12002211) and one siRNA against EGFP (GI 1373318) (Table 1, Fig. 1). Chinese hamster ovary (CHO) cells were transfected with an EGFP plasmid (pAD3) [23] or cotransfected with pAD3 and the GFP-2 siRNA. The ratio of positive cells was determined by fluorescence microscopy. The ratio of EGFP-positive cells decreased from 55% in cells transfected with plasmid alone to 6% in cells cotransfected with the GFP-2 siRNA (Figs. 2A and B). We also transfected CHO cells with an erbB1–EGFP plasmid [24], in which the coding sequence of EGFP is fused to that of the C-terminus of erbB1 (Fig. 2C). Cotransfection of the erbB1–EGFP plasmid with the erbB1-1 siRNA resulted in a 90% reduction in the number of positive cells compared to transfection with the plasmid alone (Fig. 2D). We conclude that the GFP-2 and the erbB1-1 siRNAs can efficiently inhibit the expression of EGFP and erbB1–EGFP, respectively, in transfected CHO cells.

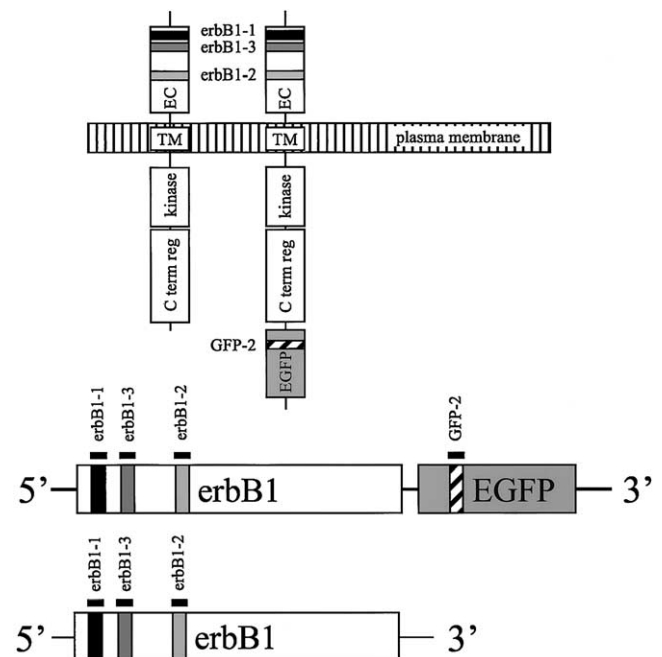
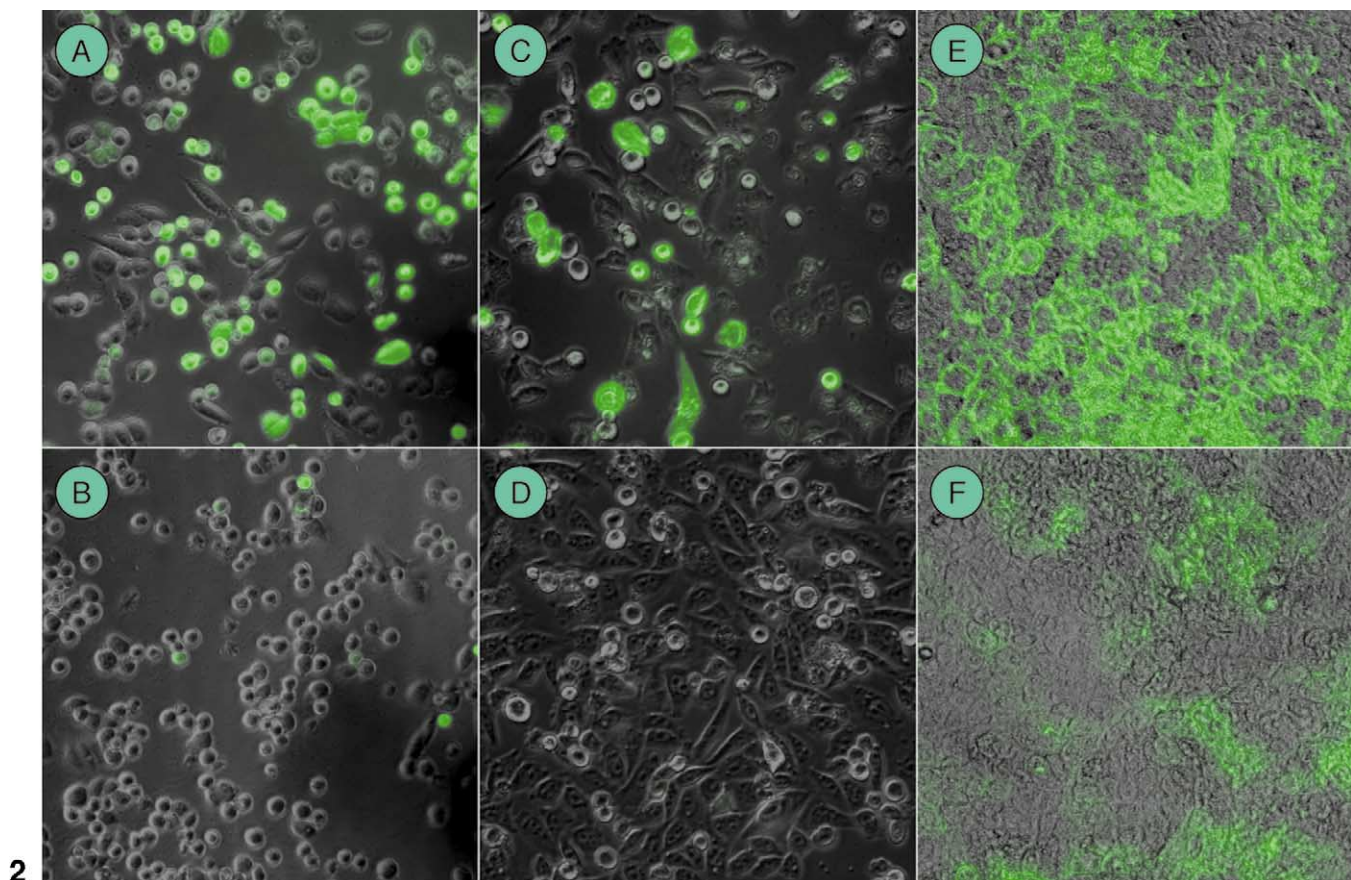
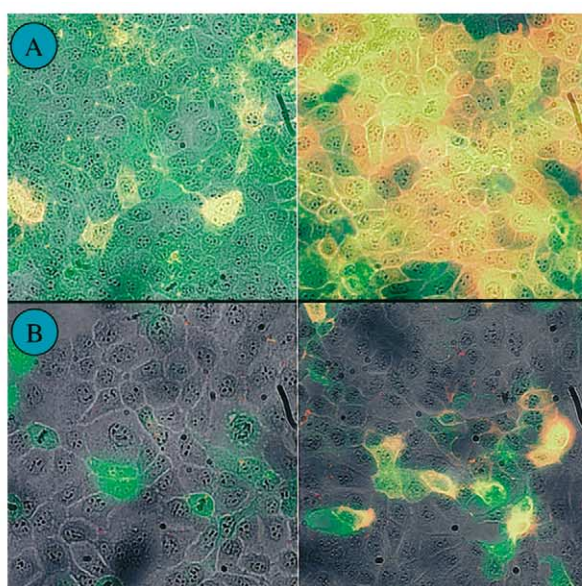


Fig. 1. Sequences and protein domains targeted by the erbB1 and GFP siRNAs. Simplified structures of the endogenous erbB1 and the erbB1–EGFP protein (top) and mRNA (bottom) are shown (EC, extracellular domain; TM, transmembrane domain; C term reg, C terminal regulatory domain). The target sequences of the siRNAs and the corresponding segments in the proteins are indicated.



2



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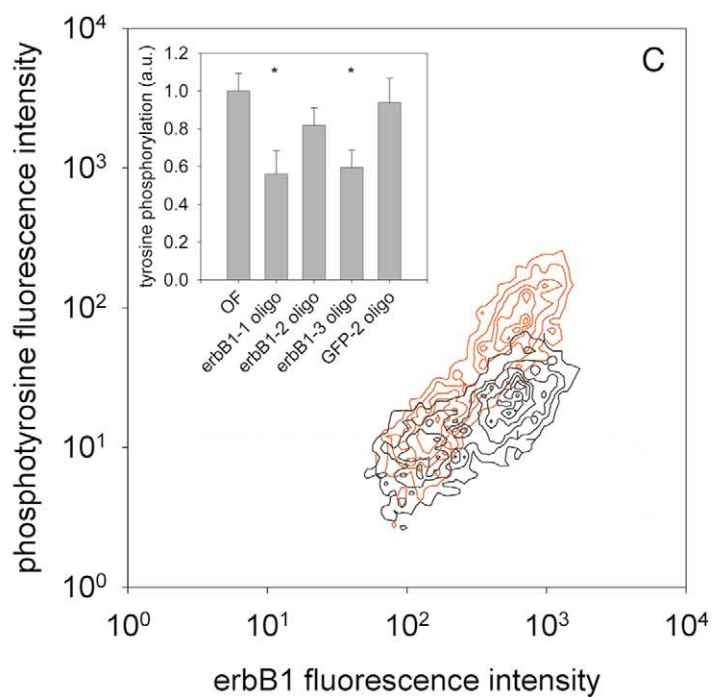


Fig. 2. siRNA-mediated inhibition of the expression of EGFP, erbB1, and erbB1-EGFP in CHO and A431 cells. Phase-contrast and overlaid fluorescence images taken 48 h after transfection are shown. CHO cells were transfected with the plasmid for EGFP (A) or erbB1-EGFP (C) or cotransfected with the EGFP plasmid and GFP-2 siRNA (B) or with erbB1-EGFP plasmid and erbB1-1 siRNA (D). A431 cells (E) and erbB1-1 siRNA-transfected A431 cells (F) were stained with an erbB1-specific antibody.

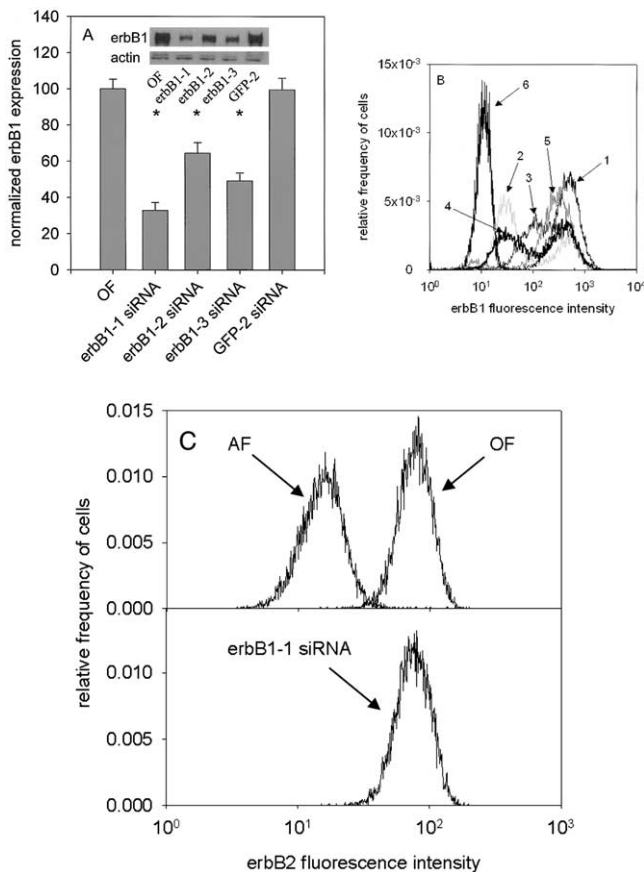


Fig. 3. Quantitative evaluation of inhibition of erbB protein expression in A431 cells evaluated by Western blotting and flow cytometry. (A, B) A431 cells were treated with oligofectamine (curve 1 in part B) and transfected with erbB1-1 siRNA (curve 2), erbB1-2 siRNA (curve 3), erbB1-3 siRNA (curve 4), or an irrelevant siRNA (curve 5). ErbB1 expression was measured by Western blotting (A) and by flow cytometry (B) 48 h after transfection. Curve 6 in B displays cells stained with the secondary antibody alone. The Western blot was stained with an erbB1-specific antibody, then stripped, and re probed with an antibody against actin. The expression of erbB1 was normalized by that of actin (A). Asterisks indicate significant difference from the oligofectamine-treated sample ($P < 0.05$). (C) A431 cells were transfected with the erbB1-1 siRNA or treated with oligofectamine only (OF) and stained with an anti-erbB2 antibody 48 h after transfection. The curve marked with AF displays oligofectamine-treated cells stained with secondary antibody only.

Inhibition of the expression of endogenous erbB1 in A431 cells

RNAi also served to suppress the expression of endogenous erbB1 in A431 epidermoid carcinoma cells. We trans-

fected A431 cells with the three different erbB1 siRNAs, each of which was effective in reducing the expression of erbB1 according to fluorescence microscopy (Figs. 2E and F), Western blotting (Fig. 3A), and flow cytometric analysis (Fig. 3B). Western blotting applied to a heterogeneous cell population can be misleading in such a system due the residual fraction of untransfected cells. In contrast, flow cytometry has the virtue of yielding information about protein expression on a cell-by-cell basis with high statistical accuracy. Thus, it was possible to distinguish between untransfected and transfected (suppressed) cells and to accurately evaluate the specific RNAi effect on the latter (Fig. 3B). With both erbB1-1 and erbB1-3 siRNAs the histograms of cells with normal and inhibited erbB1 expression were well separated, permitting the determination of the fraction of suppressed cells and the expression levels of erbB1. The erbB1-1 and erbB1-3 siRNAs suppressed erbB1 expression in 69 ± 5 and $48 \pm 4\%$ (mean $\pm$ SD) of the cells, respectively. Compared to control A431 cells expressing $2 \times 10^6 \pm 5 \times 10^4$ erbB1 molecules per cell, the erbB1-1 and erbB1-3 siRNA-suppressed populations displayed only $1.6 \times 10^5 \pm 3 \times 10^4$ ($8 \pm 2\%$ of control A431 cells) and $1.8 \times 10^5 \pm 4 \times 10^4$ ($9 \pm 2\%$ of control cells) erbB1 receptors per cell, respectively. The number of receptors per cell was determined for the various cell populations by flow cytometric analysis based on calibrated microspheres (Qifikit, DAKO). Control experiments with the transfecting agent alone or a nonspecific siRNA demonstrated a negligible effect on erbB1 expression (Figs. 3A and B). Although the sequences of the erbB1 siRNAs were chosen so as to be specific for erbB1, we determined the effect of transfection with erbB1 siRNAs on the expression of a closely related protein, erbB2 (Fig. 3C), in A431 cells. None of the siRNAs had any significant influence on the expression of erbB2 (data shown for the erbB1-1 siRNA only). From the above control experiments we conclude that the RNAi effects were specific.

EGF-induced tyrosine phosphorylation is inhibited in A431 cells transfected with the erbB1 siRNAs

Tyrosine phosphorylation is pivotal in erbB1-mediated signal transduction [25] and inhibition of the kinase activity leads to reduced cell proliferation [26]. Therefore, we tested whether the RNAi-mediated suppression of erbB1 expression results in reduced tyrosine phosphorylation. A431 cells transfected with the erbB1 siRNAs, grown in the absence of

Fig. 4. Inhibition of EGF-induced tyrosine phosphorylation in erbB1 siRNA-transfected cells. Control (A) and erbB1-1 siRNA-transfected (B) A431 cells were stimulated with 1 ng/ml EGF for 5 min. The left and right panels show unstimulated and EGF-stimulated A431 cells, respectively. Cells were stained with an erbB1-specific antibody (green channel) and with a phosphotyrosine specific antibody (red channel), and overlaid fluorescence and phase-contrast images are shown. (C) Quiescent (black contours) and EGF-stimulated (red contours) erbB1-1 siRNA-transfected cells were stained with erbB1- and phosphotyrosine-specific antibodies and analyzed with flow cytometry. The insert in part C shows the quantitation of the flow cytometric results. Oligofectamine-treated and siRNA-transfected cells were stimulated with 1 ng/ml EGF and the fluorescence intensity of phosphotyrosine-specific antibodies was measured. Asterisks indicate a significant difference from the oligofectamine-treated sample ($P < 0.05$). Cells were serum-starved for 48 h prior to EGF stimulation.

serum for 48 h, were stimulated with 1 ng/ml EGF for 5 min at 37°C. Staining with erbB1- and phosphotyrosine-specific antibodies revealed that all erbB1 siRNAs inhibited EGF-induced tyrosine phosphorylation significantly (Figs. 4A and B; results for erbB1-1 siRNA). In the case of erbB1-2 siRNA, the lesser inhibition of erbB1 expression and the lower statistical reliability of fluorescence microscopy led us to perform parallel experiments using flow cytometry. Control and siRNA-transfected A431 cells were trypsinized and stimulated with 1 ng/ml EGF in suspension. Only the erbB1-1 and erbB1-3 siRNA inhibited EGF-induced tyrosine phosphorylation to a significant extent (Fig. 4C). The two methods of analysis are not entirely comparable in that the cells had to be removed from their growth substrate for flow cytometry, and perturbation of the cytoskeletal/extracellular matrix is known to activate protein kinases directly [27]. However, there was a clear correlation between the basal level of tyrosine phosphorylation and the expression of erbB1 in cells transfected with the erbB1-1 siRNA (Fig. 4). Cells transfected by siRNA exhibited a reduction in both erbB1 expression and tyrosine phosphorylation. Furthermore, cells with lower erbB1 expression did not respond to EGF stimulation at all, whereas untransfected cells with a high level of erbB1 expression in the transfected sample displayed a level of tyrosine phosphorylation similar to that of control cells (Fig. 4C).

Proliferation and EGF-induced cell cycle responses are inhibited in erbB1 siRNA-transfected cells

EGF exerts a concentration-dependent effect on A431 cells, at 0.1 ng/ml promoting cell proliferation, but at 1 ng/ml inducing cell cycle arrest [28]. We determined whether siRNA-mediated inhibition of erbB1 expression interferes with the cell cycle arrest and proliferation-inducing effects of EGF and/or with cell proliferation in the presence of serum. All erbB1 siRNAs blocked the proliferation of A431 cells in 10% serum and in 0.1 ng/ml EGF equally well (by ~80%, Fig. 5A), i.e., regardless of the quantitative differences in erbB1 expression. An irrelevant siRNA did not significantly affect erbB1 expression (Fig. 3), but reduced cell proliferation to some extent (by ~30%), indicating that the transfection with siRNAs may partially and nonspecifically inhibit cell proliferation. To further investigate the specificity of the siRNA effects, we transfected HeLa cells, expressing ~70000 erbB1 molecules [29], with the siRNAs. The transfection efficiency with the erbB1 siRNAs determined by flow cytometry was very high (~100%) and resulted in a ~75% decrease in the expression of erbB1 (not shown). Each erbB1 siRNA also reduced cell proliferation by ~20–25% (not shown). Transfection of HeLa cells with the GFP-2 siRNA reduced cell proliferation by ~20%, an extent similar to that observed with A431 cells. We conclude that although the erbB1 siRNAs knock down protein expression, none exerted a specific effect on the proliferation of HeLa cells. We also tested the influence

of the erbB1 siRNAs on the proliferation of a cell line (CHO) lacking erbB protein expression. The various siRNAs had only a negligible effect on the proliferation of CHO cells (data not shown).

We next investigated the cell cycle changes induced by the siRNAs in order to elucidate the mechanism of the erbB1 siRNA-induced decrease in cell proliferation. We observed a significant increase in the apoptotic fraction in cells transfected with the erbB1-1 siRNA compared to untransfected cells (Fig. 5B), whereas the cell cycle distribution of the nonapoptotic fraction was unaffected. Similar changes were observed with the other erbB1 siRNAs (not shown).

In contrast to the effect of all erbB1 siRNAs on cell proliferation, only the erbB1-1 and erbB1-3 siRNAs prevented the cell cycle arrest induced by 1 ng/ml EGF (Fig. 5A). These data correlated well with the efficiencies with which the siRNAs inhibit tyrosine phosphorylation induced by the same concentration of EGF (Fig. 4C). EGF at 1 ng/ml preferentially arrested the cells that were not transfected in the sample exposed to the erbB1 siRNA, as shown in the flow cytometric analysis of the populations (Fig. 5C). Thus, the fraction of the population exhibiting suppression by the erbB1-1 siRNA in the EGF-treated sample was ~30% larger than that in the untreated one (Fig. 5C).

The expression of erbB1–EGFP is selectively suppressed in an endogenous erbB1-expressing background by EGFP-targeted siRNAs

Transfection of cells with GFP constructs is an established technique in cell biology. Selective manipulation of the expression of the transfected GFP constructs and of endogenous genes would be invaluable, but is difficult to achieve. We generated a clone of A431 cells (A4-erbB1), which expresses 1.3×10^6 erbB1–EGFP molecules/cell in addition to endogenous erbB1 (1.8×10^6 receptors/cell) [24]. Transfection of A4-erbB1 cells with the erbB1 siRNAs decreased the expression of both the endogenous erbB1 and the erbB1–EGFP proteins (Fig. 6B). In contrast, the GFP-2 siRNA only inhibited the expression of the erbB1–EGFP construct (Fig. 6C).

Discussion

ErbB1 has long been considered an important oncoprotein in the development of human malignancies and is one of the emerging targets in tumor therapy [2]. Although considerable progress has been made in the application of oncoprotein-targeted antibodies and small molecule tyrosine kinase inhibitors, none of these agents is curative [5]. In an effort to find new approaches to target erbB1-overexpressing cancer cells, we set out to characterize the RNAi-induced knock-down of endogenous and transfected erbB1.

We achieved a significant inhibition of erbB1 expression

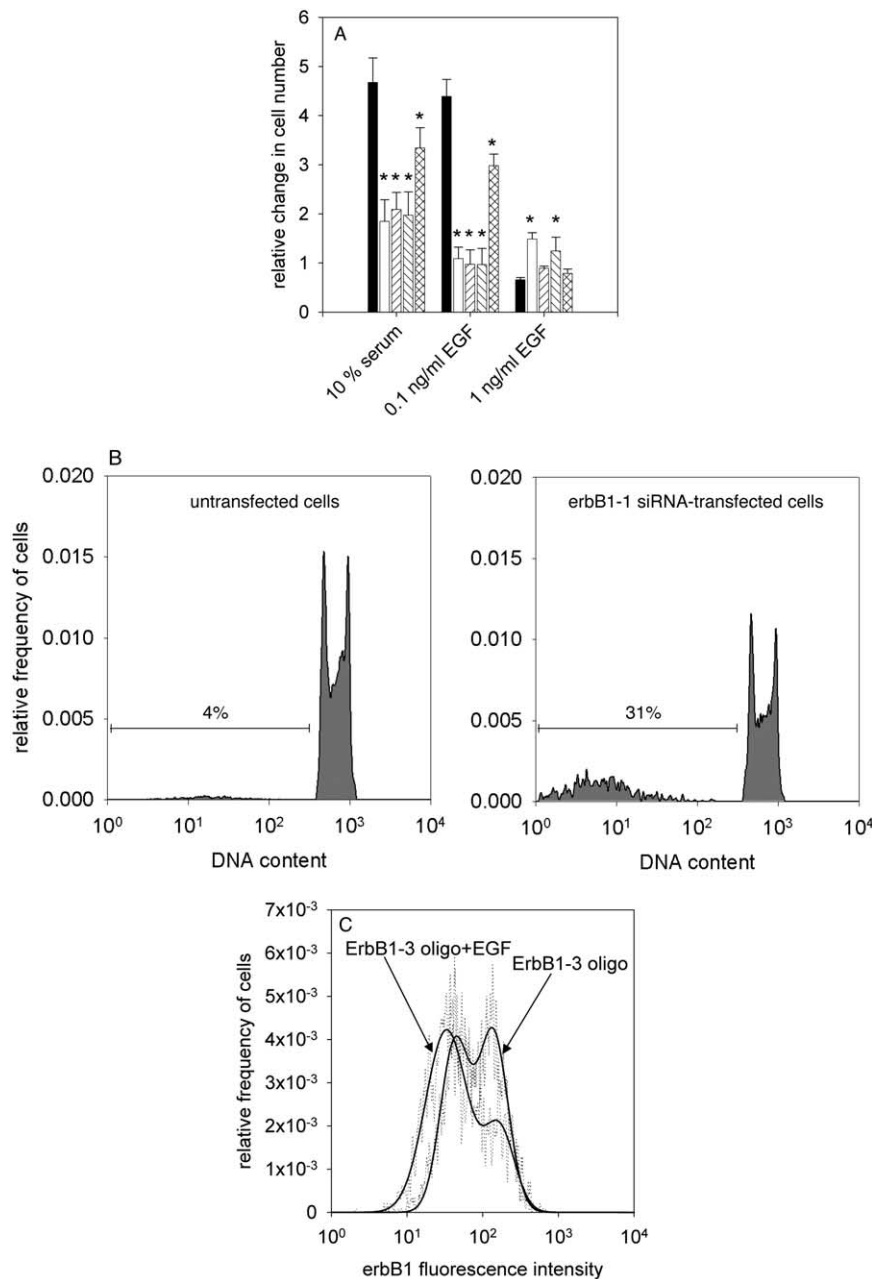


Fig. 5. ErbB1-specific siRNAs interfere with EGF-induced cell cycle changes. (A) A431 cells were treated with oligofectamine only (black) or transfected with erbB1-1 siRNA (white), erbB1-2 siRNA (left-hatched), erbB1-3 siRNA (right-hatched), or an irrelevant siRNA (GFP-2 siRNA, cross-hatched). At 12-h posttransfection cells were serum starved for an additional 24 h and then treated for 48 h as indicated on the horizontal axis before counting total cell number with a Coulter counter. The relative change in cell number ($\pm$ SD) over the 48-h period after serum starvation is plotted. Asterisks indicate a significant difference from the oligofectamine-treated sample ($P < 0.05$). (B) A431 cells were transfected with the erbB1-1 siRNA. Eight hours after transfection they were serum-starved for 24 h and then cultured in the presence of 10% FCS for another 24 h. Afterward they were stained with an anti-erbB1 antibody and propidium iodide. The graphs display the DNA content of the nontransfected (left) and knocked down (right) subpopulation, respectively. (C) Flow cytometric analysis of A431 cells transfected with erbB1-3 siRNA. At 12-h posttransfection cells were serum-starved for 24 h, and then half of the population was stimulated with 1 ng/ml EGF for 24 h. Flow cytometric histograms (dotted curves) and Gaussian fits thereof are shown for both EGF-treated and nontreated cells.

in A431 epidermoid carcinoma cells using three different siRNAs targeted to the coding region of the erbB1 mRNA (Figs. 2 and 3). Suppression of the expression of erbB1–EGFP with siRNAs against the erbB1 or EGFP part of the

mRNA was dramatic in both transiently and stably transfected cells. The degree to which the expression of a given protein lends itself to inhibition by RNA interference depends on the half-life and synthesis rate of the protein. The

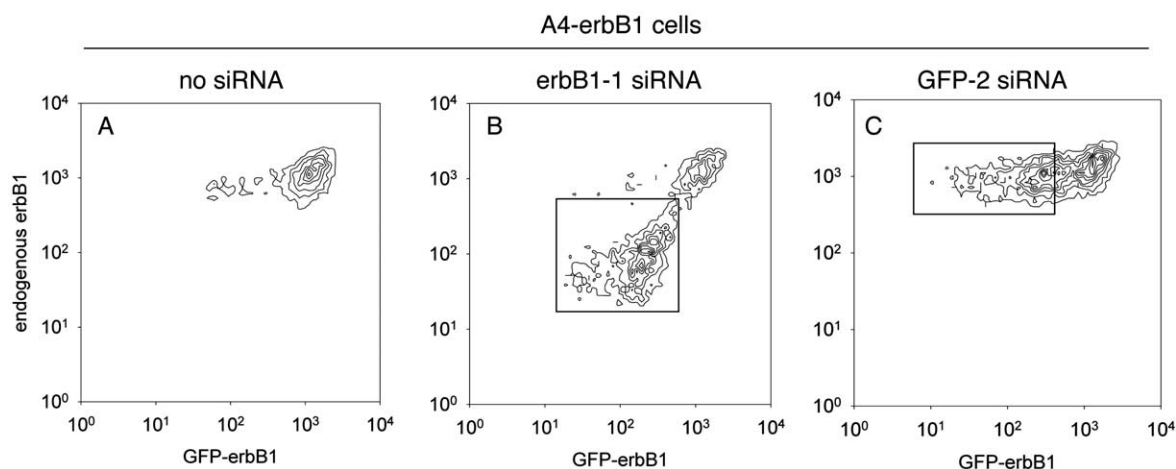


Fig. 6. Suppression of endogenous and erbB1–EGFP expression in A4-erbB1 cells. A4-erbB1 cells were treated with oligofectamine (A) or transfected with erbB1-1 siRNA (B) or GFP-2 siRNA (C). Flow cytometric contour plots were recorded 48 h after transfection. The rectangles indicate the position of the suppressed populations. In B, both endogenous erbB1 expression and EGFP–erbB1 expression were inhibited, whereas in C only the expression of erbB1–EGFP was inhibited.

turnover of erbB1 is considered to be much faster than that of other erbB proteins [30], implying that the latter may be more difficult to suppress using RNAi, because even if the biosynthesis of the protein is blocked, previously synthesized molecules persist in the plasma membrane for a relatively long time. Therefore, combination of RNAi with other approaches for downregulating erbB proteins, e.g., antibody [31] or tyrosine kinase inhibitors [32], may have additive or synergistic effects. The abundance of a target mRNA is also expected to influence the efficiency of RNAi-induced suppression of protein production. The fact that we successfully inhibited the expression of erbB1 in A431 cells in which the erbB1 gene is amplified 30-fold [33] provides evidence for the high potency of the RNA interference phenomenon [34].

We used both Western blotting and flow cytometry to measure the expression level of erbB1. The results from these two approaches were in quantitative agreement, but the heterogeneous expression of erbB1 after transfection of cells with siRNA rendered the Western blotting results misleading. Although the erbB1-1 siRNA induces a 90% reduction in erbB1 expression in cells actually transfected by the siRNA, the overall reduction of erbB1 production, detected by Western blotting, was only ~70%, reflecting the weighted average of the protein production of siRNA-transfected and nontransfected cells. Therefore, the use of single-cell measurement techniques is to be encouraged, especially in the case of cell populations in which the fraction of transfected cells is low.

Although the expression of erbB1 in A431 cells was significantly reduced by RNAi, the suppressed cells still expressed $1.5\text{--}2 \times 10^5$ erbB1 receptors per cell even in the case of the most effective siRNAs. The fact that these cells showed no increase in generalized tyrosine phosphorylation detectable with flow cytometry (Fig. 4C) suggests that an incomplete inhibition of erbB1 expression is sufficient to

abolish growth factor-mediated signaling. Growth factor receptors appear to be clustered in submicrometer membrane microdomains [35], and receptor activation is believed to be largely [36], but not exclusively [37], confined to sites where receptors encounter their respective ligands. The activities of protein tyrosine kinases are counterbalanced by protein tyrosine phosphatases [38]. Therefore, activation of the tyrosine phosphorylation response may be completely switched off if the number of erbB1 molecules drops below a given threshold, since the balance of tyrosine phosphorylation and dephosphorylation may be shifted toward the latter process to a degree that switches off downstream signaling, i.e., via the MAPK cascade [39].

Only the two most efficient siRNAs (erbB1-1 and erbB1-3) suppressed both tyrosine phosphorylation (detected by flow cytometry) and the apoptosis/cell cycle arrest induced by a high concentration of EGF (1 ng/ml; Figs. 4C and 5A). On the other hand, all erbB1 siRNAs were equally effective in blocking cell proliferation in the presence of serum or at a low concentration (0.1 ng/ml) of EGF (Fig. 5A). The induction of proliferation and apoptosis/cell cycle arrest may be differentially sensitive to the amount of residual erbB1 expression. That is, the least efficient erbB1 siRNA (erbB1-2) may block the low-dose, but not the high-dose, effects of EGF.

At first sight it may seem that the inhibitory effect of the erbB1 siRNAs on the EGF-induced proliferation inhibiting response is weak. However, the number of cells increased 1.5-fold in the erbB1-1 siRNA-transfected sample treated with 1 ng/ml EGF, which is close to the 1.8-fold increase observed in the erbB1-1 siRNA-transfected sample in the presence of 10% FCS. That is, the growth inhibitory effect of 1 ng/ml EGF was virtually completely inhibited. The protective effect of erbB1 siRNA transfection against the proliferation inhibiting effect of a high concentration of EGF is also supported by the flow cytometric measurement

of the relative ratios of the subpopulations with normal and inhibited erbB1 expressions. Cells in which erbB1 expression was low due to transfection with the erbB1 siRNA were protected from the apoptosis/cell cycle arrest-inducing effect of 1 ng/ml EGF. Therefore, the fraction of this subpopulation in the EGF-treated sample was larger than in the control (Fig. 5C).

Inhibition of the activity of erbB1 by small molecule tyrosine kinase inhibitors or antibodies reduces cell proliferation either by blocking cell cycle progression from the G1 phase [12,40] or by inducing apoptosis [41,42]. A431 cells transfected with the erbB1 siRNAs showed a rate of apoptosis significantly higher than that of control cells, implying a primarily cytotoxic rather than a cytostatic effect of these agent.

The antiproliferative effect of erbB1 siRNAs has obvious implications for cancer therapy. It is known that overexpression of erbB1 is associated with poor prognosis in many epithelial cancers including head and neck, breast, colon, and lung tumors [43]. Overexpression of erbB1 leads to activation of the MAPK pathway and a high proliferation index [44]. Although erbB1 is expressed in adult tissues and participates in physiological processes such as wound healing [45], it is expected that cells with a very high expression of the receptor show enhanced sensitivity to inhibition of erbB1 expression or function. Anti-erbB1 antibody treatment is well tolerated in patients [46], but treatment with erbB1-specific tyrosine kinase inhibitors does have side effects [47] probably related to the inhibition of erbB1 activity in normal tissues [44]. Therefore, it is important to determine how cells with different levels erbB1 expression react to siRNA-mediated inhibition of erbB1 synthesis. We found that the proliferation of siRNA-treated A431 cells with a residual expression of $\sim 2 \times 10^5$ erbB1 proteins was inhibited, whereas that of HeLa cells expressing $\sim 12 \times 10^4$ copies of the protein was not. Our results suggest that cells with high erbB1 expression show enhanced sensitivity to the knock-down of erbB1 expression compared to cells with a lower expression level. This finding is in agreement with that of Sirotnak et al. [48], who reported a correlation between erbB1 expression and the sensitivity of cell proliferation to an erbB1-specific tyrosine kinase inhibitor. However, contradictory results have also been published [12]. We did not find evidence for a significant effect of erbB1 siRNAs on the proliferation of CHO cells that do not express erbB1. This finding suggests that the erbB1 siRNAs do not inhibit cell proliferation nonspecifically. Taken together, these results imply that although erbB1 is essential for physiological processes in adults, cancer cells with deregulated erbB1 expression exhibit an increased sensitivity to erbB1-targeted drugs, thereby increasing the therapeutic index of such treatments. This circumstance may render erbB1 siRNAs effective agents in the treatment of erbB1-overexpressing cancers.

Selective suppression of the expression of a GFP-tagged transfected protein is a desirable experimental goal in cases

where modulation of the expression level of the GFP construct is required. In this study, we were able to selectively inhibit erbB1–EGFP expression in A4-erbB1 cells expressing endogenous erbB1 (Fig. 6). This result would seem to argue against the generation of secondary siRNAs specific for regions 5' from the target sequence of the original siRNA [49]. However, even if secondary siRNAs were to be generated, selective suppression of certain GFP constructs should still be feasible, given the pronounced directionality and distance dependence of the process [50].

In summary, we have shown that erbB1-specific siRNAs mediate efficient and selective inhibition of erbB1 expression in cells expressing erbB1 as an endogenous or ectopic protein. The degree of suppression was sufficient to inhibit cell proliferation and cell cycle arrest of A431 epidermoid carcinoma cells induced by low and high levels of EGF, respectively, mirroring the lack of EGF-induced tyrosine phosphorylation. RNAi-mediated inhibition of erbB1 function holds great promise for the treatment of human cancer in conjunction with tyrosine kinase inhibitors and monoclonal antibodies [5]. We are presently extending the approach to other members of the erbB family.

Acknowledgments

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Quantum dot ligands provide new insights into erbB/HER receptor-mediated signal transduction

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The erbB/HER family of transmembrane receptor tyrosine kinases (RTKs) mediate cellular responses to epidermal growth factor (EGF) and related ligands. We have imaged the early stages of RTK-dependent signaling in living cells using: (i) stable expression of erbB1/2/3 fused with visible fluorescent proteins (VFPs), (ii) fluorescent quantum dots (QDs) bearing epidermal growth factor (EGF-QD) and (iii) continuous confocal laser scanning microscopy and flow cytometry. Here we demonstrate that EGF-QDs are highly specific and potent in the binding and activation of the EGF receptor (erbB1), being rapidly internalized into endosomes that exhibit active trafficking and extensive fusion. EGF-QDs bound to erbB1 expressed on filopodia revealed a previously unreported mechanism of retrograde transport to the cell body. When erbB2-monomeric yellow fluorescent protein (mYFP) or erbB3-monomeric Citrine (mCitrine) were coexpressed with erbB1, the rates and extent of endocytosis of EGF-QD and the RTK-VFP demonstrated that erbB2 but not erbB3 heterodimerizes with erbB1 after EGF stimulation, thereby modulating EGF-induced signaling. QD-ligands will find widespread use in basic research and biotechnological developments.

The erbB family of RTKs includes erbB1 (the classical EGF receptor, EGFR), erbB2, erbB3 and erbB4¹. Activation of these transmembrane proteins initiates signaling cascades controlling numerous cellular processes such as DNA replication and division^{1,2}. Ligand binding leads to auto- and transactivation of cytoplasmic protein kinase domains in response to the formation³ or reorientation^{4–6} of homo- and heteroassociated RTK monomers. The fate of the activated receptors is complex: endocytosis via coated pits, covalent modification (deactivation by enzymatic dephosphorylation and ubiquitination), and endosomal trafficking leading to proteosomal and/or lysosomal degradation or recycling to the plasma membrane. The overexpression and/or unrestrained activation of the erbB family, particularly erbB2, are implicated in many types of cancer^{1,7}. We show here that QDs bearing natural ligands as effector molecules provide the means for prolonged real-time visualizations of the multiple steps in signaling mechanisms in living cells, resulting in detailed movies of the processes involved. This capability has important implications for biotechnological screening and assay strategies.

For the studies reported here, biotinylated EGF was bound to commercial QDs with bioconjugated streptavidin⁸. QDs provide unique advantages for cellular imaging: (i) a very high and uniform brilliance, permitting detection down to the single nanoparticle level and reliable quantification of binding and transport phenomena, (ii) photostability, allowing imaging over prolonged periods, (iii) a broad excitation spectrum, allowing the simultaneous excitation of enhanced green fluorescent protein (eGFP) and QDs, (iv) a very narrow emission band

conveniently red-shifted to the fluorescence of the VFPs and (v) the high biochemical stability and specificity, low background and adjustable stoichiometry (ligands/QD) afforded by the biotin-streptavidin system. This study adds to the list: (vi) ligand compliance, the noninterference of the QD with the biochemical specificity of an attached ligand and (vi) steric compatibility, for example, the ability of two QD-ligands to bind to the two monomers of a dimeric RTK.

The formation of homo- and heterodimers and higher order oligomers of the erbB proteins is regarded as an essential feature of the signaling mechanism^{3,9,10}, although characterization of these protein-protein interactions on and in living cells has proven difficult. The central dogma featuring a ligand-induced dimerization as an obligatory intervening step³ has been reinforced by recent crystallographic structures of isolated^{11–14} and growth factor-liganded¹⁵ erbB ectodomains, revealing homodimers stabilized by interdomain and multiple ligand-ligand contacts (erbB1). Crystallographic evidence for heterodimers is as yet unavailable. However, numerous studies of intact RTKs in their cellular environment^{4,5,16–18} provide evidence for the existence of pre-formed, that is, ligand-free, homo- and heterodimers. Thus, in addition to the dramatic ligand-induced conformational transitions, it is possible that the activation mechanism may also involve monomer-monomer rearrangements—for example, via transverse rotational motions^{4,5}—in preexisting dimeric or heterotetrameric^{6,19} complexes. The latter could be stabilized via interactions between the transmembrane receptor domains²⁰. These considerations and reports of the preferential formation of heterodimers²¹ with a reduced internaliza-

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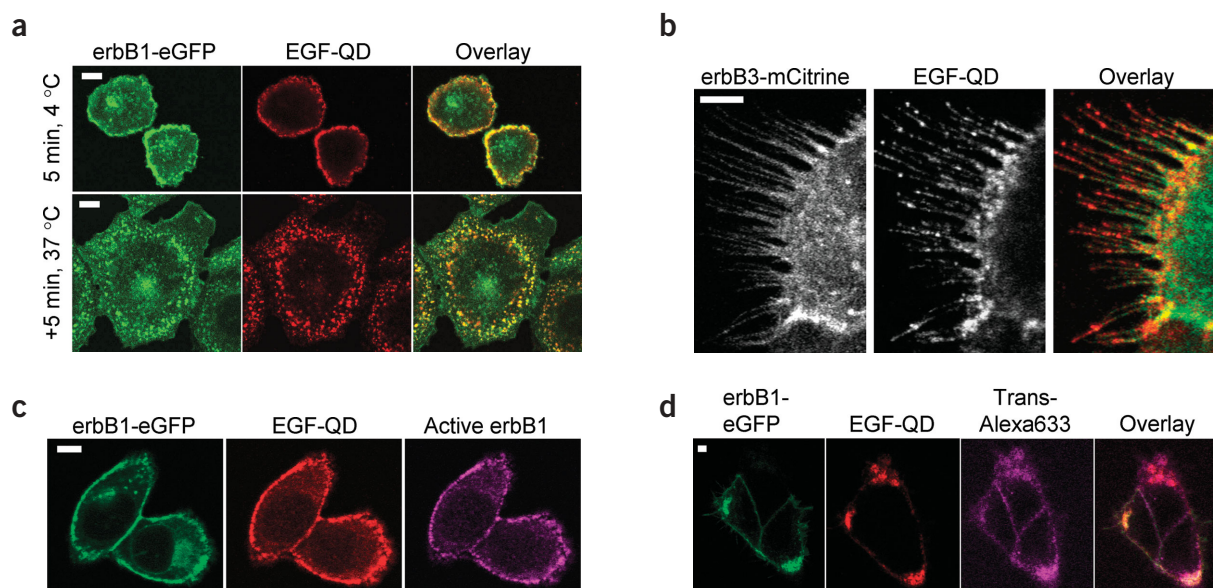


Figure 1 QDs bind and activate EGFR, and are endocytosed into living cells. **(a)** CHO cells expressing erbB1-eGFP, exposed to 7 nM biotin-EGF for 10 min at 4 °C, and then labeled with QDs. Lower panels, additional 5 min at 37 °C. Extended focus overlays of four 0.3 μm optical sections. Resolution: 0.31 μm/pixel. Scale bars, 20 μm. **(b)** Binding of 200 pM preformed 6:1 EGF-QDs to A431 cells expressing erbB3-mCitrine for 5 min at room temperature followed by methanol fixation at -20 °C. Images low pass-filtered. Resolution: 0.1 μm/pixel. Scale bar, 5 μm. **(c)** CHO cells expressing erbB1-eGFP exposed to purified 1 nM preformed 6:1 EGF-QDs at 23 °C for 15 min, fixed and stained with Cy5-anti-activated erbB1. Resolution: 0.11 μm/pixel. Scale bar, 5 μm. **(d)** A431 cells expressing erbB1-eGFP, 20 min after simultaneous addition of EGF-QD and transferrin-Alexa633, showing colocalization of erbB1-eGFP, EGF-QD and transferrin-Alexa633 in early endosomes. Scale bar, 5 μm.

tion rate²² and high ligand affinity²³ have prompted further studies of structure-function relationships in the erbB family. We have addressed these issues by using a combination of VFP-tagged erbB1/2/3 and a quantum dot-ligand complex (EGF-QD) to track by *in vivo* microscopy the binding of EGF to erbB1 and the dependence of its subsequent fate on interactions with erbB2 and erbB3. We will refer to such interactions generically as “homo- or heterodimerization.” The functional conclusions derived from these studies are independent of the presence and detection of potential higher order oligomers.

RESULTS

EGF-QDs bind and activate erbB1 and are internalized

We labeled cell surface erbB1-eGFP with streptavidin-QDs according to two protocols. In the first, we exposed cells to biotinylated EGF, then washed and labeled them with QDs (Fig. 1a). At 4 °C (upper row), we observed extensive colocalization of the red QDs with the complex of biotin-EGF and erbB1-eGFP. A brief exposure to 37 °C (lower row) led to rapid and extensive endocytosis of both erbB1-eGFP and QDs, which colocalized in endosomal structures. Whereas residual, unlabeled erbB1-eGFP remained on the cell surface, the membrane was cleared completely of QDs. In the second protocol, used in the remainder of this study, we preloaded the QDs with biotin-EGF. Each QD carries 10–15 streptavidin molecules, according to the manufacturer. Thus, the very high inherent stability of the biotin-streptavidin complex ensures that the desired degree of loading with a ligand is obtained by simply adjusting the mixing stoichiometry, which varied between 0.1 and 30 biotin-EGF/QD in our experiments. The required purity was achieved by pre- and/or post-separation of the loaded QDs (see Methods). The sensitivity of detection extended down to individual QDs, enabling the use of very low (e.g., 2 pM) bulk concentrations of the reagent and visualization of the very earliest stages of binding

and clustering. Single QDs were detected bound to cell filopodia (Fig. 1b) on human epidermoid carcinoma A431 cells expressing erbB3-mCitrine and high levels (>10⁶ molecules/cell) of endogenous erbB1 (ref. 24). Individual QD detection was confirmed by the observation of QD ‘blinking’ and by intensity comparisons with known single QD samples.

EGF-QDs readily activated erbB1 (Fig. 1c), establishing that the small nanoparticle did not impair the biological function of the attached growth factor. In the absence of either conjugated EGF or cell-surface expressed erbB1, QDs did not associate with the living cells. That is, the background arising from the nonspecific interactions generally observed with fixed cells was virtually absent (data not shown).

We confirmed that endocytosis of EGF-QD-erbB1 was via clathrin-coated pits by simultaneously adding transferrin-Alexa633 and EGF-QD to live cells. The two ligands were colocalized on the cell surface and after internalization, even in cells lacking the exogenous erbB1-eGFP (Fig. 1d).

Dynamic processes coupled to binding and uptake of EGF-QDs

Numerous dynamic cellular processes were monitored in real-time by continuous observation of erbB1-eGFP and EGF-QDs in the confocal microscope (Fig. 2). Very low occupancy of the receptor sufficed to induce extensive membrane ruffling in the erbB1-expressing Chinese hamster ovary (CHO) cell line (Fig. 2a, first frame, 330 s), indicating the global nature of this cellular response mediated by pathways coupled to the cytoskeleton. Endocytosis of both components of the EGF-QD-erbB1-eGFP complex proceeded rapidly and the colocalization was largely maintained inside the cell, demonstrated at the level of individual endosomes (Fig. 2a, last panel). **Supplementary Movies 1–4** online provide depictions of the entire process extending from bind-

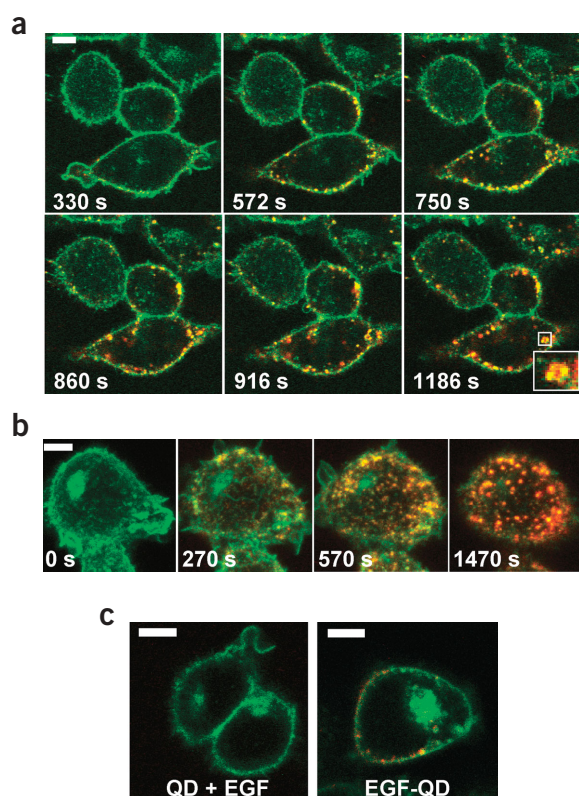


Figure 2 Live cell activation by EGF-QDs. (a) Selected time points of CHO cells expressing erbB1-eGFP after addition of 200 pM 6:1 EGF-QDs. Single optical sections at a resolution of 0.14 $\mu\text{m}/\text{pixel}$. Inset in the last panel: 4 $\times$ zoom of a large vesicle. (b) CHO cells expressing erbB1-eGFP after addition of 250 pM 30:1 EGF-QDs. Images are maximum intensity projections of 12 optical sections taken at 30 s intervals ($\Delta x, y, z$: 0.14, 0.14, 0.64 μm). (c) CHO cells expressing erbB1-eGFP. Left panel, 15 min after addition of 10 nM QDs plus 1 nM free EGF. Right panel, 23 min after addition of 0.1:1 biotin-EGF-QDs. For this loading ratio, >95% of labeled QDs were monovalent assuming a Poissonian distribution. All scale bars, 5 μm . Movies of the extended times series for **a** and **b** are available as **Supplementary Movies 1 and 2** online.

precise linear relationship to the biotin-EGF/EGF mixing ratio x ($y = 0.003 + 1.05x$, $R^2 = 0.995$; see **Supplementary Fig. 1** online). We conclude that QDs can bind in a 1:1 ratio to an EGF-erbB1 complex, regardless of the oligomeric state of the receptor.

Filopodial uptake and retrograde transport of EGF-QDs

EGF-QDs bound to the filopodia and were detected even at the single QD level (Fig. 1b). In cells expressing endogenous unlabeled erbB1 and erbB3-mCitrine, EGF-QDs bound rapidly to the cell surface and then were internalized, leaving erbB3-mCitrine behind (Fig. 3a; see discussion below and **Supplementary Movie 3** online). The image sequence of a single filopodium of this cell is depicted in **Figure 3b**. It depicts the uniform migration of the EGF-QDs toward the cell body with a velocity of ~ 10 nm/s. This retrograde movement, but not the binding of EGF-QD, was inhibited by application of 1 μM PD153035, a specific erbB1 kinase inhibitor²⁵ (data not shown). We surmise that this novel form of retrograde transport involves complexes of EGF-QD-erbB1 with treadmilling actin filaments and/or actin-localized motor proteins^{26,27}. The detection of this previously unreported process was made possible only by the use of the EGF-QD ligand.

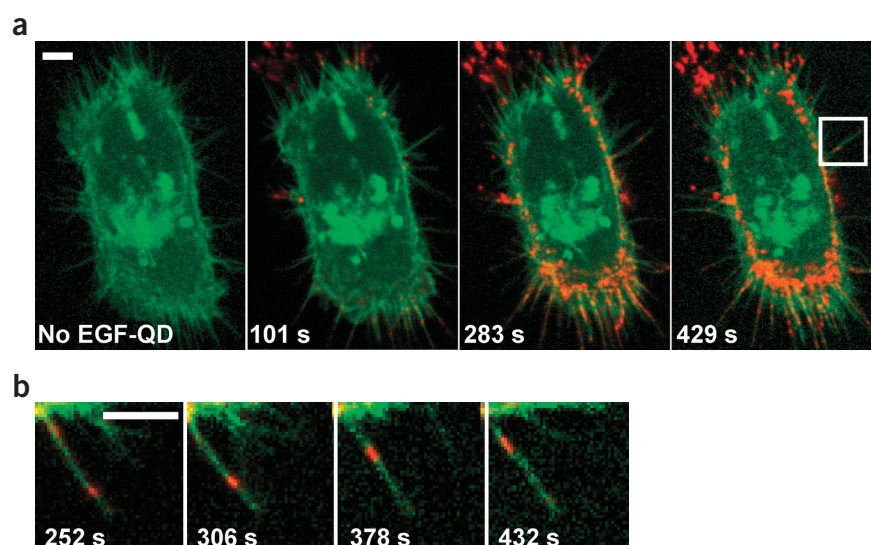
Endosomal trafficking of EGF-QDs

Internalized EGF-QDs were also very dynamic in space and time. Endocytic vesicles containing erbB1-eGFP surrounding the EGF-QD underwent Brownian movement, a directed linear motion mediated by microtubule-associated motor proteins, and vesicular fusion. In the sequence featured in **Figure 4** (see also **Supplementary Movie 4** online), the designated vesicle (red arrow) remained at one position

ing to cytoplasmic trafficking. Increasing the EGF loading of the QDs resulted in greater accumulation of QDs within the cell (Fig. 2b and **Supplementary Movie 2** online), possibly due to more extensive clustering on the cell surface before endocytosis and/or the extensive fusion of highly packed endosomes. To confirm that EGF-QD internalization was in direct response to EGF-QD binding to erbB1, free EGF and unconjugated QDs were added simultaneously to the cells. Whereas EGF induced the ruffling response, the QDs neither bound nor internalized (Fig. 2c, left panel), confirming that endocytosis of EGF-QD requires binding to and activation of the receptor. It is noteworthy that a single EGF ligand was sufficient in binding and endocytosis, as demonstrated with QDs containing a single EGF (0.1:1 EGF-QD) (Fig. 2c, right panel). This experiment also demonstrates that the QD nanoparticle did not interfere sterically with the binding and function of the ligand.

A quantitative test of QD-ligand binding specificity, linearity and stoichiometry was performed by flow cytometry. Solutions of biotin-EGF and EGF in various molar ratios were applied to A431 cells expressing erbB1-eGFP, followed by QDs. The normalized mean flow cytometric signals y exhibited a

Figure 3 Retrograde transport of EGF-QDs on filopodia. (a) A431 cell expressing erbB3-mCitrine; maximum intensity projection of four 0.5 μm confocal sections as a function of time. (b) Magnified image of filopodium indicated in the last panel of **a**. All scale bars, 5 μm . Movie of the extended times series for **a** available as **Supplementary Movie 3** online.



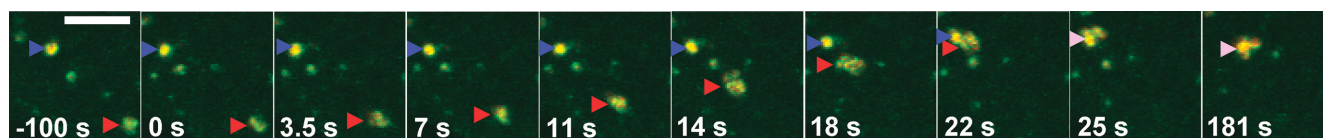


Figure 4 Dynamics of endosomal fusion. Selected time points in the cytoplasm of a CHO cell expressing erbB1-eGFP, 30 min after addition of 200 pM 6:1 EGF-QD. The vesicles show Brownian movement interrupted by directed movement and fusions. At time 0, the lower-right vesicle (red arrow) moved towards the upper-left vesicle (blue arrow), with which it fused irreversibly at 25 s (purple arrow). Scale bar, 5 μ m. Movie of the extended times series is available as **Supplementary Movie 4** online.

for 3 min (185 s), after which it rapidly (within 26 s) moved (at a velocity of 0.4 μ m/s) towards another vesicle with which it finally fused.

Quantitation of EGF-QD binding and internalization

The combination of photostable QDs and image processing techniques permitted a quantitative analysis of the coupled kinetics of ligand binding and internalization. Time series of live cells exposed to EGF-QDs were acquired and subjected to image segmentation procedures to define membrane-associated and internalized compartments (masks, right panel of Fig. 5a). The kinetics of EGF-QD accumulation on and internal to the cell surface were quite different. That is, whereas surface binding followed an exponential course, internalization was linear. We conclude that the cellular endocytotic machinery and not ligand binding constitutes the rate-limiting process in the endocytosis of the receptor-ligand complex. The plateau in the binding curve also indicates that a steady-state equilibrium was established between erbB1 internalization and recycling at 23 $^{\circ}$ C. In the absence of a constant external ligand reservoir, endocytosis was accompanied by a reciprocal depletion of EGF-QDs from the cell surface, as demonstrated with cells incubated with EGF-QDs at 4 $^{\circ}$ C and washed before raising the temperature.

Detection of erbB1 heterodimers with erbB2- or erbB3-VFP

We investigated whether RTK heterodimerization occurs in response to EGF binding to erbB1 by following the QD internalization rate and

extent of endocytosis of coexpressed erbB2 or erbB3 upon addition of EGF-QD. The A431 cells used in these experiments expressed the usual high complement of endogenous erbB1 (ref. 24) and either erbB1-eGFP, erbB2-mYFP or erbB3-mCitrine. As described above, addition of EGF-QD to A431 or CHO-erbB1-eGFP cells led to rapid internalization of EGF-QD and erbB1-eGFP (see also Fig. 5b).

In A431 cells expressing erbB2-mYFP or erbB3-mCitrine, EGF-QD was internalized, whereas erbB2 and erbB3 were retained either partially (erbB2) or totally (erbB3; Figs. 3a, 5c and **Supplementary Movie 3** online) on the cell surface. These effects were quantified by determining the amount of erbB-VFP internalization with respect to the EGF-QD internalization (see Methods; Fig. 6a). The values determined between 15 and 30 min of incubation at 23 $^{\circ}$ C were significantly different ($P < 0.015$): erbB1 (CHO, $37 \pm 5\%$; A431, $34 \pm 4\%$), erbB2 ($16 \pm 3\%$), and erbB3 ($5 \pm 3\%$). Treating cells expressing erbB2 with 2C4, an antibody that disrupts erbB2 heterodimer formation²⁸, reduced erbB2-mYFP internalization down to $6 \pm 3\%$.

We also measured by flow cytometry the endocytosis of EGF-QD in A431 cells devoid of exogenous receptor or expressing either erbB2-mYFP or erbB3-mCitrine (Fig. 6b). In A431 cells the internalized (acid-wash insensitive) fractions of EGF-QD after 5 and 20 min of stimulation with 1 nM EGF-QD were $31 \pm 4\%$ and $48 \pm 4\%$, respectively. The extent of EGF-QD uptake was unaltered in A431 cells expressing erbB3-mCitrine (5 min, $37 \pm 5\%$; 20 min, $55 \pm 6\%$), but it was significantly reduced in cells expressing erbB2-mYFP (5 min, 20 $\pm$

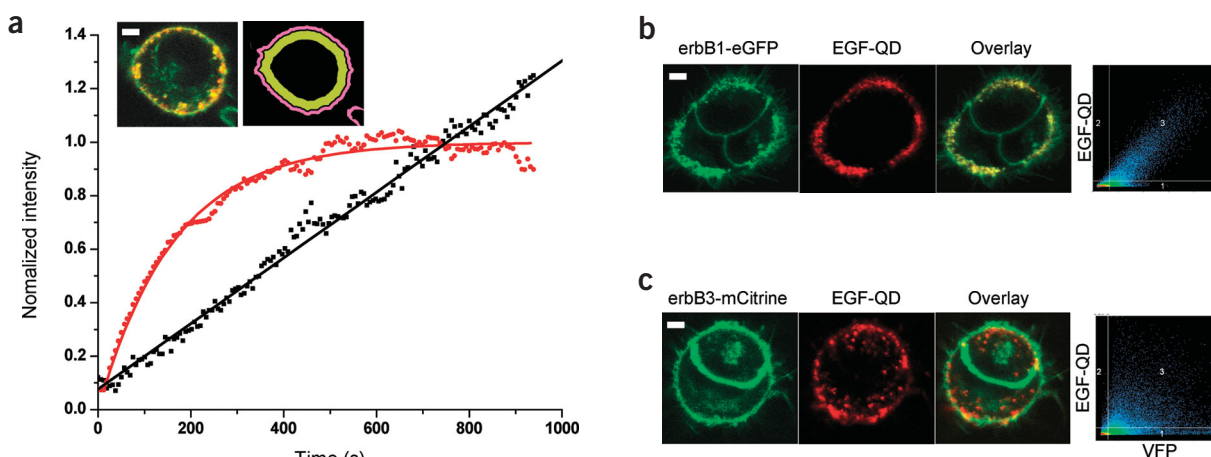


Figure 5 Quantitative analysis of real-time EGF-QD binding and internalization. **(a)** Comparative kinetics of binding and internalization on CHO-erbB1-eGFP. Inset: cellular image (left) and masks (right) corresponding to cell surface and internal regions of interest (defined automatically during analysis; see Methods). Plot of total EGF-QD signal on the cell membrane (red circle) or inside the cell (black square) as a function of time (solid lines are fits). Both plots were normalized to the amplitude of the fit of the membrane accumulation. **(b,c)** Differences in internalization between erbB1/3 and EGF-QD. **(b)** A431 cell expressing erbB1-eGFP. Right panel, 2D histogram of EGF-QD signal vs. erbB1-eGFP signal. The small amount of dispersion indicates high colocalization. **(c)** A431 cell expressing erbB3-mCitrine. Note that the endosomes are red here, not yellow. The high dispersion in the 2D histogram (right panel) is indicative of little colocalization. All scale bars, 5 μ m.

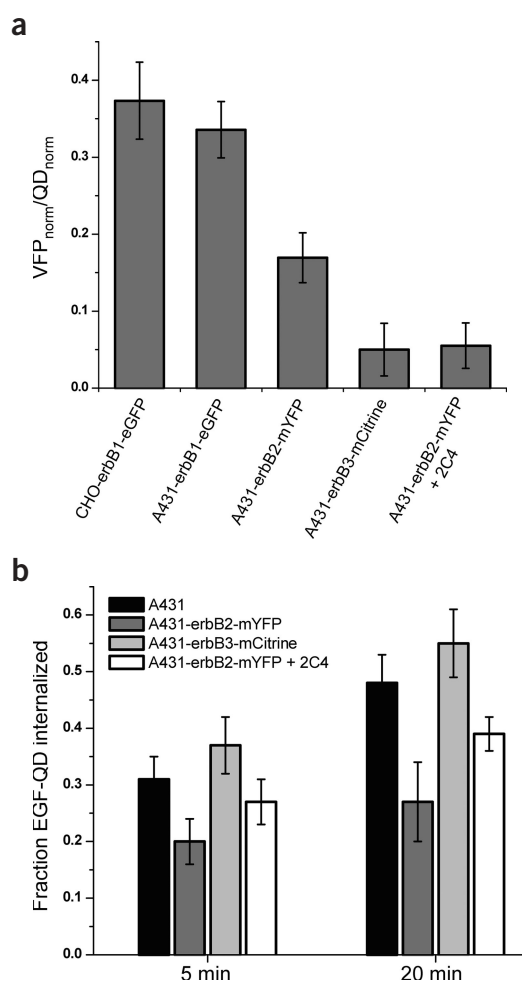


Figure 6 Quantitative analysis of EGF-QD-induced erbB1/2/3 internalization. (a) Comparison of amount of erbB-VFP internalization with respect to EGF-QD internalization (VFP_{norm}/QD_{norm}) determined by quantitative image processing (see Methods and **Supplementary Methods** online). (b) Plot of EGF-QD internalization rate for cells expressing different combinations of erbBs determined by flow cytometry (see Methods).

4%; 20 min, $27 \pm 7\%$). The EGF-QD uptake increased when erbB2-mYFP cells were treated with 2C4 (5 min, $27 \pm 4\%$; 20 min, $39 \pm 3\%$), implying that heterodimerization with erbB2 was responsible for the reduced endocytosis of erbB1.

The very low internalized fraction of erbB3 and the lack of an effect of erbB3 on the EGF-QD internalization rate indicated the absence of substantial erbB1/erbB3 heterodimers before or in response to EGF binding. In contrast, the significant internalization and effect on EGF-QD uptake of coexpressed erbB2-mYFP pointed to the existence of a substantial erbB1/erbB2 heterodimer population that was not internalized readily and confirmed the central role ascribed to erbB2 as an essential coreceptor in normal and oncogenic states^{1,7}. It follows that the heterodimer may play a significant role in EGF-induced signaling by modulating the fate of activated erbB1.

DISCUSSION

This study confirms that monomer-dimer-oligomer equilibria on the cell surface are important features of RTK signaling mechanisms. Although QDs have been loaded into cells by invasive and noninvasive

techniques^{8,29}, they have not been employed previously in combination with receptor-specific, natural ligands. This study shows that QD ligands are extremely valuable reagents for *in vivo* studies of signal transduction, particularly in combination with the expression of fluorescent target proteins with functional ectodomains incorporating ligand binding sites, such as the RTKs. The *in vivo* measurements reported here revealed new insights into processes and interactions that could previously only be studied on fixed cells or by biochemical fractionation, and demonstrated the existence of a novel retrograde transport mechanism in filopodia. An understanding of receptor-mediated transduction mechanisms is essential for rational receptor-targeted therapeutics. Quantitative approaches based on multiple combinations of QDs and ligands will be invaluable for such investigations.

METHODS

Reagents. All reagents were of analytical grade. Biotin-EGF, EGF and transferrin-Alexa633 were purchased from Molecular Probes. Qdot Streptavidin Conjugate 605 nm was from Quantum Dot. Live cell labeling was carried out in Tyrode's buffer with 20 mM glucose and 0.1 % BSA.

RTK-VFP fusion proteins: gene construction and transfected cell lines. Plasmids for the erbB proteins were the kind gift of Y. Yarden and for Citrine of R. Tsien. ErbB1-eGFP was generated as described previously³⁰. ErbB2 fused to eYFP at the C terminus was produced by PCR amplification and introduced into the *XhoI/HindIII* sites of pEYFP-N1 (Clontech). The nondimerizing, that is, monomeric, YFP-A206K mutation³¹ was made using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) (further details provided in **Supplementary Methods** online). *KpnI* and *NotI* sites were created in erbB3, and *XbaI* and *ApaI* sites were created in monomeric (A206K) Citrine by PCR amplification and introduced into pcDNA3 (Invitrogen) (details are provided in **Supplementary Methods** online). Stable CHO or A431 cells transfected with erbB1-eGFP, erbB2-mYFP or erbB3-mCitrine were established and cultured in DMEM with 10% fetal calf serum and 500 ng/mL geneticin (Gibco). Absolute expression levels were determined by flow cytometry (Qifitkit, DAKO).

Microscopy. Confocal laser scanning microscopy was performed with a Zeiss LSM 310- or 510-meta systems using a 40 $\times$ or 63 $\times$ 1.2 NA water immersion objective. Excitation and filters were as follows: VFP, 488 nm excitation, emission BP 520 $\pm$ 12 nm filter; QD, 488 nm excitation, emission LP 585 filter; Alexa633, 633 nm excitation, emission LP 650 filter. Multitrack LSM 510 imaging of QDs with erbB2-mYFP or erbB3-mCitrine expressing cells used 488 nm excitation of the VFPs and QD excitation at 458 nm of QDs to eliminate crosstalk of the VFPs in the QD channel.

EGF and quantum dot labeling of cells. Consecutive binding of biotin-EGF and QDs. Cells were labeled with 40 ng/ml biotin-EGF on ice in Tyrode's buffer for 5 min, washed and incubated for 10 min with 2 nM QDs, washed and either fixed directly in cold methanol or incubated at 37 $^{\circ}$ C for various times before fixation. Rehydrated cells were mounted in glycerol or PBS and imaged. Early lots of the commercial QDs were stable after rehydration from methanol for several days but subsequent lots showed a time-dependent decay of fluorescence.

Preparation and application of EGF-QD. EGF-QD complexes were formed by incubation of biotin-EGF with QDs at 4 $^{\circ}$ C with mixing for >30 min. For most experiments a 6:1 molar ratio of EGF:QDs was used. To remove unbound ligand, the complexes were purified by chromatography over P30 size exclusion spin columns (Biorad). In cases of a low loading ratio (0.1:1), it was necessary to pre-purify the QDs using an S200 size exclusion spin column (Amersham Biosciences) to remove small amounts of free streptavidin shed from the QDs upon storage. Cells were grown in 8-well Lab-Tek chambers (Nunc) and imaged in Tyrode's buffer containing glucose and BSA. EGF-QD was added at 23 $^{\circ}$ C to cells being imaged in the confocal laser scanning microscope, at a concentration of 0.1–2 nM (some experiments were performed at 2 pM). Transferrin-Alexa633 was used at 60 nM.

Data analysis. Background subtraction from both channels was performed before quantitative manipulations of the images. Segmentation of the cells was achieved by global thresholding in the green channel. Circumferential areas located 3–10 and 15–35 pixels distance to the background border (using a Euclidian distance transform), that is, toward the cell interior, were denoted as the membrane and inside rim regions, respectively (see Fig. 4). Values inside the QD-mask (>96% quantile) were summed separately for the red (QD) and green (VFP) channels in the membrane region and inside rim regions (QD_{mem} , QD_{in} , VFP_{mem} , VFP_{in}). The internal signal was then normalized to the amount of signal in the membrane ($VFP_{norm} = VFP_{in}/VFP_{mem}$, $QD_{norm} = QD_{in}/QD_{mem}$). The degree of VFP internalization relative to QD internalization was given by VFP_{norm}/QD_{norm} . (Further details of the image processing procedures are given in the **Supplementary Methods** online.).

Determination of EGF-QD internalization by flow cytometry. Trypsinized cells were labeled with EGF-QD for 30 min on ice in Tyrode's buffer containing glucose and 1% BSA followed by incubation at 37 °C. Half of the sample was then treated with 15-fold excess volume of acid strip buffer (0.5 M NaCl, 0.1 M Na-glycine, pH 2.5) for 5 min on ice followed by washing and resuspension in Tyrode's buffer. Cells were analyzed on a Coulter Epics Elite flow cytometer. Simultaneous detection of QD and VFP fluorescence was achieved by excitation at 488 nm with a BP605/20 filter for QD emission and a BP525/45 filter for VFP. The internalized fraction of EGF-QD was determined by dividing the QD fluorescence intensity of the acid stripped sample with that of the unstripped one.

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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Decreased Accessibility and Lack of Activation of ErbB2 in JIMT-1, a Herceptin-Resistant, MUC4-Expressing Breast Cancer Cell Line

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Abstract

Overexpression of erbB2 in breast tumors is associated with poor prognosis and is a target of receptor-oriented cancer therapy. Trastuzumab (Herceptin), a monoclonal antibody against a membrane-proximal epitope in the extracellular region of erbB2, shows a therapeutic effect against a fraction of *erbB2*-amplified breast tumors. Unfortunately, resistance to Herceptin is common, and its cause is as yet unclear. Here we investigated the properties of erbB2 in a Herceptin-resistant cell line, JIMT-1, established from a breast cancer patient showing *erbB2* gene amplification and primary resistance to Herceptin. The expression profile of erbB proteins, Herceptin-induced erbB2 internalization, and down-regulation in JIMT-1 were similar to those in Herceptin-sensitive lines. However, the mean number of Herceptin Mab binding sites in JIMT-1 was 1/5 that of the expressed erbB2 molecules, although 5% to 10% of the cells showed a ~10-fold higher Herceptin binding than the main population. Herceptin Fab and Mab 2C4, an antibody binding to an epitope in the ectodomain further removed from the membrane, bound more efficiently to JIMT-1 cells than Herceptin Mab, implying that erbB2 was partly masked. The expression of MUC4, a membrane-associated mucin that according to reports contributes to the masking of membrane proteins, was higher in JIMT-1 than in Herceptin-sensitive lines, and its level was inversely correlated with the Herceptin binding capacity of single cells. Knockdown of MUC4 expression by RNA interference increased the binding of Herceptin. Western blotting showed a low level of proteolytic processing, shedding, and tyrosine phosphorylation of erbB2 in JIMT-1. The latter finding may explain its Herceptin-resistant phenotype characterizing both the low and high Herceptin binding subpopulations. We conclude that masking of erbB2 in JIMT-1 leads to diminished Herceptin binding and isolation of erbB2 from its normal interaction and activation partners. (Cancer Res 2005; 65(2): 473-82)

Introduction

The erbB/HER family of transmembrane receptor tyrosine kinases consists of four proteins: erbB1 [epidermal growth factor

(EGF) receptor, EGFR/HER1], erbB2/HER2, erbB3/HER3, and erbB4/HER4 (1). The limited signal transduction and transforming ability of single erbB proteins is significantly increased by extensive homoassociations and heteroassociations (2). The flexibility and scope of the network is further enhanced by the large number of activating peptide growth factors (3, 4). ErbB2 has no soluble high-affinity ligand (5) but fulfills a central role in the erbB signal transducing network by increasing the ligand binding spectrum (6) and affinity (7) of erbB1, erbB3, and erbB4. ErbB2 has a very potent intracellular tyrosine kinase domain conferring signaling superiority to its heterodimers by virtue of strong activation of both the mitogen-activated protein kinase and phosphatidylinositol 3-kinase (PI3K) pathways. The erbB2/erbB3 heterodimer is a very efficient "oncogenic unit" (8, 9). ErbB2-containing heterodimers are internalized less efficiently (10, 11) and evade lysosomal degradation (12), an effect which is even more pronounced upon erbB2 overexpression (13) also leading to ligand-independent constitutive activation of erbB2 homodimers (14, 15).

The overexpression of erbB2 observed in 20% to 30% of breast cancers identifies a patient group with poor prognosis (16). In addition to its prognostic value, erbB2 is the target of receptor-oriented cancer therapy (17), including trastuzumab (Herceptin), the first immunotherapeutic drug for the treatment of breast cancer (18, 19); 2C4, an antibody blocking heterodimerization of erbB2 (20); and CI-1033, a pan-erbB tyrosine kinase inhibitor (21).

Herceptin binds to a membrane proximal epitope in the extracellular region of erbB2 (22, 23). Its mode of action has been extensively studied, but the conclusive mechanism of action is still unknown. Herceptin induces internalization and down-regulation of erbB2. Although these phenomena are presumed to contribute to the therapeutic action (24), reports demonstrating the lack of correlation between the rate of internalization and the antibody's antiproliferative effect cast doubt on this model (25, 26). ErbB2 undergoes proteolytic cleavage by a metalloprotease, generating a soluble extracellular domain and a kinase active, tyrosine-phosphorylated 95-kDa intracellular fragment. This process is inhibited by Herceptin (27). Additionally, Herceptin treatment down-regulates phosphorylated Akt and increases the nuclear concentration of the cyclin-dependent kinase inhibitor p27KIP (28). Herceptin is a partial agonist of erbB2, an effect that requires further investigation (24, 29). Recently, genes have been identified that are either up-regulated or down-regulated during Herceptin treatment (30). In addition to these direct effects, antibody-dependent cellular cytotoxic reaction against Herceptin-targeted cells mediated by Fc receptors has also been invoked (31).

Although Herceptin constitutes a breakthrough in the treatment of advanced breast cancer, 70% of erbB2-overexpressing breast

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Table 1. Expression levels of erbB proteins and Herceptin-induced internalization and down-regulation of erbB2

	JIMT-1	SKBR-3	BT-474	MDA-453
Expression level ($\times 10^3$)				
erbB1	160	190	16	60
erbB2	620	1,100	1,450	620
erbB3	10	42	20	18
erbB4	0	0	0	2
IGF-1R	5	4	9	5
Herceptin internalization (%)	33 $\pm$ 4	25 $\pm$ 1	22 $\pm$ 2	16 $\pm$ 2
Herceptin-induced erbB2 down-regulation (%)	40 $\pm$ 5	31 $\pm$ 2	27 $\pm$ 2	38 $\pm$ 3

NOTE: The expression levels of proteins were determined by flow cytometry. The following antibodies were used: Ab1-Clone 528 (erbB1), Ab3/OP15 (erbB2), Ab4-Clone H3.90.6 (erbB3), Ab1-Clone H4.77.16 (erbB4), and Ab1-Clone 24-31 (IGF1R). For the determination of Herceptin-induced effects, cells were treated with 10 μ g/mL Herceptin. Herceptin internalization and Herceptin-induced erbB2 down-regulation were determined at 3 and 48 hours of Herceptin treatment, respectively. Herceptin internalization is expressed as the percentage of the initial level of bound Herceptin. ErbB2 down-regulation values represent the percentage by which erbB2 expression was reduced after 48 hours of Herceptin treatment, compared with untreated samples.

cancers show primary resistance to Herceptin as a single agent (32). Although the response rate to Herceptin when combined with chemotherapy is somewhat higher (33), continued administration of the antibody inevitably leads to secondary resistance.

The molecular mechanisms accounting for Herceptin resistance in patients are currently unknown. Autocrine production of EGF-like ligands (34), overexpression of insulin-like growth factor 1 receptor (IGF-1R; ref. 35) and production of an alternatively spliced, intracellularly retained extracellular domain of erbB2 (36) have been invoked as possible causes. The common denominator is the presence of an erbB2-independent means for the constitutive activation of the PI3K pathway.

Blocking of Herceptin binding by MUC4, a cell surface mucin, has also been implicated in Herceptin resistance. It has been shown that overexpression of rat Muc4 reduces binding of Herceptin to erbB2-expressing tumor cells (37). However, it remains unclear whether human MUC4 fulfills a similar role. MUC4 is a membrane-associated, highly glycosylated mucin consisting of two parts: MUC4 β , a 90-kDa transmembrane subunit containing EGF-like domains and MUC4 α , a noncovalently associated, heavily *O*-glycosylated soluble mucin subunit (38). The latter contains a large tandem repeat domain varying in length between 3285 and 7285 amino acids (39, 40). Consequently, the molecular weight of the soluble subunit varies between 500 and 900 kDa, and the whole molecule extends ~ 1 to 2 μ m from the cell surface. Mucins provide a protective coat to epithelia (38). Cancers often overexpress MUC4 and usurp its protective function to inhibit cellular and antibody-mediated immune attack (37, 41) or to increase their metastatic potential (42). Inhibition of MUC4 expression suppresses the growth and metastatic potential of pancreatic cancer cells (43). Furthermore, Muc4 of rat origin is reported to be a membrane-associated ligand of erbB2 (44, 45).

In the current paper, we examine the mechanisms of Herceptin resistance in JIMT-1 cells, a line recently established from a Herceptin-resistant breast cancer patient. It has been shown that JIMT-1 retained erbB2 amplification and overexpression as well as Herceptin resistance as a stable phenotype.⁵ We show that the Herceptin binding epitope of erbB2 in JIMT-1 was masked, probably by MUC4, leading to diminished binding of Herceptin.

RNA interference (RNAi)-mediated suppression of MUC4 expression increased Herceptin binding to the cells. Both shedding and tyrosine phosphorylation of erbB2 were low in JIMT-1 cells, presumably due to sequestration of erbB2 away from its natural interaction partners. Although a minor subpopulation of JIMT-1 showed ~ 10 times higher Herceptin binding than the main subpopulation, both were Herceptin resistant.

Materials and Methods

Cells. SKBR-3, BT-474, and MDA-453 cell lines were obtained from the American Type Culture Collection (Rockville, MD) and cultured according to their specifications. The JIMT-1 breast cancer line isolated from the pleural effusion of a Herceptin-resistant breast cancer patient was grown in F12/DMEM (1:1) supplemented with 10% FCS, 60 units/L insulin and antibiotics.⁵ For flow cytometric experiments, cells were harvested with a PBS-based enzyme-free cell dissociation buffer (Invitrogen, Carlsbad, CA).

Antibodies, Growth Factors, and Chemicals. Staining of cell surface erbB1, erbB3, erbB4, and IGF-1R was carried out with Ab1-Clone 528, Ab4-Clone H3.90.6, Ab1-Clone H4.77.16, and Ab1-Clone 24-31 (all from LabVision, Fremont, CA), respectively, followed by fluorescent secondary antibody. Labeling of erbB2 was either carried out with the Ab3/OP15 antibody (Calbiochem-Merck Biosciences, Schwalbach, Germany) on fixed (3.7% HCHO, 30 minutes) and permeabilized (0.1% Triton X-100) cells followed by secondary labeling or with fluorescent 2C4 antibody, Herceptin antibody or Fab (all from Genentech, South San Francisco, CA) on nonpermeabilized cells. Labeling of primary antibodies or Fabs with Alexa dyes was carried out according to the manufacturer's specifications (Molecular Probes, Eugene, OR). Calibration of fluorescent signals in terms of number of binding sites per cell was done with Qifikit (DakoCytomation, Glostrup, Denmark). The 1G8 antibody against the membrane-associated β subunit of MUC4 was developed in one of our laboratories (KLC). It has been shown that 1G8 recognizes a band corresponding to the expected molecular weight of human MUC4 β in Western blotting, and it specifically stained cells expressing human MUC4 in immunofluorescence experiments (46). 1G8

⁵ Rennstam et al., submitted for publication.

was applied to fixed and Triton X-100-treated cells followed by secondary labeling with Cy2-goat anti-mouse Fab (Jackson ImmunoResearch, West Grove, PA). All steps of primary and secondary labelings were carried out on ice. Heregulin- β 1 was purchased from R&D Systems (Minneapolis, MN), and was used at a concentration of 100 ng/mL. Aminophenylmercuric-acetate (APMA) was from Sigma (Schnelldorf, Germany).

Flow and Image Cytometry. A Coulter Epics Elite flow cytometer was used to quantitate expression levels of proteins labeled by fluorescent antibodies. Analysis of flow cytometry data was carried out with FCS Express V2 (De Novo Software, Thornhill, Ontario, Canada). Optical sections of cells were acquired with a Zeiss LSM510 confocal laser scanning microscope. General image analysis (smoothing, registration) and quantitation of fluorescence intensities were done with Scil-Image (University of Amsterdam, The Netherlands). Pixel-by-pixel analysis of colocalization between MUC4 and Herceptin was done with a program written in LabView 5.1 (National Instruments, Austin, TX).

Calculation of the Relative Number of Antibody Binding Sites on Different Cell Lines. Cells were labeled with OP15, Herceptin Mab (in the presence or absence of Triton X-100), Herceptin Fab, 2C4 Mab as described above, and fluorescence intensities were determined using flow cytometry. The mean fluorescence intensities of JIMT-1 and BT-474 cells were divided by the corresponding mean intensities of SKBR-3. The resultant ratio was divided by the erbB2 expression ratio for the given cell pair (JIMT-1/SKBR-3 = 0.56; BT-474/SKBR-3 = 1.32; determined by Qifit), so that the normalized ratio reflected whether the given epitope was equally exposed on the two cell lines (i.e., a value of 1 corresponded to equal exposure).

Internalization of Herceptin. Cells in suspension were incubated with 10 μ g/mL Alexa633-labeled Herceptin at 37°C for 0 to 3 hours. Subsequently, 1/2 of the samples were treated with a 15-fold excess of acid strip buffer [0.5 mol/L NaCl, 0.1 mol/L glycine (pH 2.8)] for 3 minutes on ice followed by washing and resuspension in PBS. Cells were analyzed by flow cytometry, and the internalized fraction of Herceptin was calculated by dividing the mean fluorescence intensity of the acid stripped sample with that of the untreated control.

Western Blotting. Whole cell lysates were prepared in 2 $\times$ SDS sample buffer [125 mmol/L Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 100 mmol/L DTT, 0.02% bromophenolblue] or in lysis buffer [20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 10% glycerol, 1 mmol/L EGTA, 1 % Triton X-100, 1 Complete Mini (Roche, Mannheim, Germany) protease inhibitor cocktail tablet/10 mL, 1 mmol/L Na₃VO₄, 1 mmol/L phenylmethylsulfonyl fluoride, 10 mmol/L NaF, 10 mmol/L β -glycerol phosphate, 10 mmol/L Na₂P₂O₇]. Immunoprecipitation of erbB2 was carried out with Herceptin or Ab3-OP15 for 1 hour on ice and Sepharose 4B Fast Flow Protein G beads (Sigma). Immunoprecipitates or whole cell lysates were resolved on 4% to 12% Novex Bis-Tris SDS-polyacrylamide gradient gels (Invitrogen) and blotted to nitrocellulose membranes. The following antibodies were used for primary labeling of the membranes at dilutions suggested by the manufacturers: Ab3-OP15 and Ab20 (L87 + 2ERB19; LabVision) for the intracellular and extracellular parts of erbB2, respectively; Ab18-PN2A (LabVision) for erbB2 phosphorylated at Y1248, PY99-sc7020 (Santa Cruz Biotechnology, Santa Cruz, CA) for phosphotyrosine; and 1G8 for MUC4 and A4700 (Sigma) for actin. Peroxidase-conjugated goat anti-mouse IgG and an enhanced chemiluminescence kit (Amersham, Freiburg, Germany) were used for detection.

Detection of ErbB2 Ectodomain Shedding by ELISA. The shed ectodomain of erbB2 was analyzed from undiluted cell culture media using a commercially available enzyme immunoassay kit (Bender MedSystems, Vienna, Austria) according to the manufacturer's instructions. The detection threshold for soluble erbB2 was 0.1 ng/mL.

Reverse Transcription-PCR of MUC4 mRNA. Reverse transcription-PCR was used to study MUC4 gene expression in JIMT-1 and control cells lines (SKBR-3 and BT-474). Lung tissue was used as a known positive control. Total RNA was extracted using GenElute Mammalian

Total RNA kit (Sigma Chemical Co., St. Louis, MO). Reverse transcription of MUC4 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reference gene was done using 2.5 μ g of total RNA as a template according to the manufacturer's instructions (Superscript First-Strand Synthesis System for reverse transcription-PCR, Invitrogen). The first strand cDNA synthesis product (1/10 volume) was used as a starting material in subsequent PCR amplification. PCR for MUC4 was done as follows: initial denaturation (95°C for 4 minutes), denaturation (94°C for 1 minute), annealing (58°C for 1 minute), and extension (72°C for 1 minute), 30 cycles. PCR for GAPDH: denaturation (94°C for 30 seconds), annealing (62°C for 45 seconds), and extension (72°C for 45 seconds), 30 cycles. The primers for MUC4 were 5'-CTGGATGGT-CATCTCGGAGT-3' and 5'-TCGAGTTTCATGCTCAGGTG-3' and for GAPDH were 5'-TCCTGGAAGATGGTGATGGGAT-3' and 5'-TGAAGTCGGAGTCAACGGATT-3'. MUC4 expression levels were evaluated relative to GAPDH.

RNAi. Twenty-one-nucleotide double-stranded small interfering RNA (siRNA) with two-nucleotide 3' overhangs (antisense strand GUGAA-GUCCGAUGCUUGCGTT and sense strand CGCAAGCAUCGGACUUCACCT) against human MUC4 was synthesized by Qiagen (Hilden, Germany). The above sequence is present in all splice variants of MUC4 characterized thus far. A control siRNA against green fluorescent protein (ref. 47; antisense strand GUUACCUUGAUGCC GUUCTT and sense strand GAACGGCAUCAAGGUGAACTT) was synthesized by T. Tuschl (currently at Rockefeller University, New York, NY). A BLAST analysis revealed no known unintended targets of the above siRNAs. Transfection of JIMT-1 cells with 100 nmol/L siRNA was carried out with Oligofectamine (Invitrogen) according to the manufacturer's specifications and protein expression and the phenotype of cells were analyzed 48 hours after transfection.

Results

The Expression of ErbB Family Members and Herceptin-Induced ErbB2 Internalization and Down-Regulation Are Not Significantly Different in JIMT-1 Cells from Herceptin-Sensitive Lines. Altered expression of erbB proteins is thought to be causally related to the malignant phenotype. Therefore, we investigated whether the expression levels of erbBs in the Herceptin-resistant JIMT-1 line were different from those in three Herceptin-sensitive lines, SKBR-3, BT-474 and MDA-453. Although the differences between the cell lines were substantial (Table 1), they probably cannot account for the Herceptin resistance of JIMT-1 because the expression levels of erbB proteins in JIMT-1 were in the same range as in the other cell lines. In particular, the expression profile of MDA-453 was very similar to that of JIMT-1 (Table 1). IGF-1R, the overexpression of which has been suggested to induce Herceptin resistance, was present in JIMT-1 at approximately the same level as in the other cell lines. We also measured the internalization of Herceptin and down-regulation of erbB2 induced by Herceptin (Table 1). Both of these processes took place as efficiently in JIMT-1 as in the Herceptin-sensitive cell lines. We conclude that impaired Herceptin internalization or erbB2 down-regulation does not account for the Herceptin resistance of JIMT-1.

The Epitope of Herceptin Was Masked in JIMT-1 Cells. Inasmuch as the number of erbB2 molecules in JIMT-1 cells was determined with an antibody specific for an intracellular epitope, it was still possible that Herceptin binding was impaired. Indeed, although the expression of erbB2 in JIMT-1 was ~50% that of SKBR-3 cells, the relative Herceptin binding capacity of JIMT-1 was ~10% that measured in SKBR-3, implying that only 1 of 5 erbB2 proteins constitute a Herceptin binding site in JIMT-1 cells (Fig. 1A). The diminished binding of Herceptin to JIMT-1 cells was

unique to this line, because it was not observed in BT-474. We also determined the dissociation constant for Herceptin binding to erbB2 on the three breast cancer cell lines. These measurements also showed that Herceptin binding to JIMT-1 was compromised, but the dissociation constants were not strongly correlated with the Herceptin-resistant phenotype [JIMT-1, 1.5 $\mu\text{g/mL}$ (10 nmol/L); SKBR-3, 0.6 $\mu\text{g/mL}$ (4 nmol/L); BT-474, 1 $\mu\text{g/mL}$ (6 nmol/L)]. Next, we tested whether the binding of Herceptin to JIMT-1 could be increased by fixing and permeabilizing the cells before labeling. Triton X-100-permeabilized JIMT-1 cells showed a remarkably higher Herceptin binding capacity than their unpermeabilized counterparts, whereas the binding of Herceptin to SKBR-3 or BT-474 cells was not substantially affected (Fig. 1A and B). Furthermore, JIMT-1 cells showed a binding capacity for both Herceptin Fab and 2C4 Mab exceeding that of Herceptin Mab. Labeling with Herceptin Fab and 2C4 Mab was done on nonpermeabilized cells, excluding that increased Herceptin Mab binding to JIMT-1 after permeabilization cells was caused by the sequestration of erbB2 in an intracellular pool. Sequencing of the entire coding sequence of erbB2 in JIMT-1 revealed no mutations,⁶ from which we tentatively conclude that masking of the Herceptin epitope in JIMT-1 accounted for the diminished binding of the antibody.

The Local Density of the Membrane-Associated Mucin, MUC4, Was Negatively Correlated with Herceptin Binding. It had been reported previously that overexpression of rat Muc4 results in reduced binding of antibodies against the extracellular region of erbB2 (37). Fluorescence microscopy (Fig. 1B), Western blotting (Fig. 2A), and reverse transcription-PCR (Fig. 2B) showed that MUC4 was expressed at a substantially higher level in JIMT-1 than in the Herceptin-sensitive breast tumor cell lines. On Western blots, the anti MUC4 Mab 1G8 recognized a ~120-kDa protein, corresponding to the approximate molecular weight of the membrane-bound subunit of MUC4. JIMT-1 cells labeled with 1G8 and Herceptin showed an inverse correlation between the local density of MUC4 and Herceptin binding (Fig. 2C). A two-dimensional histogram of Herceptin and 1G8 fluorescence intensities revealed that the negative correlation manifested itself primarily in the high intensity pixels, which displayed either high MUC4 density or high Herceptin binding but not both, implying that high MUC4 expression interfered with the binding of Herceptin (Fig. 2D).

RNAi-Mediated Suppression of MUC4 Expression Increased Herceptin Binding. RNAi is a highly efficient means for the selective suppression of gene expression (48). We designed a siRNA against human MUC4 that is homologous to all known splice variants of the protein. The MUC4 siRNA proved to be an efficient suppressor of MUC4 expression when transfected to JIMT-1 (Fig. 3A). Although the fraction of cells transfected with the siRNA was low (~15%), the suppression efficiency was ~100% in these cells, as reflected by the superposition of the peaks corresponding to the transfected cells and the unlabeled control. Specificity of the RNAi effect is supported by the absence of inhibition of MUC4 expression by a nonspecific siRNA (Fig. 3A) and by the lack of knockdown of actin expression by the MUC4 siRNA (Fig. 3B). Herceptin staining of

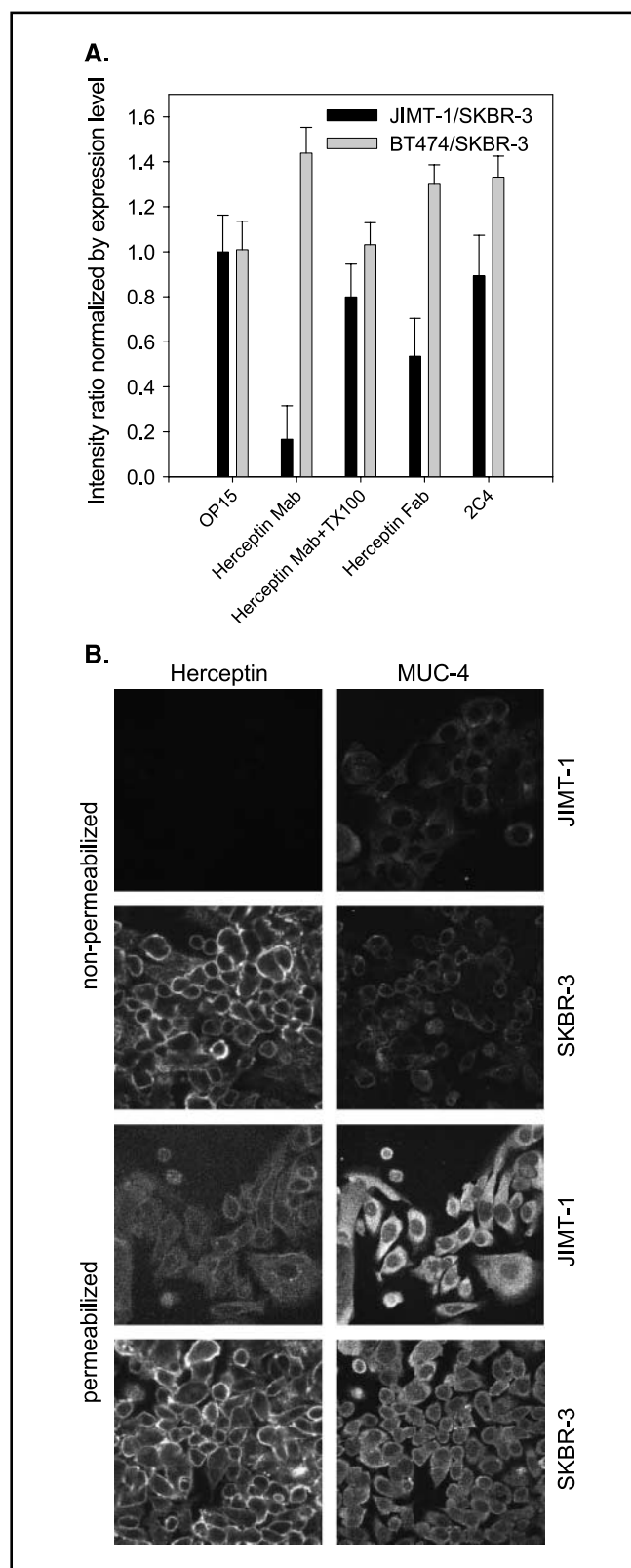
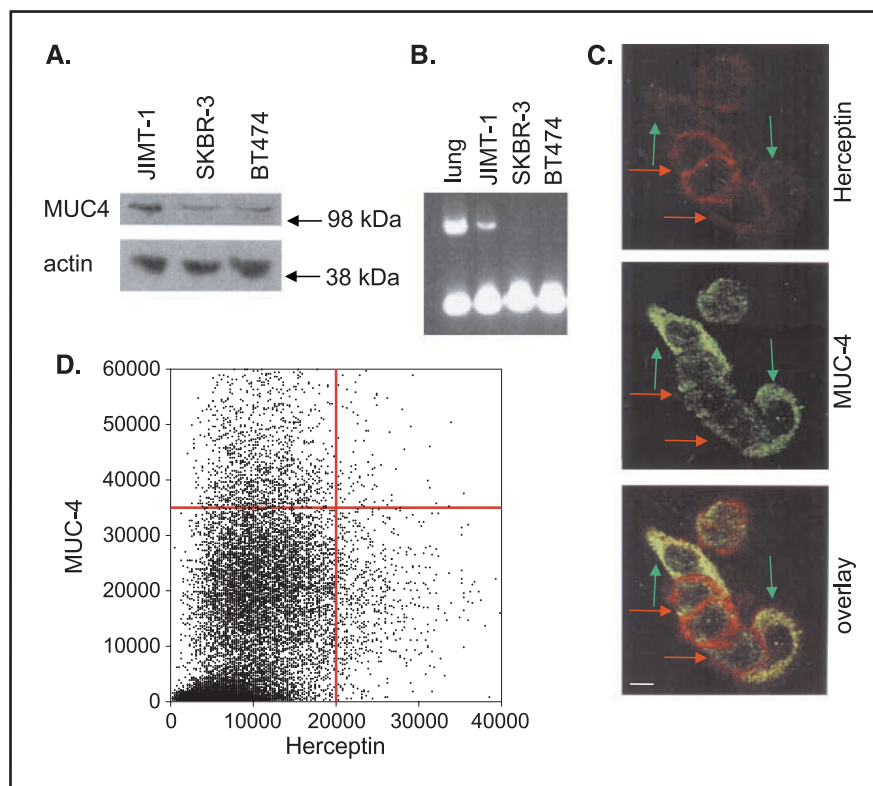


Figure 1. Masking of the Herceptin epitope on erbB2 in JIMT-1 cells. **A**, cells labeled with the indicated antibodies and analyzed by flow cytometry. Intensity ratios JIMT-1/SKBR-3 and BT-474/SKBR-3 were determined and normalized as described in MATERIALS AND METHODS. Bars, SE. **B**, cells cultured on glass coverslips and stained with Herceptin or 1G8 (against MUC4) with or without permeabilization with 0.1% Triton X-100. Imaging was with identical microscope settings.

⁶ Tanner et al., unpublished observation.

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Figure 2. MUC4 was highly expressed in JIMT-1 cells and its local density was negatively correlated with Herceptin binding. *A*, whole cell lysates separated by SDS-PAGE, and blotted with 1G8 antibody against MUC4. Membranes were stripped and reprobed with A4700 antibody against actin. *B*, reverse transcription-PCR amplification of MUC4 (top bands, 414 bp) and GAPDH (bottom bands, 204 bp). *C*, JIMT-1 cells grown on coverslips, fixed in 3.7% HCHO and permeabilized with 0.1% Triton X-100. Cells were labeled with Cy5-Herceptin (red channel) and with 1G8 against MUC4 followed by secondary labeling with Cy2-goat anti-mouse Fab (green channel). Green arrows, regions with high MUC4 and low Herceptin intensity; red arrows, low MUC4 and high Herceptin regions. Bar, 10 μ m. *D*, two-dimensional histogram of 1G8 intensity versus Herceptin intensity. Cells double-labeled as in *C* were thresholded to yield a mask containing pixels with higher than background Herceptin intensity. The two-dimensional histogram was calculated for pixels in the Herceptin mask.



nonspecific siRNA-transfected or -untransfected JIMT-1 cells yielded a bimodal distribution; ~10% of the cells showed high Herceptin binding. The size of the subpopulation with bright Herceptin staining was higher in the MUC4 siRNA-transfected sample, and the increase (~15%) corresponded well with the fraction of cells with no MUC4 expression (Fig. 3C). We did fluorescence-activated cell sorting on Herceptin-stained cells and separated the two subpopulations. Western blotting of the sorted

cells revealed that MUC4 expression was negatively correlated with the Herceptin binding capacity (Fig. 3C).

ErbB2 Was Less Tyrosine Phosphorylated in JIMT-1 Cells than in Herceptin-Sensitive Lines. Overexpressed erbB2 is phosphorylated on tyrosine residues (13). Although JIMT-1 overexpressed erbB2, we could detect only very low levels of tyrosine phosphorylation of erbB2 in whole cell lysates of normally cultured JIMT-1 cells using an antibody specific for erbB2 phosphorylated on

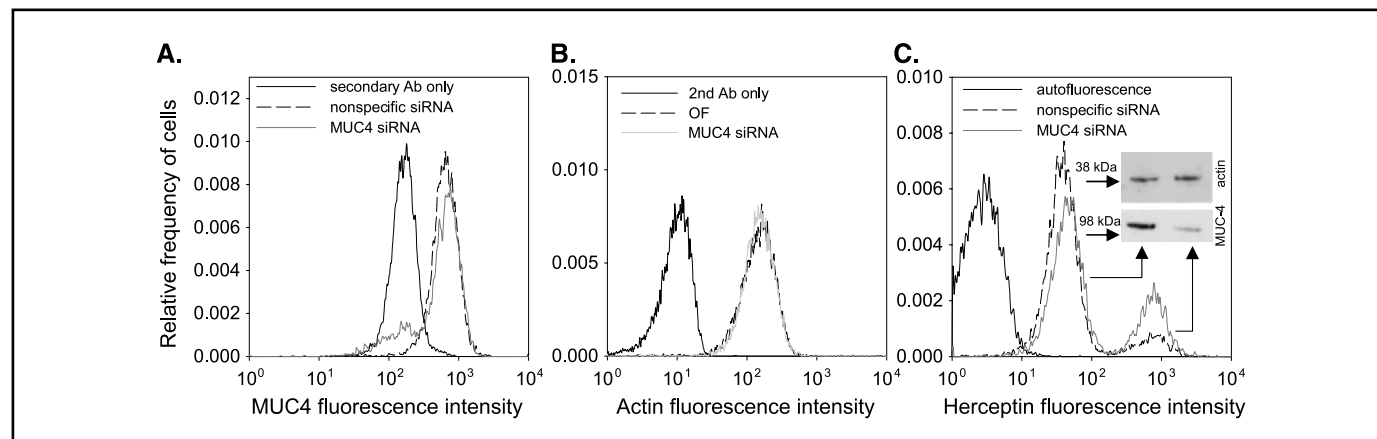


Figure 3. RNAi-mediated knockdown of MUC4 expression increased Herceptin binding. *A* and *B*, flow cytometry of JIMT-1 cells transfected with a nonspecific siRNA or a siRNA against MUC4. Cells were removed from the plates with enzyme-free cell dissociation buffer 48 hours after transfection or treatment with oligofectamine (OF) only, and MUC4 (*A*) or actin (*B*) expression was analyzed by flow cytometry. *C*, flow sorting and Western blotting of siRNA-transfected cells. Cells transfected with a nonspecific siRNA or MUC4 siRNA were stained with Cy5-Herceptin and analyzed by flow cytometry. An equal number (20,000) of cells in the subpopulations with high and low Herceptin binding were sorted, and their MUC4 and actin expressions were determined by Western blotting.

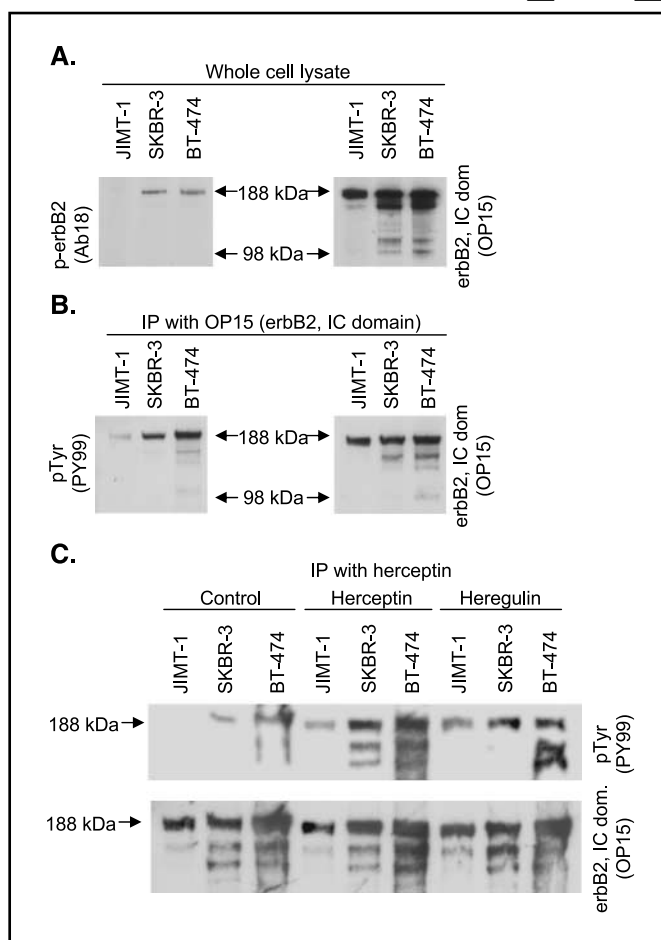


Figure 4. Low level of tyrosine phosphorylation of erbB2 in JIMT-1 cells. **A**, whole cell lysates of breast cancer cell lines. Lysates of cells kept under standard culture conditions were electrophoresed by SDS-PAGE and transferred to nitrocellulose. Membranes were blotted with an antibody against erbB2 phosphorylated on Tyr1248, then stripped and reprobed with OP15 (anti-erbB2 Mab) to show equal loading of full-length erbB2. **B**, ErbB2 immunoprecipitated with OP15 from whole cell lysates probed with an antiphosphotyrosine antibody followed by stripping and reprobing with OP15. Cells were kept under normal culture conditions. **C**, ErbB2 immunoprecipitated from lysates of cells exposed to Herceptin or heregulin, electrophoresed and probed with an antiphosphotyrosine Mab (PY99) and then stripped and reprobed with OP15. Cells were starved in the absence of serum for 24 hours. Control samples and cells treated with 10 μ g/mL Herceptin or with 100 ng/mL heregulin for 10 minutes at 37°C were lysed, and erbB2 was immunoprecipitated with Herceptin, and was resolved by SDS-PAGE.

Tyr 1248 (Fig. 4A) or on Herceptin-immunoprecipitated erbB2 with a phosphotyrosine-specific Mab (Fig. 4B). Under the same experimental conditions, erbB2 was heavily tyrosine phosphorylated on SKBR-3 and BT-474. Reprobing the membranes with an anti-erbB2 antibody showed approximately equal loading of full-length erbB2. A 24-hour starvation in the absence of serum decreased but did not eliminate erbB2 tyrosine phosphorylation in SKBR-3 and BT-474 (Fig. 4C). Stimulation with Herceptin or heregulin led to elevated erbB2 tyrosine phosphorylation in all three breast cancer lines, but the increase in JIMT-1 was much smaller than in SKBR-3 or BT-474.

Shedding of ErbB2 Ectodomain Was Reduced in JIMT-1. Bands corresponding to proteolytic products, including the NH₂-terminally truncated p95^{erbB2}, were absent from or very weak on Western blots of JIMT-1 but were prominent on blots from

SKBR-3 and BT-474 (Fig. 4). To establish that the lower molecular weight bands were NH₂-terminally truncated intracellular fragments of erbB2, we blotted whole cell lysates of the breast cancer cell lines with OP15, an antibody recognizing an intracellular epitope on erbB2, followed by stripping and reprobing with Ab20 against the extracellular region of erbB2 (Fig. 5A). The failure of Ab20 to recognize the low molecular weight bands in the SKBR-3 and BT-474 lanes proved that these were indeed NH₂-terminally truncated fragments. We confirmed these results with similar experiments done on immunoprecipitated erbB2 (Fig. 5B). In addition, despite the fact that bands corresponding to full length erbB2 on the OP15 blots showed comparable intensities in the case of all three breast cancer lines, the signal was very weak or absent in the JIMT-1 lane on the Ab20 blots (Fig. 5A and B), indicating that the epitope recognized by Ab20 was altered in JIMT-1. This finding needs further investigation.

ELISA detection of the shed erbB2 ectodomain showed a reduced level of soluble erbB2 in the conditioned medium of JIMT-1 compared with SKBR-3 or BT-474 (Fig. 5C). ErbB2 was shed from JIMT-1 to a lesser extent even taking into consideration the 2-fold lower expression level of erbB2 in JIMT-1.

Metalloprotease-Mediated Cleavage Unmasked the Herceptin Binding Site on ErbB2. APMA is an activator of metalloproteases. SKBR-3 and BT-474 cells were treated with 1 mmol/L APMA, and subsequently stained with fluorescent 2C4 to quantitate the amount of erbB2 on the cell surface. These experiments revealed that APMA decreased the cell surface expression of erbB2 in SKBR-3 and BT-474 by ~20% (Fig. 6A) in accordance with previous results (27). APMA treatment of JIMT-1 cells resulted in a ~40% increase in 2C4 binding. APMA induced a ~10-fold increase in Herceptin binding to SKBR-3 and BT-474 (Fig. 6B and D), and a ~40-fold increase in JIMT-1 (Fig. 6B and C). APMA-activated metalloproteases also cleaved membrane proteins other than erbB2 including those that mask it (e.g., MUC4). This may have lead to increased exposure of erbB2 epitopes, although the total number of membrane-bound erbB2 extracellular domains decreased. These findings corroborate that the Herceptin epitope was masked in JIMT-1 and to a lesser degree in Herceptin-sensitive lines as well. The enhanced 2C4 binding to APMA-treated JIMT-1 cells indicated that the membrane-distant 2C4 epitope was also masked to some extent.

Double labeling of untreated breast cancer cell lines with Mabs 2C4 and Herceptin showed that the two subpopulations of JIMT-1 cells showing different capacities for Herceptin binding (Figs. 3C and 6C) expressed erbB2 equally on the cell surface (Fig. 6E) providing further evidence for masking of the Herceptin epitope. The heterogeneity in SKBR-3 and BT-474 with respect to Herceptin binding capacity was negligible compared with that observed in JIMT-1 (Fig. 6E and F).

The Subpopulations Showing High and Low Herceptin Binding Capacity Are Equally Resistant to Herceptin. JIMT-1 cells were treated with 10 μ g/mL Herceptin for 5 days with replenishment of Herceptin containing medium every 2nd day. Cell counting revealed that Herceptin did not slow the proliferation of JIMT-1 (2.9 ± 0.4 - and 3.3 ± 0.4 -fold increase in cell number in the absence and presence of Herceptin, respectively). The size of the high Herceptin binding subpopulation determined by flow cytometry in Herceptin-treated ($8 \pm 2\%$) and control cells ($7 \pm 1\%$) was not significantly different from each other. Had the subpopulation with high

Herceptin binding capacity been sensitive to the antibody treatment, its representation would have been less in the Herceptin-treated sample. Thus, we conclude that both subpopulations of JIMT-1 are Herceptin resistant.

Discussion

A cell line, JIMT-1, has been established from the pleural effusion of a Herceptin-resistant breast cancer patient. JIMT-1 retains *erbB2* oncogene amplification and its Herceptin-resistant phenotype *in vitro*.⁵ In a search for the mechanism behind the Herceptin resistance of this cell line, we characterized its *erbB* expression profile and the expression of IGF-1R implicated in Herceptin resistance. We found no biologically significant alteration in the absolute numbers of proteins expressed, and thus their relative ratios, compared with three Herceptin-sensitive breast cancer cell lines. We concluded that differences in the *erbB* and IGF-1R expression levels could not account for the Herceptin resistance of JIMT-1 cells.

Binding of Herceptin to *erbB2* is essential for its therapeutic action. We observed a diminished binding of Herceptin Mab to intact, nonpermeabilized JIMT-1 cells; the number of Herceptin binding sites was $\sim 1/5$ the number of *erbB2* proteins expressed. This discrepancy was not the result of intracellular retention of *erbB2*, because another anti-*erbB2* antibody, 2C4, was able to bind to *erbB2* much more efficiently. The preservation of the binding efficiency of 2C4, which recognizes a membrane-distant epitope in the extracellular part of *erbB2*, suggested that the more membrane-proximal Herceptin epitope may have been masked. The binding capacity of Herceptin Fab was significantly higher than that of the whole antibody, confirming the hypothesis that a molecular block selectively hindered access of the larger IgGs to the Herceptin epitope. Detergent pretreatment increased the number of Herceptin Mab binding sites in JIMT-1, presumably by loosening the molecular associations preventing Herceptin binding. We gathered several additional lines of evidence supporting the hypothesis that the Herceptin epitope in JIMT-1 was masked: (a) APMA-induced a

significantly higher level of increase in Herceptin-binding to JIMT-1 than in Herceptin-sensitive lines. (b) APMA treatment also increased the 2C4-binding capacity of JIMT-1 cells, but to a much smaller extent than that of Herceptin. This finding paralleled the higher level of 2C4 binding compared with Herceptin in untreated JIMT-1. We assume that in addition to *erbB2*, APMA-induced metalloproteases cleave other cell surface proteins, probably including MUC4, thereby decreasing the masking of epitopes on *erbB2*. The balance between APMA-induced cleavage and unmasking of *erbB2* determines whether the binding of an antibody will increase or decrease upon APMA treatment. (c) We identified two subpopulations in JIMT-1 showing different Herceptin binding whereas expressing equal levels of *erbB2*. The low Herceptin-binding subpopulation expressed a higher level of MUC4, the molecule we propose to be responsible for masking the Herceptin epitope.

Rat Muc4 blocks the binding of anti-*erbB2* antibodies, including Herceptin (37). We showed that MUC4 was expressed at a higher level in JIMT-1 compared with Herceptin-sensitive lines. Additionally, pixel-by-pixel analysis of MUC4 density and Herceptin fluorescence intensity revealed an inverse correlation between the two quantities. RNAi-mediated knockdown of MUC4 expression significantly increased the binding of Herceptin to the transfected subpopulation. Taken together, these results present strong evidence that MUC4 expression was the cause of the diminished binding of Herceptin to JIMT-1. It has been shown that overexpression of rat Muc4 induces tyrosine phosphorylation of *erbB2* through a specific interaction between *erbB2* and one of the EGF-like domains in the membrane-bound subunit of MUC4 (44). However, phosphorylation of *erbB2* was weak in JIMT-1, although human MUC4 also contains EGF-like domains (40). One possibility is that the level of MUC4 expression was not high enough to cause *erbB2* activation. The size of human MUC4 (4,500-8,500 amino acids; ref. 40) is significantly larger than that of the rat protein (3,000 amino acids; ref. 49). Thus, it is possible that the EGF-like domains in human MUC4 cannot orient properly and consequently cannot bind to *erbB2* due to the

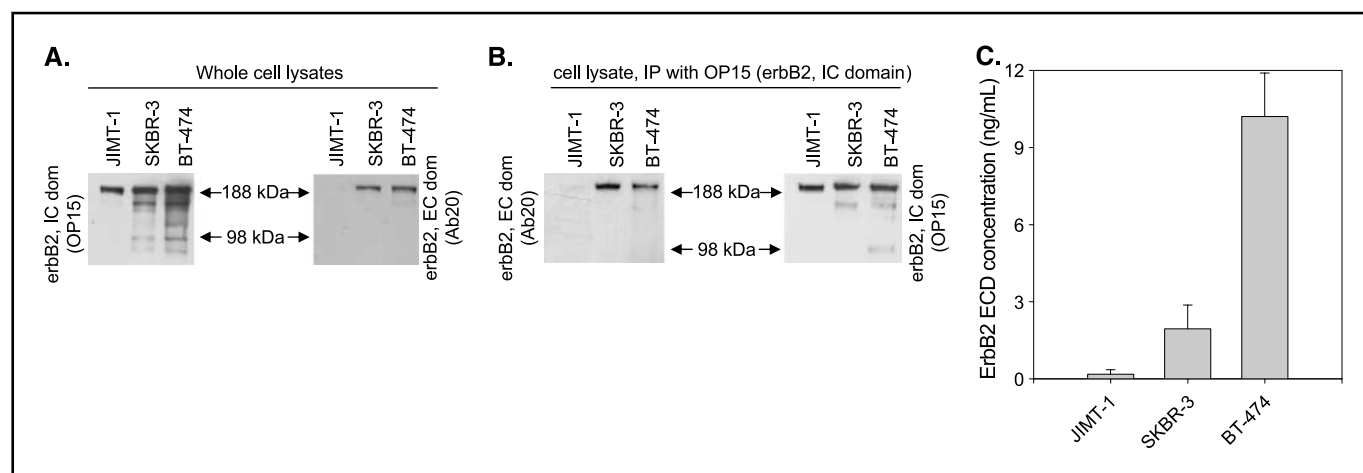


Figure 5. ErbB2 underwent less limited proteolysis in JIMT-1 cells and was recognized less efficiently by Ab20 than in Herceptin-sensitive lines. **A**, whole cell lysates probed with OP15, an antibody against an intracellular epitope on *erbB2*, then stripped and reprobed with Ab20, a cocktail of two antibodies against the extracellular region of *erbB2*. **B**, ErbB2 immunoprecipitated with OP15, separated by SDS-PAGE, and transferred to nitrocellulose. Membranes were first blotted with Ab20, then stripped and reprobed with OP15. **C**, Detection of the shed extracellular domain (ECD) of *erbB2* in conditioned media. Cells were seeded at 80% confluency and conditioned media were assayed for the shed ECD of *erbB2* using an ELISA kit. Bars, SE.

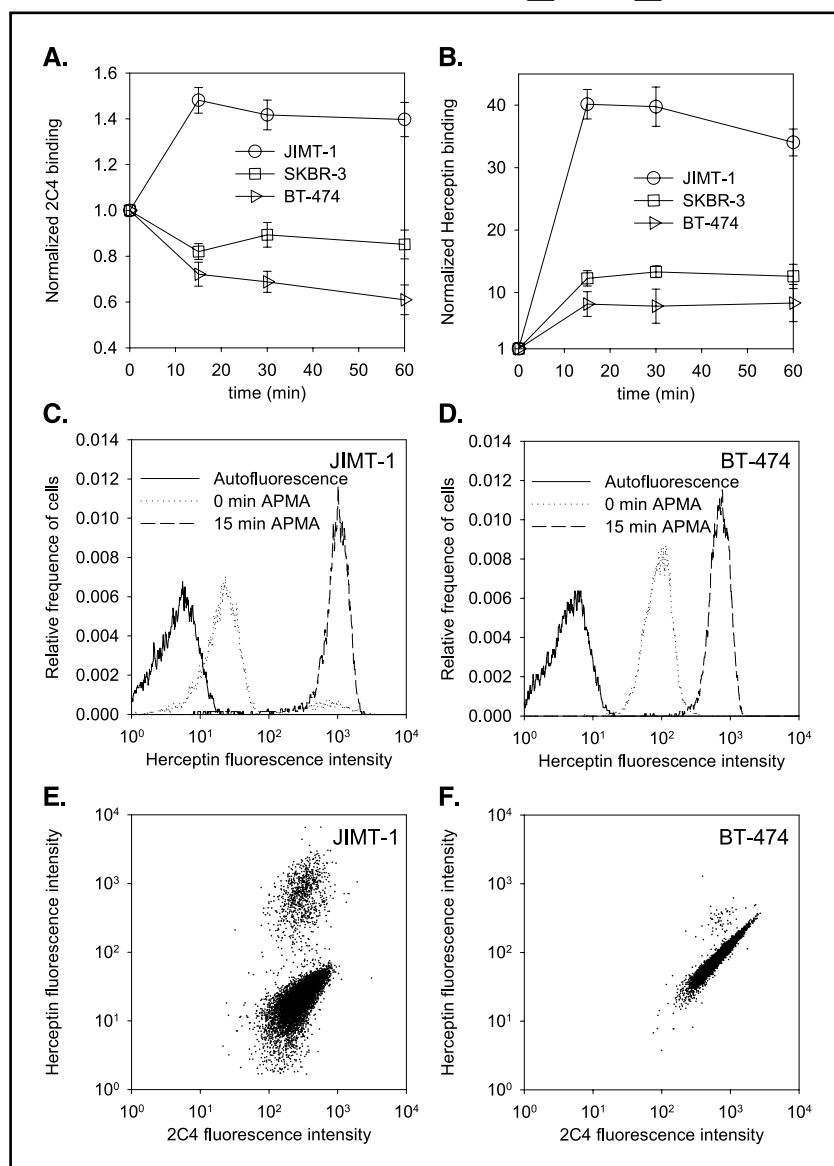


Figure 6. APMA-induced cleavage and unmasking of erbB2. *A* and *B*, normalized 2C4 or Herceptin binding to APMA-treated cells. Cells were treated with 1 mmol/L APMA followed by trypsinization and labeling with fluorescently tagged Mab 2C4 (*A*) or Herceptin (*B*). Mean fluorescence intensities determined by flow cytometry were normalized by the untreated intensity of the respective cell line. *Bars*, SE. *C* and *D*, flow cytometric histograms of JIMT-1 cells (*C*) and BT-474 cells (*D*) treated with APMA followed by labeling with fluorescent Herceptin. Histograms of untreated cells and cells treated with 1 mmol/L APMA for 0 and 15 minutes. The fluorescence histograms of SKBR-3 cells were very similar to those of BT-474 cells and are not shown. *E* and *F*, flow cytometric dot plots of untreated JIMT-1 (*E*) and BT-474 (*F*) cells double-labeled with Alexa488-conjugated 2C4 and Alexa633-conjugated Herceptin showing the correlation of Herceptin and 2C4 binding. The dot plot of SKBR-3 cells was very similar to that of BT-474 cells and is not presented.

presence of the bulky MUC4 α domain. On the other hand, even if MUC4 β can activate the kinase domain of erbB2, MUC4 α may sterically hinder dimerization and cross-phosphorylation of erbB2. Other cell type-specific factors, including increased tyrosine phosphatase activity, may also inhibit or decrease activation of erbB2.

Herceptin-induced erbB2 internalization and down-regulation are thought to be causally related to the mechanism of action of the antibody, although formal proof for this hypothesis is lacking. We found that erbB2 is internalized and down-regulated by Herceptin treatment in JIMT-1 to an extent similar to that observed in Herceptin-sensitive lines. It is noteworthy that in the case of a cell line expressing 5 to 10×10^5 erbB2 per cell, a 20% to 40% down-regulation, the range usually observed, still leaves 3 to 8×10^5 erbB2 proteins on the cell surface, a number substantially higher than the normal expression level. Although it cannot be ruled out that even a relatively small decrease in erbB2 expression can have profound effects on a cell line that is "addicted" to

overexpressed level of an oncogene (50), it is plausible that other actions of Herceptin also contribute to its therapeutic efficiency. The very low level of erbB2 activation in cultured JIMT-1 implies that erbB2 is not used and is therefore not required for the proliferation of JIMT-1. Thus, Herceptin-induced erbB2 down-regulation did not result in a decreased activation state of key signal transducing pathways. This circumstance explains the Herceptin-resistant phenotype of both the low and high Herceptin binding subpopulations of JIMT-1. We believe that the lack of erbB2 activation in JIMT-1 is the result of "molecular isolation" (i.e., a deprivation of erbB2 of its normal interaction and activation partners by MUC4). Previous studies have shown that rat Muc4 can localize erbB2 to the apical surfaces of polarized epithelial cells (51) thus sequestering it from other receptors and ligands present at the basolateral surface. Whether MUC4 can also sequester erbB2 in nonpolarized cells remains to be investigated. The low level of proteolytic processing of erbB2 is probably also caused by blocking access of metalloproteases to

erbB2 by MUC4. Thus, the constitutively active p95^{erbB2} fragment (14) is absent in JIMT-1 and therefore cannot account for Herceptin resistance.

We propose the following model for the evolution of JIMT-1 cells. ErbB2 became overexpressed at an early stage of cancer progression and provided a survival/proliferation advantage. Later MUC4 became overexpressed, increasing the metastatic potential of the cells and protecting them from the immune system. Because MUC4 prevented association of erbB2 with its normal interaction partners, JIMT-1 cells required an alternative pathway leading to the activation of the mitogen-activated protein kinase and PI3K pathways. In this sense, overexpressed erbB2 is a "molecular fossil" reflected in its lack of activation. An important inference is that mere detection of erbB2 overexpression or amplification does not suffice for concluding that a cell line or patient is Herceptin responsive calling for func-

tional assays in the selection of patients for Herceptin treatment. Indeed, it has been observed that patients with erbB2-phosphorylated breast cancer benefit more from Herceptin therapy than those without activated erbB2 (52). Diagnostic and treatment approaches based on a molecular understanding of the activation pathways of receptor tyrosine kinases will eventually lead to a more efficient and patient-specific treatment of malignant diseases.

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Novel Calibration Method for Flow Cytometric Fluorescence Resonance Energy Transfer Measurements Between Visible Fluorescent Proteins

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Background: The combination of fluorescence resonance energy transfer (FRET) and flow cytometry offers a statistically firm approach to study protein associations. Fusing green fluorescent protein (GFP) to a studied protein usually does not disturb the normal function of a protein, but quantitation of FRET efficiency calculated between GFP derivatives poses a problem in flow cytometry.

Methods: We generated chimeras in which cyan fluorescent protein (CFP) was separated by amino acid linkers of different sizes from yellow fluorescent protein (YFP) and used them to calibrate the cell-by-cell flow cytometric FRET measurements carried out on two different dual-laser flow cytometers. Then, CFP-Kip1 was coexpressed in yeast cells with YFP and cyclin-dependent kinase-2 (Cdk2) and served as a positive control for FRET measurements, and CFP-Kip1 coexpressed with a random peptide fused to YFP was the negative control.

Results: We measured donor, direct, and sensitized acceptor fluorescence intensities and developed a novel

way to calculate a factor (α) that characterized the fluorescence intensity of acceptor molecules relative to the same number of excited donor molecules, which is essential for quantifying FRET efficiency. This was achieved by calculating FRET efficiency in two different ways and minimizing the squared difference between the two results by changing α . Our method reliably detected the association of Cdk2 with its inhibitor, Kip1, whereas the nonspecific FRET efficiency between Cdk2 and a random peptide was negligible. We identified and sorted subpopulations of yeast cells showing interaction between the studied proteins.

Conclusions: We have described a straightforward novel calibration method to accurately quantitate FRET efficiency between GFP derivatives in flow cytometry.

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Key terms: flow cytometry; fluorescence resonance energy transfer; green fluorescent protein

The conformation and association state of proteins are recognized as important factors in biological regulation. Fluorescence resonance energy transfer (FRET) is a physical phenomenon that can be used to measure in a quantitative way the average separation distance between two molecules (1). Although it was described in the 1940s by Dexter (2) and Förster (3), it has become a widely accepted biological research tool only in the past decade. In FRET, energy is transferred in a non-radiative fashion among an excited fluorescent molecule, the donor, and a nearby acceptor molecule that is usually, but not necessarily, fluorescent (4). Energy transfer efficiency (E) is

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defined as the fraction of excited donor molecules undergoing FRET and can be expressed in the following way:

$$E = \frac{R_0^6}{R_0^6 + R^6} \quad (1)$$

where R is the separation distance between the donor and the acceptor and R_0 is a distance at which E is 50%. R_0 is characteristic of a given donor-acceptor pair and its value is typically in the 3- to 8-nm range. The rate of FRET efficiency depends on the sixth power of the separation distance between the donor and the acceptor, thus providing a sensitive tool for the measurement of protein associations in the 2- to 10-nm range.

Before the introduction of green fluorescent protein (GFP) and its variants, which we collectively refer to as visible fluorescent proteins (VFPs), FRET measurements were limited to intrinsically fluorescent proteins or to proteins labeled with fluorescent ligands or antibodies. The advent of VFP-tagged proteins made FRET measurements in unperturbed systems much more widely applicable. Cyan and yellow fluorescent proteins (CFP-YFP) and blue fluorescent protein (BFP) and GFP are good donor-acceptor pairs based on their R_0 values and the overlap between donor and acceptor excitation and emission wavelengths (5). Many methods are available for the measurement of FRET. Several recent papers have been devoted for a comprehensive review of the topic (6–8), so we only briefly refer to flow cytometrically based FRET methods and to problems related to reliable quantification of FRET experiments.

In many cases, investigators have calculated FRET intensities or indices instead of reporting FRET efficiencies. FRET intensity is the fluorescence intensity measured in the FRET channel after correction for spectral overspill (9). It is sensitive for the amount of fluorophores expressed and thus is very error-prone. FRET indices are functions of the FRET efficiency only, but their interpretation is made difficult by the absence of a clear physical meaning of the calculated parameter (10). In addition, some of them may show interlaboratory variation. FRET_N (11) and N-FRET (12) are FRET indices that take the donor and acceptor concentrations into account and are supposed to be true measurements of protein-protein interactions (11,12). Lately, their utility has been questioned based on their non-monotonous dependence on the acceptor concentration (13). Although methods based on the detection of increased donor anisotropy in the presence of FRET have a solid physical background, their results are difficult to interpret in terms of protein associations without resorting to complicated modeling (14–16).

A plethora of approaches exists that yields the FRET efficiency itself. We previously reported one such method based on the combined detection of donor and acceptor fluorescence intensities in cells labeled with fluorescent ligands or antibodies using flow cytometry (17,18). Later, we established a similar method in fluorescence microscopy (19). These methods require a factor, variably called α (17–19) or G (11,13), which relates the intensity lost on the donor side due to donor quenching to the enhanced

emission of the acceptor due to sensitized emission. Methods for the determination of α have been reported for antibody-labeled cells in flow cytometry (17,18) and microscopy (19) and lately for VFP-expressing cells in microscopy (13). To the best of our knowledge, successful determination of α has not been reported for VFP-expressing cells using flow cytometry. A popular approach for the determination of FRET efficiency is based on the release of donor quenching upon photodestruction of the acceptor (20,21). Although this method has been applied to conventional fluorophores (20) and VFP-expressing cells (21), it cannot be used in flow cytometry because detection of donor fluorescence intensity before and after acceptor photobleaching is usually not feasible.

Because flow cytometry is statistically superior to fluorescence microscopy, it is an attractive method for FRET measurements. As discussed above, accurate determination of the FRET efficiency in VFP-expressing cells has not been accomplished in flow cytometry. We present such an approach, and its novelty lies in the determination of α in cells expressing VFP using flow cytometry. We demonstrate the reliability of the approach using cells with and without interaction between the donor and acceptor VFP derivatives.

MATERIALS AND METHODS

Cells

Saccharomyces cerevisiae INV Sc1 cells with the auxotroph markers *trp1*, *ura3*, *leu2*, and *his3* were used. Cells were transformed using polyethylene-glycol/lithium acetate and heat shock (42°C for 15 min) and then plated on glucose-containing selection plates. pYES2- and pYES3-based constructs complemented for the *ura3* and *trp1* auxotroph markers, respectively. To induce protein expression under the control of the GAL1 promoter in pYES2 or pYES3, 2- to 3-day-old colonies were transferred to galactose/raffinose-containing plates, and VFP expression was checked every hour with a fluorescence microscope. Best expression results were obtained 6 to 8 h after induction.

Plasmids

A detailed description of the cloning strategy has been published elsewhere (22); therefore we give a brief summary only.

Cloning of VFP variants. GFP (GFP-S65G-S72A) *mut-3* optimized for codon usage in the yeast (yGFP) (23) was provided in a pUC19 vector by Dr. Alistair J. P. Brown (Institute of Medical Science, University of Aberdeen). To make purification of the expressed proteins easier, a hexa-His tag was added to the N-terminus of yGFP using polymerase chain reaction (PCR). Because every construct was derived from yGFP, all contained the hexa-His tag. One additional mutation was introduced (T203Y) to convert yGFP to yYFP. Six mutations (F64L, S65T, Y66W, N146I, M153T, V163A, and N212K) were needed to generate yCFP from yGFP. In addition to the spectral shift, these mutations increased water solubility, temperature resistance, and maturation rate of the protein (24–29). yYFP and yCFP were cut out of pUC19 with *HindIII* and *EcoRI*

and cloned into pYES2 (Invitrogen, Karlsruhe, Germany) using the same restriction sites.

Cloning of the CFP-30Pro-YFP construct. Synthetic oligonucleotides (Gibco-Invitrogen, Karlsruhe, Germany) coding for 30 prolines with *EcoRI/BamHI* sticky ends (forward: AATTC CCA CCA CCT CCA CCA CCT CCA CCA CCC CCA CCA CCT CCA CCA CCT CCA CCA CCC CCA CCA CCT G; reverse: GATCC AGG TGG TGG GGG TGG TGG AGG TGG TGG AGG TGG TGG GGG TGG TGG AGG TGG TGG AGG TGG TGG G) were annealed and ligated into a modified pGAD T7 vector (Clontech, Palo Alto, CA, USA) in which the *HindIII* restriction site at position 2280 was destroyed. A *HindIII/EcoRI* fragment of pUC19 containing yECFP was cloned in front of the linker. Then the yCFP+30 Pro segment was cut out with *HindIII/BamHI* and ligated into pYES2 into which yYFP had already been cloned.

Cloning of the CFP-3Gly-YFP construct. Three glycine (Gly) residues were added to the C-terminus (3' end) of yCFP by PCR, and the yCFP+3 Gly segment was inserted into pYES2-yYFP via *HindIII/BamHI* 5' of yYFP.

Cloning of the CFP-25 AA-YFP construct. The donor and the acceptor were linked by the trypsin-sensitive linker SSMTGGGQMGRLYDDDDKDPPAEF (26) using two PCR steps. First, yYFP-containing pUC19 was used as a template and the sequence GRDLYDDDDKDPPAEF was added to the 5' end of yYFP and a *PstI* site to the 3' end. Then this PCR product was used as a template and an *EcoRI* restriction site and the rest of the linker (SSMTGGGQM) was added to the 5' end. The final PCR product was cloned into yCFP-containing pUC19 via *EcoRI/PstI* 3' of yCFP and cut out of pUC19 via *HindIII/XbaI* and cloned into pYES2.

Cloning of CFP-Kip1 and YFP plus cyclin-dependent kinase-2. Kip1 (accession no. U10906, with V109G mutation) and cyclin-dependent kinase-2 (Cdk2; accession no. X61622, with S177C mutation) were provided by Otagene USA (Seattle, WA, USA), cut out of pGAD T7 with *EcoRI/XbaI*, and inserted in-frame to the 3' end of the yCFP-containing pYES2 plasmid or the yYFP-containing pYES3 plasmid using the above restriction sites.

Purification of His-Tagged CFP-YFP Constructs

Yeast cells were grown on selective plates without galactose. A preculture was prepared in 5 ml of basic drop-out medium without galactose. The main culture was started at an OD₆₀₀ of 0.2 and grown overnight until an OD₆₀₀ of ~2 was reached. Protein expression from the GAL1 promoter was induced by 2% galactose for 6 h. Cells were pelleted, resuspended in breaking buffer (11.3 mM NaH₂PO₄, 38.7 mM Na₂HPO₄, pH 7.4, 1 mM ethylenediaminetetraacetic acid, 5% glycerol, 1 mM phenyl methyl sulfonyl fluoride), and lysed using glass beads (G8772, Sigma, Budapest, Hungary). His-tagged proteins were purified from the cell lysate on an Äkta Basic high-performance liquid chromatographic system using HisTrap HP columns (Amersham Biosciences, Freiburg, Germany).

Flow Cytometry

A modified Becton-Dickinson Facstar Plus flow cytometer was used for energy transfer measurements. The 457- and 514-nm lines of two argon ion lasers were used to excite CFP and YFP, respectively. Donor and acceptor emissions were separated by a 525-nm dichroic mirror and were detected through 487/37-nm and 560/70-nm bandpass mirrors, respectively. Donor, acceptor, and FRET intensities were recorded as described previously (17,18). Briefly, two fluorescence intensities were detected for the donor excitation at 457 nm (donor channel I₁, detection at 487 nm; acceptor channel I₂, emission corresponding to the FRET channel at 560 nm), and one intensity was measured for the acceptor excitation at 514 nm (I₃, acceptor emission at 560 nm). For experiments with BFP and GFP, the ultraviolet lines (334 to 364 nm) and the 488-nm line of two argon ion lasers were used. BFP and GFP fluorescence emissions were separated by a 495-nm dichroic mirror and detected through 440/50-nm and 525/50-nm bandpass filters, respectively. Two fluorescence intensities (I₁, I₂) and one fluorescence intensity (I₃) were recorded from the intersection points corresponding to the donor and acceptor excitations, respectively, as described above.

In other experiments a Becton-Dickinson FACS Vantage flow cytometer was used to measure FRET between CFP and YFP. CFP and YFP were excited by argon-krypton and argon ion lasers at 406 to 415 nm and 514 nm, respectively. The emitted fluorescence was collimated and photons originating from the two laser lines were separated by a half-mirror that reflected only the beam coming from the 406- to 415-nm excitation. This reflected beam was further divided by a 510-nm dichroic mirror, and donor and acceptor fluorescence intensities were measured through 450/50-nm and 560/70-nm bandpass filters, respectively, corresponding to I₁ and I₂. The beam from the 514-nm excitation was measured through a 575/26-nm bandpass filter (I₃).

Mathematical Background of Flow Cytometric FRET Calculations

There are three unknowns in an experimental system in which the interaction of a protein pair separately labeled with donor and acceptor is investigated: the densities or amounts of the donor and acceptor and the FRET efficiency. Therefore, three independent measurements have to be taken that correspond to the donor (I₁), FRET (I₂), and acceptor (I₃) channels (17,18):

$$I_1(\lambda_{D,ex}, \lambda_{D,em}) = I_D(1 - E) \quad (2)$$

$$I_2(\lambda_{D,ex}, \lambda_{A,em}) = I_D(1 - E)S_1 + I_A S_2 + I_D E \alpha \quad (3)$$

$$I_3(\lambda_{A,ex}, \lambda_{A,em}) = I_D(1 - E)S_3 + I_A + I_D E \alpha \frac{S_3}{S_1} \quad (4)$$

Subscripts D and A refer to donor and acceptor, respectively, and subscripts ex and em stand for excitation and emission, respectively. I_D and I_A are the unquenched

donor and direct acceptor intensities, respectively; and E represents FRET efficiency. The constants S_1 to S_3 correct for the spectral overspill between the detection channels. S_1 corrects for the overspill of donor fluorescence from I_1 to I_2 , S_3 for the overspill of donor fluorescence from I_1 to I_3 , and S_2 for the overspill of acceptor emission from I_3 to I_2 . The constants S_1 to S_3 are determined on single-labeled samples as described previously (17,18) and can be expressed by the following equations:

$$S_1 = \frac{\eta_D(I_2)}{\eta_D(I_1)} \quad (5)$$

$$S_2 = \frac{\rho(\lambda_{D,ex})\varepsilon_A(\lambda_{D,ex})\eta_A(I_2)}{\rho(\lambda_{A,ex})\varepsilon_A(\lambda_{A,ex})\eta_A(I_3)} \quad (6)$$

$$S_3 = \frac{\rho(\lambda_{A,ex})\varepsilon_D(\lambda_{A,ex})\eta_D(I_3)}{\rho(\lambda_{D,ex})\varepsilon_D(\lambda_{D,ex})\eta_D(I_1)} \quad (7)$$

where $\eta_D(I_x)$ and $\eta_A(I_x)$ represent the detection efficiencies of donor and acceptor fluorescences, respectively, in the I_x fluorescence channel; $\rho(\lambda_{D,ex})$ and $\rho(\lambda_{A,ex})$ denote the photon fluxes of the lasers used for donor and acceptor excitation, respectively; ε_D and ε_A refer to the molar absorption coefficients of the donor and acceptor, respectively, at the wavelengths in parentheses. $\eta_A(I_2)$ and $\eta_A(I_3)$ are equal if the same detector is used for the measurement of I_2 and I_3 .

It can be seen from equations 2 to 7 that the constant α is necessary for the calculation of FRET efficiency on a cell-by-cell basis. The α value relates the fluorescence intensity of acceptors in detection channel I_2 to the intensity of the same number of excited donor molecules in detection channel I_1 and can be expressed according to the following equation:

$$\alpha = \frac{\Phi_A \eta_A(I_2)}{\Phi_D \eta_D(I_1)} \quad (8)$$

where Φ_D and Φ_A represent the fluorescence quantum efficiencies of the donor and acceptor, respectively. For determination of α in flow cytometry, two samples with an equal number of donor and acceptor molecules are needed. Alternatively, one sample expressing the same number of non-interacting ($E = 0\%$) donor and acceptor molecules is necessary. Such samples are very difficult to prepare, so an alternative approach had to be developed.

RESULTS

Theory of Determining α and the Absorption Ratio

We used two different methods to calculate FRET from flow cytometric data. According to the method involving α as described in Materials and Methods, FRET can be calculated from equations 2 to 4:

$$\frac{E}{1-E} = \frac{1}{\alpha} \left(\frac{I_2 - S_2 I_3}{\left(1 - \frac{S_2 S_3}{S_1}\right) I_1} - S_1 \right) = \frac{1}{\alpha} R_1 \quad (9)$$

$$E = \frac{R_1}{\alpha + R_1} \quad (10)$$

FRET calculation can alternatively be based on the following equation:

$$\frac{F_{AD}}{F_A} = 1 + \frac{\varepsilon_D c_D}{\varepsilon_A c_A} E = R_F \quad (11)$$

where F_{AD} and F_A are the fluorescence intensities of the acceptor in the presence and absence of the donor, respectively; and c_D and c_A are the molar concentrations of the donor and acceptor, respectively. F_{AD} and F_A can be expressed according to the notation of equations 2 to 4:

$$F_{AD} = I_2 - I_1 S_1 \quad (12)$$

$$F_A = I_A S_2 \approx I_3 S_2 \quad (13)$$

The above approximation holds if S_3 is negligible and $S_1 > S_3$. In our case, $S_3 < 0.01$ and $S_1 > 1$; therefore, neglecting the terms containing S_3 in equation 4 is substantiated. FRET efficiency can be isolated from equation 11:

$$E = \frac{R_F - 1}{\frac{\varepsilon_D c_D}{\varepsilon_A c_A}} \quad (14)$$

The drawback of the first approach (equation 10) is that it involves α , and the disadvantage of the second method (equation 14) is that it involves the absorption ratio $(\varepsilon_D c_D)/(\varepsilon_A c_A)$, a parameter also difficult to determine experimentally in flow cytometry. If there are a series of samples with different FRET efficiencies in which the donor and the acceptor are expressed in a 1:1 ratio, one of these samples is selected as a reference (displayed with index 1), and the FRET efficiencies of the rest of the samples are divided by the FRET efficiency of the reference sample eliminating the absorption ratio. These ratios are separately calculated according to methods 1 (equation 10) and 2 (equation 14); their difference is calculated and summed for all the samples:

$$\sum_{j=2,3,\dots,n} \left(\frac{R_{F,1} - 1}{R_{F,j} - 1} - \frac{R_{1,1}}{\alpha + R_{1,1}} \frac{\alpha + R_{1,j}}{R_{1,j}} \right)^2 \quad (15)$$

The fact that the donor and the acceptor are expressed in a 1:1 ratio eliminates the donor and acceptor concentrations from equation 15, the minimum of which is found by changing α to yield α itself, because the two approaches are expected to give identical FRET efficiencies. A 1:1 donor:acceptor ratio is not required for this method, but a constant

Table 1
Calibration of FRET Values Using the Three CFP-YFP Constructs*

	I ₁	I ₂	I ₃	R _I	R _F	FRET (%)		I _D	I _A	ε _D /ε _A
						From R _I	From R _F			
30 Pro	143	425	225	0.62	1.54	9.8	10.1	158	224	5.9
25 AA	179	615	297	1.05	1.87	15.6	16.3	210	296	6.0
3 Gly	109	586	233	2.82	2.79	33.1	33.5	159	231	5.8

*Fluorescence intensities in the donor, FRET, and acceptor channels were measured. Autofluorescence-corrected values are presented in columns I₁ to I₃. R_I and R_F values calculated from flow cytometric data and the FRET values derived from these ratios according to equations 10 and 14 are presented. The α value and the absorption ratio (ε_D/ε_A) were calculated from R_I and R_F and were 5.7 and 5.3, respectively. I_D and I_A values were determined by solving equations 2 to 4 using the constants S₁ = 1.31, S₂ = 0.68, and S₃ = 0.004, and the absorption ratio was determined according to equation 18 for every sample.

donor:acceptor ratio in every sample is necessary. We opted for a 1:1 ratio because this seemed to be the easiest to accomplish. If F_{AD}/F_A calculated according to equations 12 and 13 is plotted against E calculated according to equation 10 using α derived as described above, the slope of the graph yields the absorption ratio (see equation 11).

By substituting equation 10 into equation 11 the relationship between R_F and R_I can be determined:

$$R_F = 1 + \frac{\varepsilon_D C_D}{\varepsilon_A C_A} \frac{R_I}{\alpha + R_I} \quad (16)$$

The linear form of equation 16 can also be used to derive the absorption ratio and α:

$$\frac{1}{R_F - 1} = \frac{\varepsilon_A C_A}{\varepsilon_D C_D} \left(1 + \frac{\alpha}{R_I} \right) \quad (17)$$

A plot of 1/(R_F - 1) against 1/R_I yields a line with an intercept of (ε_AC_A)/(ε_DC_D) and a slope of α × intercept. Similar to the above described minimization approach, the linear regression also provides the absorption ratio and α.

If the donor and the acceptor are expressed in a 1:1 ratio and α is known after minimizing equation 15, the unquenched donor (I_D) and direct acceptor (I_A) intensities can be calculated by solving the set of equations 2 to 4 (18). The only compensation necessary to get the fluorescence intensities of an equal number of donor and acceptor molecules is the correction for the different molar extinction coefficients of the donor and the acceptor, because the same laser line is used for the donor (I₁) and FRET (I₂) channels. Therefore, α can be calculated according to the following equation:

$$\alpha = \frac{I_A S_2 \varepsilon_D(\lambda_D)}{I_D \varepsilon_A(\lambda_D)} \quad (18)$$

The absorption ratio can be easily calculated from equation 18.

Determining α and the Absorption Ratio Using Three CFP-YFP Constructs

We generated three CFP-YFP chimeras in which the fluorophores were separated by three glycines, 30 prolines, or a trypsin-sensitive linker consisting of 25 amino acids (26), and the proteins were expressed in yeast cells. The α value was determined by minimizing equation 15, and the absorption ratio was calculated from the slope plotted according to equation 11. The absorption ratio was also calculated for each sample by using equation 18. In addition, the absorption ratio and α were determined according to equation 17 in one step. The calculations yielded similar results (Table 1, Fig. 1). FRET efficiencies were calculated according to equations 10 and 14 (Table 1, Fig. 2). In addition, the FRET efficiency of the CFP-YFP construct in which the fluorophores were separated by the trypsin-cleavable linker were determined by trypsin-induced de-quenching (26), yielding a FRET efficiency of 20%, a value very close to our flow cytometric results. FRET calculated by the two flow cytometric methods are identical within experimental error, and their magnitude changes as expected: the FRET efficiency of the CFP-3Gly-YFP sample is the highest, followed by the CFP-25AA-YFP and CFP-30Pro-YFP samples. The 30-Pro linker is thought to form a rigid helix between CFP and YFP (see Discussion for more details), and the 25-AA and 3-Gly linkers maintain a shorter separation distance according to the length of their amino acid chains. A similar series of BFP-GFP chimeras were also generated. Unfortunately, we could not carry out a reliable determination of α using this FRET pair for reasons presented in the Discussion.

Reliability of the Determination of α

The mean squared error (MSE) of the minimized norm can be determined according to the following equation:

$$MSE = \frac{\sum_{j=2,3..n} \left(\frac{R_{F,j} - 1}{R_{F,j} - 1} - \frac{R_{I,j}}{\alpha + R_{I,j}} \frac{\alpha + R_{I,j}}{R_{I,j}} \right)^2}{n - 1} \quad (19)$$

The MSE was calculated from the data presented in Table 1, and it is shown in Figure 3A. In a separate experiment, α,

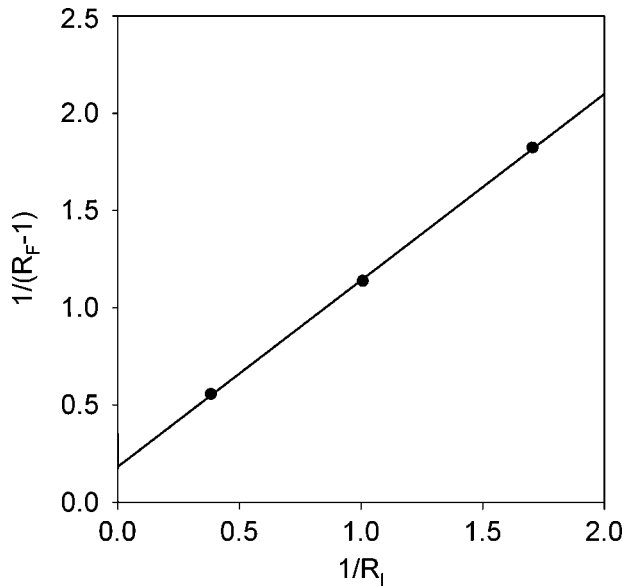


FIG. 1. Determination of α and the absorption ratio using linear regression. R_F - R_I pairs measured on the three calibration samples were plotted according to equation 17, and a line was fitted, yielding a slope of 0.96 and an intercept of 0.18. From these values the absorption ratio and α were determined to be 5.48 and 5.26, respectively.

the absorption ratio, and the FRET efficiencies were determined using different optical alignment and detector settings. The α value was much smaller (0.615 instead of 5.7), and the MSE increased much faster around the minimum, especially in the case of large α values (Fig. 3B). Noisy measurements can give rise to weak dependence of MSE on α , leading to unreliable determination of the minimum. Therefore, we investigated whether the significantly different shape of the $MSE(\alpha)$ curve is related to noise or to the different values of α determined by the experimental setup. A computer simulation was carried out assuming an absorption ratio of 5 and FRET efficiencies of 5%, 15%, and 30% for the calibration samples. R_F values were calculated from equation 14, and R_I values were determined according to equation 10 using four different α values (0.5, 2, 5, and 10). The MSE was then calculated using equation 19 (Fig. 3C). It can be seen that the MSE increases much faster around the minimum if α is small. We concluded that the different dependences of the MSE on α shown in Figures 3A and 3B are at least partly caused by optical and other instrumental factors unrelated to noise.

Confidence Interval of α

Given the weak dependence of the MSE on α , if the minimum of the function is at a large value of α , we wanted to determine the 95% confidence interval of the calculation of α . Twenty thousand normally distributed triplets of I_1 , I_2 , and I_3 were generated according to the means and standard deviations of the intensities corresponding to the two experimental setups presented in Figures 3A ($\alpha = 5.7$) and 3B ($\alpha = 0.615$). Then, α was determined from the simulated datasets, and a histogram was calculated from the 20,000 α values (Fig. 4). The histogram corresponding to α

$= 0.615$ is somewhat narrower, but it has a long tail right of the peak. This results in an insignificant difference between the standard deviationss of the histograms (0.71 and 0.67 corresponding to $\alpha = 0.615$ and $\alpha = 5.7$, respectively). Similarly, the widths of the 95% confidence intervals of the calculations are not substantially different for the two cases (between -0.06 and 3.067 for $\alpha = 0.615$; between 4.72 and 7.56 for $\alpha = 5.7$). Equation 10 shows that the relative rather than the absolute error of α determines the error of FRET calculations. Therefore, we calculated the coefficient of variation (standard deviation divided by the mean) of the histograms that yielded 0.91 and 0.11, corresponding to $\alpha = 0.615$ and $\alpha = 5.7$, respectively. We concluded that the weak dependence of the MSE on α in the case of large α values does not deteriorate the reliability of FRET calculations.

Correspondence Between Calculated and Experimentally Determined R_F - R_I Plots

Using the values of α and the absorption ratio presented in Table 1, simulated R_I and R_F values were calculated according to equations 10 and 11, respectively, corresponding to a range of E values. The simulated R_F - R_I curve and the experimentally determined three pairs of R_F - R_I were plotted in the same graph (Fig. 5A), which can be regarded as a fit of equation 16 to the measurement. The correspondence between the fitted and the measured data was remarkably good.

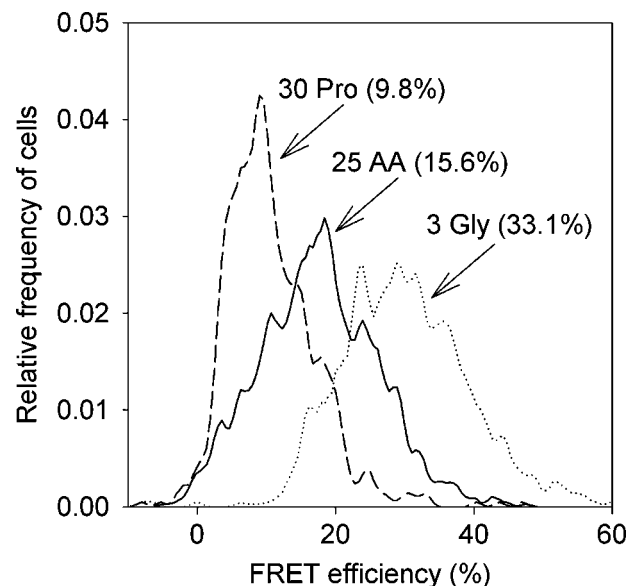


FIG. 2. Flow cytometric FRET histograms of three different CFP-YFP constructs. Yeast cells expressing constructs in which CFP and YFP were separated by three glycines, the 25 AA linker, or 30 prolines were analyzed by flow cytometry. The α value necessary for the calculation of FRET efficiency was determined, and FRET was determined on a cell-by-cell basis. The mean values of the three FRET histograms are displayed in parentheses.

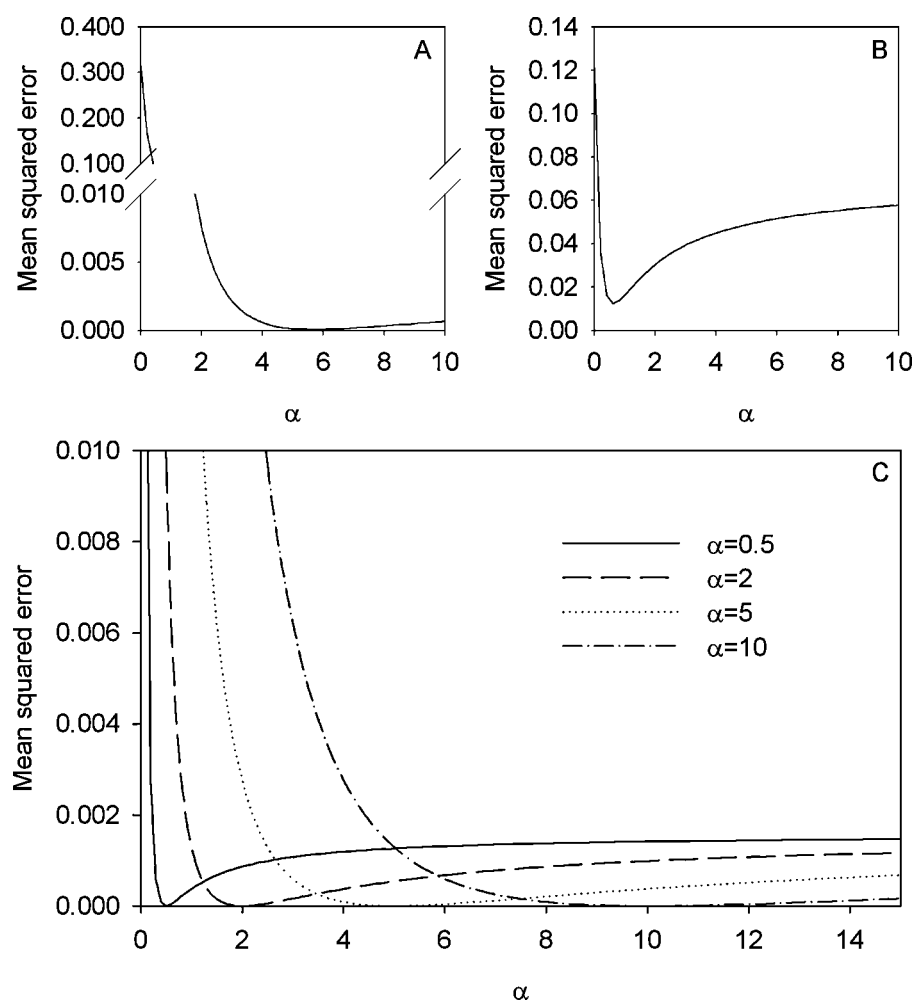


FIG. 3. Experimental and simulated mean squared errors of the minimization process as a function of α . **A, B:** Mean squared error of the minimization of equation 15 determined from flow cytometric data. R_I and R_F values displayed in Table 1 were substituted into equation 15, the minimization of which yielded $\alpha = 5.7$. The mean squared error was plotted as a function of α (A). An identical calculation was carried out after measuring the same samples with flow cytometric data obtained with different instrument parameters yielding $\alpha = 0.615$, and the mean squared error was plotted against α (B). **C:** Simulation of experimental data. Calculations were carried out by assuming that the FRET efficiencies of the measured samples were 5%, 15%, and 30% and the absorption ratio ($\epsilon_D C_D / \epsilon_A C_A$) was 5. R_I and R_F values were calculated for four different values of α (0.5, 2, 5, and 10), and the mean squared error was determined according to equation 19 and plotted as a function of α .

Measurement of the Interaction Between p27-Kip1 and Cdk2

Cdk2 interacts with the inhibitor p27-Kip1 (30); therefore, we used them as a positive control to test the FRET system described above. A CFP-Kip1 chimera and a YFP-Cdk2 chimera were coexpressed in yeast cells and a FRET histogram was calculated from 10,000 cells (Fig. 6). The average FRET efficiency was calculated from the means of three such histograms and was $9 \pm 2\%$. If YFP-Cdk2 was coexpressed with a random peptide fused to CFP, FRET was $1 \pm 1\%$. Next, the CFP-Kip1 and YFP-Cdk2 coexpressing sample were subjected to sorting. Two sorting gates were defined as shown in Figures 7A and 7B. The gate in Figure 7A was created so that only cells expressing high levels of donor and acceptor are sorted and their relative expression ratio is not substantially different from 1. The second gate was placed on cells in which the intensity in the FRET channel was relatively high; therefore, an interaction between the two proteins can be expected. We must note that the separation between the excitation and emission wavelengths in the FRET channel (406 to 415 nm and 560/70 nm, respectively) were large; therefore, spectral overspill from direct donor or acceptor emission to

the FRET channel was negligible. However, even in this case the intensity in the FRET channel is determined by the concentration of the donor and the FRET efficiency ($I_D \cdot E \cdot \alpha$), so offline FRET calculation was necessary to show that cells containing interacting proteins had been sorted. We first established that cells corresponding to the sorting gates were indeed sorted (Fig. 7C,D). Then, α was calculated using the method described in this report, and FRET was determined on a cell-by-cell basis for the unsorted and sorted populations. This calculation clearly showed that the sorted population was enriched in cells containing interacting proteins (Fig. 7E).

DISCUSSION

We have developed a novel flow cytometric method for the quantitative evaluation of the FRET efficiency between different VFPs on a cell-by-cell basis. A flow cytometric FRET calculation must involve a constant, designated α in the present report, that relates the fluorescence intensity of acceptor molecules to that of an equal number of excited donor molecules. The major merit of the present study is the elaboration of a novel method for the flow cytometric determination of α . In microscopy the determination of α can be based on the comparison of the inten-

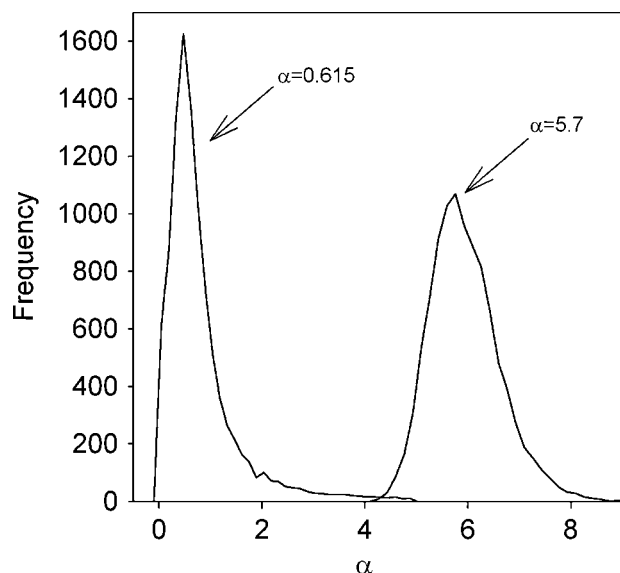


FIG. 4. Confidence interval of the determination of α . Means and standard deviations of parameters I_1 , I_2 , I_3 , S_1 , and S_2 corresponding to the flow cytometer settings yielding $\alpha = 5.7$ and $\alpha = 0.615$ were used to generate 20,000 normally distributed I_1 , I_2 , I_3 , S_1 , and S_2 variables from which R_1 and R_F were calculated. Then, minimization of the mean squared error in equation 19 was carried out, and the distributions of the resultant α values were plotted. The 95% confidence intervals are 4.72 to 7.56 and 0.06 to 3.067 for the histograms corresponding to $\alpha = 5.7$ and $\alpha = 0.615$, respectively.

sity lost on the acceptor side due to acceptor bleaching to the consequent increase in donor intensity due to release of quenching (13). Because photobleaching measurements are difficult to carry out in flow cytometry, the usual flow cytometric way to determine α is to compare the fluorescence intensity of an equal number of excited donor and acceptor molecules. When antibody- or ligand-labeled cells are used, this is usually done by separately labeling an equal number of epitopes with donor- and acceptor-tagged antibodies. Identical expression of two YFPs serving as a donor and acceptor pair can be achieved only if the two are part of a single protein. If there is no FRET between the donor and acceptor, α can be directly calculated using equation 18 provided the absorption ratio is known. We wanted to create such a CFP-YFP chimera by placing a linker consisting of 30 prolines between the two YFPs (31). If there had been no FRET between CFP and YFP in such a construct, it could have been used for the determination of α directly as described previously (17,18). However, initial measurements showed that FRETn and N-FRET parameters were not 0, indicating that FRET took place. The lack of correlation between the FRET efficiency and the expression level of the fusion construct indicated that intermolecular FRET did not play a role. Taking into account that the R_0 for the CFP-YFP pair is 4.9 nm (5), a FRET efficiency of 10% corresponds to a donor-acceptor separation distance of 7 nm. In a typical polyproline helix in an aqueous environment (polyproline helix type II), the helical rise per residue is 0.31 nm, yielding 9.3 nm for the separation between the N- and C-termini of the polyproline linker expected to result in a FRET

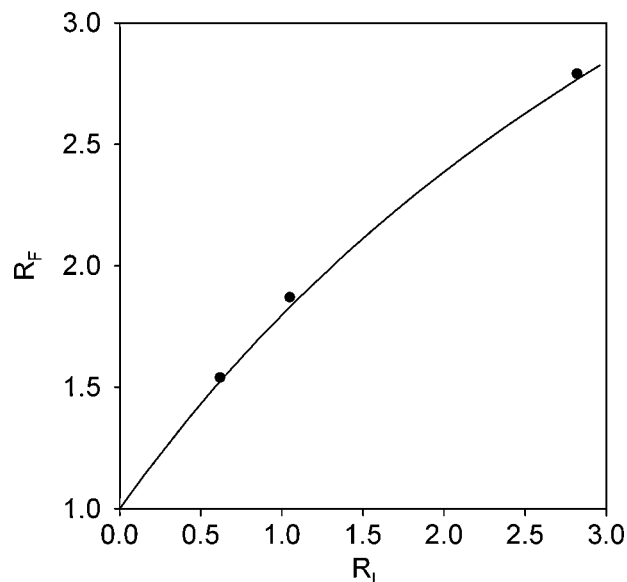


FIG. 5. Simulated and experimentally determined R_1 and R_F values. The α value and the absorption ratio ($\epsilon_D C_D / \epsilon_A C_A$) determined from data in Table 1 were used to generate pairs of R_1 and R_F values according to equations 10 and 11 by changing the FRET efficiency, and they were plotted together with the experimentally determined R_1 and R_F values in Table 1.

efficiency of 2%. If the fluorophores are assumed to be at the end of the helix, the polyproline linker has to be significantly bent (with a curvature radius of 3.8 nm). Although polyproline helices can be distorted by asymmetric hydrogen bonding with surrounding water molecules, such a significant bending is unlikely. In contrast, CFP and YFP attached to the termini of the linker may

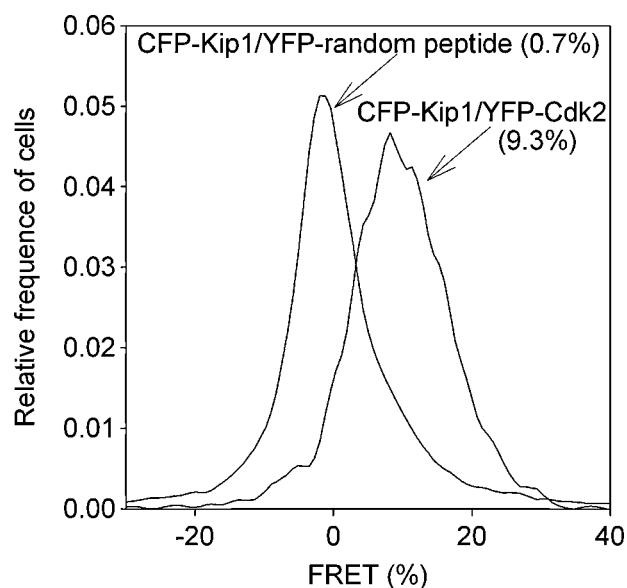


FIG. 6. Flow cytometric measurement of the interaction of p27-Kip1 with Cdk2 and a random peptide. Yeast cells expressing CFP-Kip1 and YFP-Cdk2 or CFP-Kip1 and YFP fused to a random peptide were analyzed by flow cytometry. FRET between CFP and YFP was quantitated and histograms of 10,000 cells were plotted. The mean FRET efficiencies are displayed in parentheses.

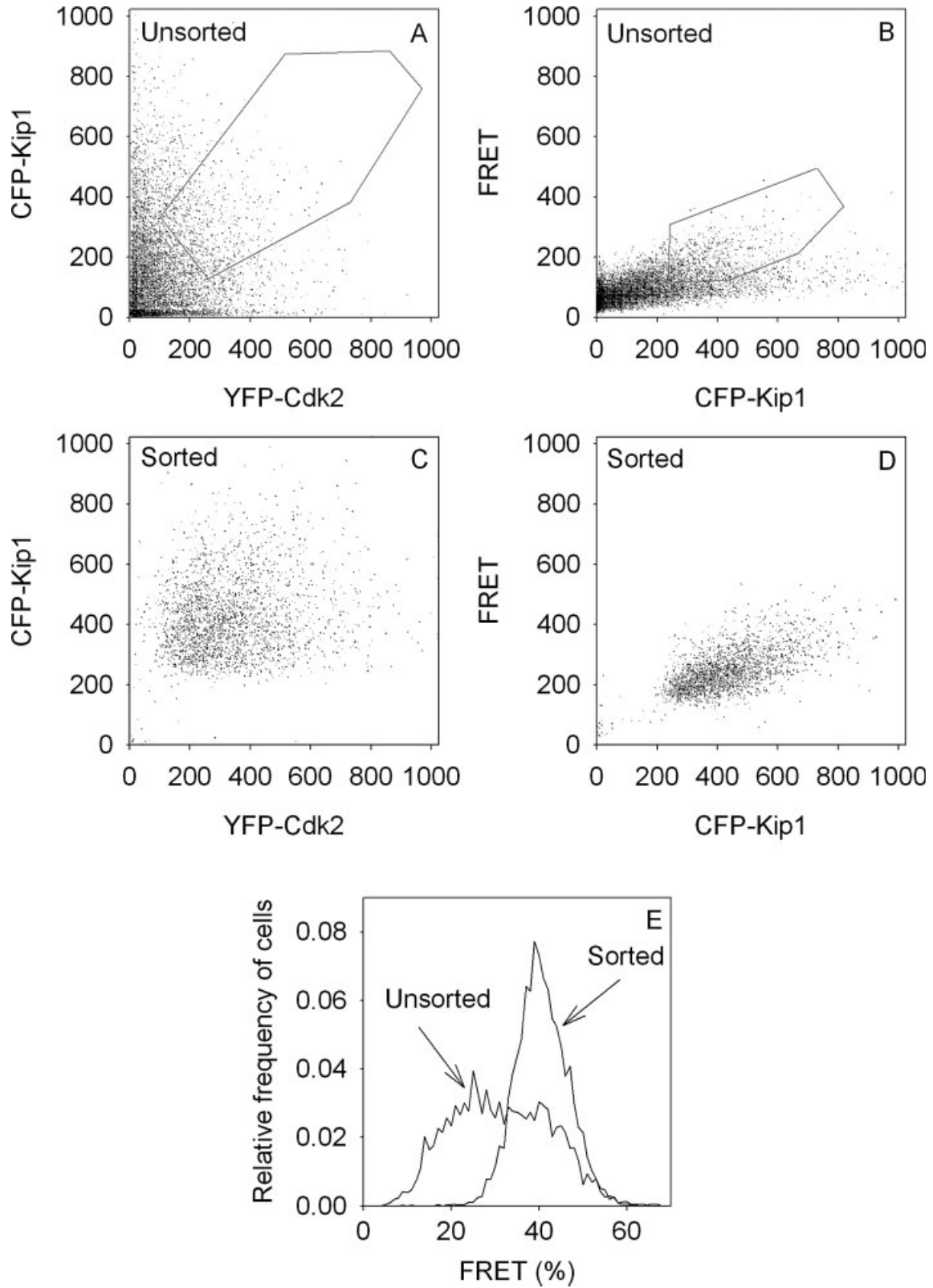


FIG. 7. Sorting of yeast cells based on the interaction between CFP-Kip1 and YFP-Cdk2. Yeast cells coexpressing CFP-Kip1 and YFP-Cdk2 were analyzed by flow cytometry, and fluorescence intensities in the donor (CFP-Kip1), acceptor (YFP-Cdk2), and FRET channels were measured. Numbers on the FRET axes in B and D are fluorescence intensity values and not calibrated FRET efficiencies as in E. **A, B:** Dot plots of unsorted cells. Cells expressing large amounts of CFP-Kip1 and YFP-Cdk2 at ~1:1 ratio displaying high fluorescence intensity in the FRET channel were sorted. The sorting gates are indicated in A and B. **C, D:** Two-parameter dot plots of sorted cells. **E:** FRET histograms were made by cell-by-cell FRET calculations carried out on unsorted and sorted cells.

swing toward each other, thus decreasing the separation distance between the fluorophores below the length of the linker. The presence of FRET in the CFP-30Pro-YFP sample was the major motive to elaborate the method for the determination of α described in this report, which does not rely on the lack of FRET in the calibration samples or knowledge of the absorption ratio.

Our method requires at least two samples with different FRET efficiencies expressing donor-acceptor chimeras strictly generating a donor to acceptor ratio of 1. The reliability of the method is significantly enhanced if there are at least three calibration samples. FRET is calculated using two different approaches (equations 10 and 14) that yield two FRET-related ratios (R_I and R_F). The relation between R_I and R_F is determined by the absorption ratio and α according to equations 16 and 17. These equations can be used to derive the absorption ratio and α in a single step by fitting equation 16 or 17 to the R_F - R_I or $1/(R_F - 1) - 1/R_I$ plot, respectively. From a practical point of view, the number of calibration samples is usually small; in our case it was three, so fitting two parameters to three measurement data points is prone to errors. If there were a much larger number of calibration samples, fitting of equation 16 or 17 could be reliably used, but the requirement for a larger set of calibration samples would make the method more costly and laborious. Our method eliminates this drawback by creating a ratio norm whose squared sum can be minimized by changing only α (equation 15) and then finding the absorption ratio by linear regression (equation 11). We verified our flow cytometric method by determining the FRET efficiency of the CFP-YFP construct containing a trypsin-sensitive linker by trypsin digestion, which induces the separation of the donor and the acceptor and a concomitant de-quenching of the donor (26). This measurement yielded a FRET value not significantly different from our flow cytometric determination.

The dependence of the MSE on α was relatively weak if α was large, especially above the minimum (equation 19, Fig. 3). However, the 95% confidence interval of α was not substantially different between small and large values of α due to presence of a tail in the frequency histogram of calculated α values above the optimal value of α if α was small (Fig. 4).

We have successfully applied the described approach for the CFP-YFP pair, but we could not achieve a reliable determination of α with the BFP-GFP donor-acceptor pair. The major cause of the failure must have been the low fluorescence quantum yield and high bleaching rate of BFP (32) and the high level of autofluorescence in this spectral region. Low donor intensity severely increases the error of equation 10 (18). In addition, equation 8 shows that a low donor fluorescence quantum yield increases α , resulting in a weak dependence of the MSE on α .

Finding interaction partners of a protein is a frequent problem in the post-genomic era. Currently, yeast two-hybrid (33,34) and fluorescence complementation (35) techniques are used to find interaction partners for a protein of interest. Both approaches are based on the recon-

stitution of some kind of an activity (e.g., transcription, fluorescence) after two halves of a protein are brought together by the association of two interacting proteins the two fragments are genetically fused to. A lingering question about these methods is whether an interaction is driven by the two halves of the transcription factor (yeast two-hybrid) or VFP (fluorescence complementation) or by the proteins they are fused to. We demonstrated that cells can be sorted based on FRET. Sorting was done based on the intensity in the FRET channel, which is determined not only by FRET. Faster electronics can perform online FRET calculations according to equation 10, making sorting based on accurate FRET values possible. If a bait is fused to CFP and a library of YFP-fused proteins is generated, then FRET can be used as a marker for the interaction and cells can be sorted accordingly (36). Similarly to the split ubiquitin system (33), our approach is exquisitely suited for the study of membrane protein interactions, an area that was not amenable to yeast two-hybrid experiments.

The deficiency of calculating fluorescence intensities in the FRET channel instead of real FRET efficiencies is demonstrated by a recently published report in which the investigators examined the interaction between CFP- and YFP-tagged proteins in live yeast cells based on measuring FRET intensities (9). Due to the sensitivity of FRET intensity for the expression level of VFPs, the distinction between "significant" and "nonsignificant" FRET intensity is rather subjective, probably resulting in an underestimation of the fraction of cells showing FRET, as the investigators acknowledge. These investigators (9) also suggested using FRET measurements to screen VFP-tagged proteins for interactions. We believe that this could be accomplished using our quantitative approach in a much more sensitive way.

We have developed a method for the quantitation of FRET between VFPs in flow cytometry. Our approach eliminates the pitfalls of calculating FRET indices instead of FRET efficiency. We have demonstrated the successful application of the method for the CFP-YFP donor-acceptor pair. Quantitative FRET measurements have the potential to identify and characterize molecular interactions in their native environment more accurately than currently used molecular biological techniques.

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Hyaluronan-induced masking of ErbB2 and CD44-enhanced trastuzumab internalisation in trastuzumab resistant breast cancer

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ABSTRACT

Although trastuzumab, a recombinant humanised anti-ErbB2 antibody, is widely used in the treatment of breast cancer, neither its mechanism of action, nor the factors leading to resistance are fully understood. We have previously shown that antibody-dependent cellular cytotoxicity is pivotal in the *in vivo* effect of trastuzumab against JIMT-1, a cell line showing *in vitro* resistance to the antibody, and suggested that masking of the trastuzumab-binding epitope by MUC-4, a cell surface mucin, took place. Here, we further explored the role of masking of ErbB2 in connection with CD44 expression and synthesis of its ligand, hyaluronan. We show that high expression of CD44 observed in JIMT-1 cells correlates with ErbB2 downregulation *in vivo*, while siRNA-mediated inhibition of CD44 expression leads to decreased rate of trastuzumab internalisation and low cell proliferation *in vitro*. An inhibitor of hyaluronan synthesis, 4-methylumbelliferon (4-MU) significantly reduced the hyaluronan level of JIMT-1 cells both *in vivo* and *in vitro* leading to enhanced binding of trastuzumab to ErbB2 and increased ErbB2 down-regulation. Furthermore, the inhibitory effect of trastuzumab on the growth of JIMT-1 xenografts was significantly increased by 4-MU treatment. Our results point to the importance of the CD44-hyaluronan pathway in the escape of tumour cells from receptor-oriented therapy.

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1. Introduction

Overexpression of ErbB2 has been unquestionably linked to adverse prognosis in breast cancer.¹ ErbB2 heterodimerises with other members of the ErbB family of receptor tyrosine kinases (RTK) leading to enhanced ligand binding affinity, protection from lysosomal degradation and diversified signalling.² ErbB2 is viewed as a non-autonomous, ligandless, positive regulator of ErbB signalling,² but its participation in non-ErbB protein-mediated signalling is attracting more and more interest as well. Among these, β -integrins,³ MUC-4⁴ and CD44⁵ are probably the best known candidates whose

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roles in cancer progression are well documented.^{3,6,7} ErbB2 is embedded into this network of signalling and accessory molecules and by its promiscuous association profile it promotes cancer progression.⁸ CD44 is recognised as the major hyaluronan receptor having several, alternatively spliced isoforms varying in their physiological function.⁹ Binding of hyaluronan activates CD44-mediated signal transduction pathways via interactions between CD44, Grb2, Vav2 and ErbB2.¹⁰ CD44 is involved in the direct regulation of ErbB2¹¹ and multiple other RTKs.¹² It has been suggested that ligation of CD44 by endogenous hyaluronan leads to the activation of the phosphatidylinositol 3-kinase (PI3K)-Akt survival pathway,¹³ and that displacement of endogenous hyaluronan by exogenous hyaluronan oligosaccharides disrupts the activation.¹² CD44-mediated cytoskeletal rearrangements have been observed⁶ implying the involvement of CD44 in cellular adhesion, migration and invasion.¹⁴ CD44 is almost absent in normal human breast epithelial cells, emerges in benign and premalignant lesions, and is upregulated in carcinomas.¹⁵ However, a recent study suggested that CD44 opposes rather than promotes the spreading of breast carcinoma in mice.¹⁶

While the role of CD44 in breast cancer progression *in vivo* is not completely settled, the accumulation of hyaluronan around malignant cells or in adjacent stroma has been unambiguously shown to be an indicator of poor prognosis in breast cancer.¹⁷ In line with the findings on human tumours, hyaluronan synthesis facilitates the invasive growth of grafted tumour cells *in vivo*,¹⁸ and blocking hyaluronan interactions with its receptors, using soluble CD44 or hyaluronan oligomers, inhibits tumour cell growth in experimental animals.¹⁹ Besides creating signals to prevent apoptosis,¹³ hyaluronan is required for activation of the ErbB2-ErbB3 receptor leading to the formation of cardiac valves.²⁰ Thus, there is mounting evidence that ErbB2, CD44 and hyaluronan are connected both in physiological signalling and cancer pathogenesis through mechanisms largely unknown at present.^{5,11}

Being a membrane protein and a key member of survival and proliferation signalling pathways, ErbB2 is the target of receptor-oriented antibody therapy.²¹ Trastuzumab (Herceptin), a humanised monoclonal anti-ErbB2 antibody, induces objective clinical responses in 40% of patients as a single agent given as first-line treatment of ErbB2-overexpressing metastatic breast cancer.²² Although combination of trastuzumab with conventional chemotherapy increases response rates dramatically, the development of resistance seems currently inevitable.²³ Direct action of trastuzumab on ErbB2 (e.g. ErbB2 down-regulation, inactivation of Akt, inhibition of metalloprotease-mediated shedding)²⁴ and antibody-dependent cellular cytotoxicity (ADCC)^{25,26} have been invoked to explain the mechanism of action of trastuzumab. Despite intense investigations, trastuzumab resistance remains enigmatic and unpredictable in the clinical setting. Production of EGF-like growth factors,²⁷ loss of PTEN,²⁸ masking of ErbB2⁴ and impaired ADCC reaction²⁹ have all been suggested as possible mechanisms.

In the current paper we investigated JIMT-1, a cell line showing *in vitro* resistance to trastuzumab,³⁰ and found a high level of CD44 overexpression. We showed previously that the *in vivo* trastuzumab resistance of the cell line is partial, and development of complete trastuzumab resistance takes 5–10

weeks.²⁶ We suggested that masking of ErbB2 may be the culprit.⁴ Given the suggested association of CD44 with ErbB2¹¹ we asked whether CD44 plays any significant role in the survival of JIMT-1 during trastuzumab therapy. Using siRNA-mediated suppression of CD44 expression we showed that CD44 is necessary for trastuzumab-induced internalisation of ErbB2 and for the survival of JIMT-1 cells *in vitro*. 4-methylumbelliferone (4-MU), a hyaluronan synthase inhibitor, has been shown to increase the efficiency of chemotherapy.³¹ We reasoned that hyaluronan may play a role in masking of cell surface ErbB2.³² We show that *in vitro* and *in vivo* treatment with 4-MU decreased the pericellular hyaluronan concentration around JIMT-1 cells accompanied by increased binding of trastuzumab to ErbB2. 4-MU acted synergistically with trastuzumab in inhibiting the progression of JIMT-1 tumours. Elucidation of the role of CD44 overexpression in trastuzumab resistant cell lines may help understand the causes of therapeutic failures in patients with this type of breast cancer.

2. Materials and methods

2.1. Cells

JIMT-1 cells were grown in F-12/DMEM (1:1) supplemented with 10% FCS, 60 units/L insulin and antibiotics.³⁰ The SKBR-3 cell line was obtained from the American Type Culture Collection (Rockville, MD) and grown according to its specifications.

2.2. Antibodies

Trastuzumab (Herceptin) was purchased from Roche Ltd. (Budapest, Hungary). Mab 2C4 was a generous gift from Genentech (South San Francisco, CA). Monoclonal antibodies against ErbB2 (ErbB2-76.5) and CD44 (Hermes-3) were produced from their hybridoma supernatants (ErbB2-76.5 obtained from Y. Yarden, Weizmann Institute of Science, Rehovot, Israel; Hermes-3 produced by the HB-9480 hybridoma obtained from ATCC) and purified using protein A affinity chromatography. Hermes-3 was also kindly donated by Dr. Sirpa Jalkanen (University of Turku, Finland). Cy3- and Cy5-conjugated goat anti human IgG (H + L) Fab was obtained from Jackson ImmunoResearch Europe (Cambridgeshire, UK). Conjugation of primary antibodies with AlexaFluor (Molecular Probes, Eugene, OR), Cy3 and Cy5 (Amersham, Braunschweig, Germany) dyes was carried out according to the manufacturers' specifications.

2.3. Hyaluronan

Highly purified large molecular weight hyaluronan (HA-LMW) with an average molecular mass of 1.2×10^6 Da was donated by Genzyme (Cambridge, MA). Purified hyaluronan decasaccharides (HA10) were kindly provided by Seikagaku Corporation (Tokio, Japan).

2.4. Western blotting

Whole cell lysates were prepared in lysis buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM EGTA, 1% Triton X-100, 1 Complete Mini (Roche, Mannheim,

Germany) protease inhibitor cocktail tablet/10 mL, 1 mM Na_3VO_4 , 1 mM PMSF, 10 mM NaF, 10 mM β -glycerol phosphate and 10 mM $\text{Na}_4\text{P}_2\text{O}_7$. Immunoprecipitation of ErbB2 and ErbB1 was carried out with Ab3-OP15 antibody (Calbiochem-Merck Biosciences, Schwalbach, Germany) and F4 (E3138, Sigma, Schnelldorf, Germany), respectively, for 1 h on ice and Sepharose 4B Fast Flow Protein G beads (Sigma). Immunoprecipitates were resolved on SDS-polyacrylamide gels and blotted to PVDF membranes. The following antibodies were used for primary labelling of the membranes at dilutions suggested by the manufacturers: Ab3-OP15 for ErbB2, F4 for ErbB1, PY99-sc7020 (Santa Cruz Biotechnology, Santa Cruz, CA) for phosphotyrosine and Hermes-3 for CD44. Peroxidase-conjugated goat anti-mouse IgG and an enhanced chemiluminescence kit (Amersham, Freiburg, Germany) were used for detection.

2.5. Flow cytometric measurement of cell numbers and receptor expression levels

Quantitative determination of receptor expression levels was carried out on a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) using Qifikit (DakoCytomation, Glostrup, Denmark) according to the manufacturer's instructions. In proliferation assays, cells were counted with a FACSArray flow cytometer (Becton Dickinson).

2.6. Xenograft tumours

The severe combined immunodeficiency (SCID) C.B-17 scid/scid mouse population originated from the laboratory of Fox Chase Cancer Center, Philadelphia, PA, and was housed in a pathogen-free environment. Only non-leaky mice with murine IgG levels below 100 ng/ml were used in this study. Seven-week old female SCID mice were given a single subcutaneous injection of 5×10^6 JIMT-1 cells suspended in 150 μL Hank's buffer and mixed with an equal volume of Matrigel (Basement Membrane Matrigel, BD Biosciences, Bedford, MA). Tumour volumes were calculated as the product of the length, width and height of the tumour measured once a week with a caliper. Trastuzumab was administered at a dose of 5 $\mu\text{g/g}$ by weekly intraperitoneal (i.p.) injection. Control mice received weekly i.p. injection of 100 μL physiologic saline. Animals were euthanised by CO_2 inhalation. The experiments were done with the approval of the ethical committee of the University of Debrecen.

2.7. 4-methylumbelliferone treatment

4-methylumbelliferone (4-MU) (Sigma, Budapest, Hungary), an inhibitor of hyaluronan synthase, was suspended in 1% arabic gum and administered orally at a dose of 3 mg/g body weight twice daily.³³ For *in vitro* experiments, 4-MU was dissolved in PBS and added to the culture medium at a concentration of 1 mM.

2.8. Immunohistochemistry

Subcutaneous tumours were removed from anaesthetised mice, covered with Shandon Cryomatrix (Thermo Electron Corporation, Waltham, MA) and stored in liquid nitrogen.

20- μm thick fast frozen samples were made by Shandon AS-620E Cryotome (Thermo Electron Corporation) on silanised slides, fixed in 4% formaldehyde for 30 min, and washed twice in PBS (pH 7.4) supplemented with 1% BSA for 20 min at room temperature. The slides were labelled with a saturating concentration (10–20 $\mu\text{g/ml}$) of fluorophore-conjugated antibodies in 100 μL PBS containing 1% BSA (PBS-BSA) overnight on ice. The samples were washed twice with PBS-BSA and covered with 15 μL Mowiol (Merck, Budapest, Hungary). For labelling of hyaluronan, tissue sections were treated with endogenous biotin blocking kit (Molecular Probes) followed by labelling with 5 $\mu\text{g/ml}$ biotinylated HABC (hyaluronan binding complex) at 4 $^\circ\text{C}$ overnight.³⁴ Prior to staining with fluorescein-avidin the samples were washed five times in PBS-BSA.

2.9. Confocal microscopy

A Zeiss LSM 510 confocal laser-scanning microscope (Carl Zeiss AG, Göttingen, Germany) was used to image samples. AlexaFluor488 was excited at 488 nm and detected between 505–530 nm. Cy3 and AlexaFluor546 were excited with the 543 nm line of a green He-Ne laser, and emission was measured between 560–615 nm. Cy5 and AlexaFluor647 were excited with the 633 nm line of a red He-Ne laser, and their emissions were measured over 650 nm. Fluorescence images were taken as 1- μm optical sections using a $63\times$ (NA = 1.4) oil immersion objective.

2.10. Image analysis

Confocal microscopic images were analysed with the DipImage toolbox (Delft University of Technology, Delft, The Netherlands) under Matlab (Mathworks Inc., Natick, MA). The cell membrane was identified by a manually-seeded watershed algorithm³⁵ using a custom-written interactive algorithm implemented in DipImage/Matlab. The fluorescence intensity was evaluated only in pixels corresponding to the cell membrane. For the analysis of the influence of CD44 expression on the relative binding of trastuzumab to ErbB2, tissue sections were triple-labelled with AlexaFluor488-ErbB2-76.5, Cy3-anti-human IgG (to visualise trastuzumab) and AlexaFluor647-Hermes-3 (against CD44). A two-dimensional histogram (dot plot) of trastuzumab versus ErbB2 intensity was prepared using only membrane pixels. The CD44 expression level was separately evaluated in pixels corresponding to high trastuzumab/ErbB2 and low trastuzumab/ErbB2 ratios identified based on the dot plot.

Colocalisation between two different fluorescent labels was calculated according to Pearson's formula:

$$\frac{\sum_k (I_k - I_{\text{mean}})(J_k - J_{\text{mean}})}{\sqrt{\sum_k (I_k - I_{\text{mean}})^2 \sum_k (J_k - J_{\text{mean}})^2}}$$

where I_k and J_k are the intensities of the k th pixel in the first and second image, respectively, I_{mean} and J_{mean} are the average fluorescence intensities of the first and second image, respectively. Low intensity pixels were excluded from the analysis. The value of the cross-correlation coefficient ranges from +1 to –1. Values close to 1, 0 and –1 indicate high and low degree

of colocalisation, and anticorrelation, respectively. Colocalisation was evaluated using a custom-made software written in LabView (National Instruments, Austin, TX).

2.11. Fluorescence resonance energy transfer (FRET)

Flow cytometric FRET measurements were performed on a FACSVantage SE instrument with DiVa option (Becton Dickinson) equipped with three lasers emitting at 488, 532 and 633 nm. A detailed description of the method has been published elsewhere.³⁶ Cellular autofluorescence was measured in the FL1 channel (excited by the 488 nm laser) through a 530/30 nm band pass filter to discard debris and dead cells. Donor fluorescence was excited at 532 nm and recorded in the FL4 channel through a 585/42 nm bandpass filter, while the acceptor was excited at 633 nm and detected in the FL6 channel through a 650 nm long pass filter. The FRET intensity was excited at 532 nm and detected in the FL5 channel through a 650 nm long pass filter. Calculations were carried out on a cell-by-cell basis using the ReFlex software.³⁷ FRET efficiencies were normalised to a 1:1 donor-acceptor ratio³⁸ and are presented as mean values of FRET histograms of 10,000 cells.

2.12. Internalisation of trastuzumab

siRNA-transfected and control cells were incubated with 20 µg/ml AlexaFluor647-labelled trastuzumab at 37 °C. The samples were treated with acid strip buffer (0.5 M NaCl, 0.1 M glycine, pH 2.5) for 3 min on ice followed by washing and resuspension in PBS. Cells were analysed by flow cytometry, and the internalised fraction of trastuzumab was calculated by dividing the mean fluorescence intensity of the acid-stripped sample with that of the non-acid-treated control.

2.13. RNA interference (RNAi)

Small interfering RNAs (siRNA) against human CD44 were designed and synthesised by Dharmacon (Chicago, IL) using the SMARTselection rules. The sequences of the anti-sense strands of the siRNAs are: AUGUCUUCAGGAUUCGUUCUU (CD44 siRNA-4), UAUUCAAAUCGAUCUG CGCUU (CD44 siRNA-5). A siRNA against GFP was used as a negative control.³⁹ siRNA transfection of JIMT-1 was carried out with the Nucleofector device of Amaxa (Cologne, Germany) according to the manufacturer's specifications. The optimal electroporation conditions (solution V, program T-20) were selected using GFP plasmid transfection.

3. Results

3.1. CD44 is overexpressed on trastuzumab resistant JIMT-1 cells and associated with ErbB2

We compared the expression levels of CD44 in trastuzumab resistant and sensitive breast cancer cell lines in order to reveal the possible roles of CD44 in trastuzumab resistance. Flow cytometric data showed a ~35-times higher expression level of CD44 in trastuzumab resistant JIMT-1 cells (2.3 ± 0.3 million/cell) than in SKBR-3 (65000 ± 5000 /cell), their trastuzumab-sensitive counterpart (Fig. 1A). Since CD44 has been

shown to interact with ErbB2 in ovarian cancer,^{5,10} we investigated the potential interaction between them. Analysis of confocal microscopic images yielded cross-correlation coefficients of 0.612 and 0.602 between CD44 and ErbB2 on JIMT-1 and SKBR-3 cells, respectively (Fig. 1B,C). The cross-correlation coefficient between two different antibodies against ErbB2 was used as a positive control. The fact that the cross-correlation coefficient of the positive control was not substantially different from that between CD44 and ErbB2 implies a strong colocalisation between these molecules on the micrometre scale. Normalised flow cytometric fluorescence resonance energy transfer (FRET) values of $16 \pm 3\%$ and $10 \pm 3\%$ for the association of CD44 and ErbB2 in JIMT-1 and SKBR-3, respectively, show that these molecules are associated at the molecular level as well (Fig. 1B), since FRET values above 5% are considered to imply significant association.⁴⁰ The above biophysical data demonstrating association between ErbB2 and CD44 in JIMT-1 cells were reinforced by molecular biological methods. ErbB2 co-immunoprecipitated with CD44 (Fig. 1D, left panel), and its tyrosine phosphorylation was increased by large molecular weight hyaluronan, whereas hyaluronan decasaccharide inhibited ErbB2 tyrosine phosphorylation (Fig. 1D, middle panel). Neither low, nor large molecular weight hyaluronan modified the activation state of ErbB1 (Fig. 1D, right panel).

3.2. CD44 expression correlates with trastuzumab internalisation in JIMT-1 xenografts

In order to study the possible role of CD44 overexpression in trastuzumab resistance, 7-week old female SCID mice were inoculated by JIMT-1 cells with a single subcutaneous injection. Mice received trastuzumab or physiologic saline weekly starting immediately after tumour injection. Mice were treated with trastuzumab for 9 weeks, then by physiologic saline for another 6 weeks before sacrificing. Tumour sections were triple-stained against ErbB2, trastuzumab and CD44. The watershed algorithm was used to segment the images. The seeds for the watershed algorithm were placed inside cells positive for CD44 and ErbB2. Since mouse stromal cells express neither CD44 nor ErbB2, this approach ensured that the analysis was restricted to JIMT-1 cells. We observed a lack of tight correlation between trastuzumab binding and ErbB2 expression in these mice whose trastuzumab therapy had been suspended for 6 weeks (Fig. 2B,D). The trastuzumab/ErbB2 ratio was inversely correlated with CD44 expression, i.e. cells in which the relative binding of trastuzumab to ErbB2 was low displayed high CD44 expression (Fig. 2D,E). The lack of strict correlation between trastuzumab binding and ErbB2 expression could be caused by local heterogeneities in trastuzumab binding or internalisation. We excluded the role of trastuzumab binding by investigating tumour sections from mice sacrificed immediately after 15 weeks of trastuzumab treatment and showing a tight correlation between trastuzumab binding and ErbB2 expression (Fig. 2F). Unfortunately, direct quantitation of trastuzumab internalisation by measuring fluorescence intensity inside cells was not possible due to the low signal-to-noise ratio. Therefore, we used an alternative approach by analysing the correlation between CD44 and ErbB2 expressions before, during and after trastuzumab

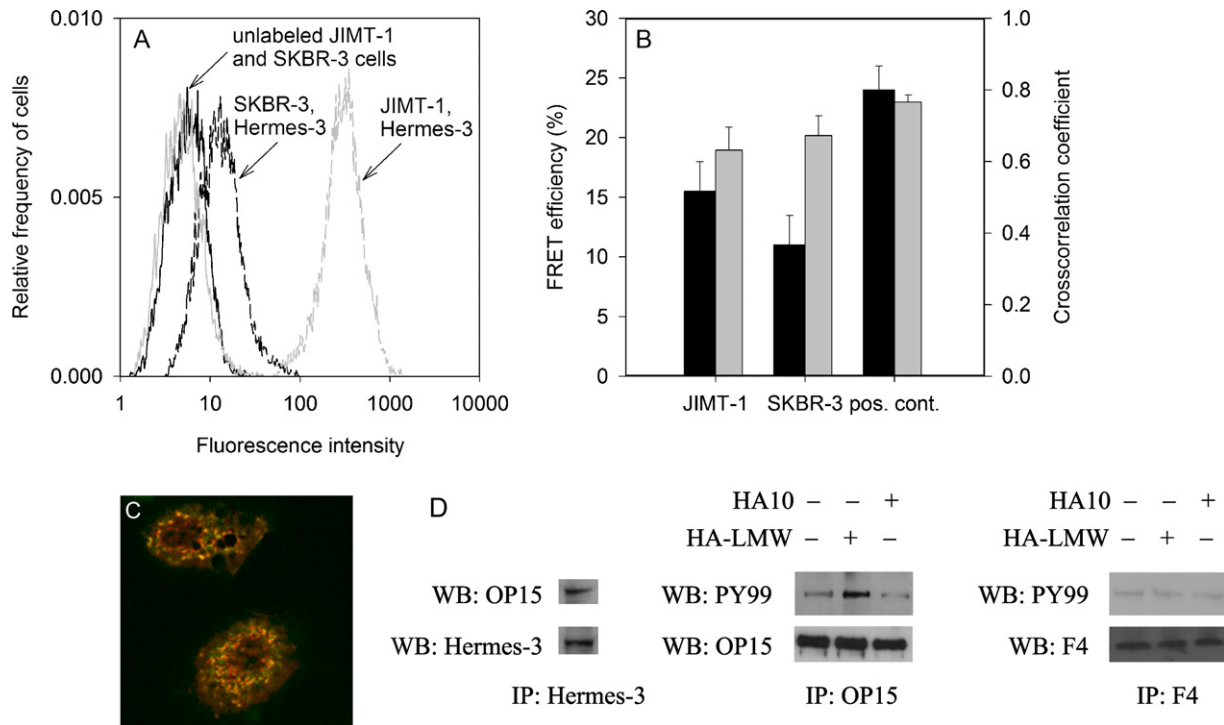


Fig. 1 – CD44 is overexpressed in JIMT-1 cells and interacts with ErbB2. (A) JIMT-1 (grey dashed line) and SKBR-3 (black dashed line) cells were labelled with AlexaFluor488-Hermes-3 antibody against CD44, and the fluorescence intensity was measured by flow cytometry. Unlabelled JIMT-1 (grey continuous line) and SKBR-3 (black continuous line) cells were used as a negative control. (B) JIMT-1 and SKBR-3 cells were labelled with Cy3-2C4 (against ErbB2) and Cy5-Hermes-3 (against CD44), and the FRET efficiency was measured by flow cytometry (black bars) and the cross-correlation coefficient by confocal microscopy (grey bars). Cells labelled with two non-competing antibodies against ErbB2 (Cy3-trastuzumab, Cy5-2C4) were used as a positive control for both FRET and cross-correlation measurements. Error bars indicate the standard error of the mean. (C) JIMT-1 cells were labelled with AlexaFluor488-2C4 and AlexaFluor647-Hermes-3 against ErbB2 and CD44, respectively. The red and green channels correspond to ErbB2 and CD44, respectively. The yellow colour represents overlap between the two fluorescence channels. Quantitative evaluation of colocalisation is shown in part B. (D) Left panel: JIMT-1 cell lysate was immunoprecipitated with Hermes-3 (against CD44), and the membrane was blotted with Hermes-3 and OP15 (against ErbB2). Middle and right panels: JIMT-1 cells were stimulated with 100 µg/ml hyaluronan decasaccharide (HA10) or large molecular weight hyaluronan (HA-LMW) for 30 min at 37 °C. ErbB2 (middle panel) and ErbB1 (right panel) were immunoprecipitated with OP15 and F4, respectively. The membranes were probed with OP15, F4 and PY99 to detect ErbB2, ErbB1 and phosphotyrosine, respectively.

treatment, and found that they show a positive correlation in control mice (Fig. 2G) and in mice in which trastuzumab treatment has been stopped (Fig. 2I). Strikingly, CD44 and ErbB2 expression levels were inversely correlated with each other during trastuzumab treatment (Fig. 2H), i.e. pixels with high CD44 expression displayed low ErbB2 intensity and vice versa. This finding can be rationalised by assuming that ErbB2 is internalised and down-regulated more efficiently in cells with high CD44 expression.

3.3. RNA interference (RNAi)-mediated suppression of CD44 expression inhibits trastuzumab internalisation

The *in vivo* results supporting the role of CD44 in trastuzumab-mediated ErbB2 internalisation were tested by *in vitro* RNAi experiments. Two siRNAs against CD44 elicited efficient and specific inhibition of CD44 expression in JIMT-1 supported by the lack of effect of an irrelevant siRNA on CD44 expression, and the lack of CD44 siRNA-induced sup-

pression of the expression of an irrelevant protein (Fig. 3A). Quantitative flow cytometric analysis confirmed that CD44 siRNAs inhibited trastuzumab internalisation by ~50% (Fig. 3B).

3.4. 4-methylumbelliferone (4-MU) synergises with trastuzumab in inhibiting the growth of JIMT-1 xenografts

We showed that CD44 plays a role in trastuzumab internalisation both *in vivo* and *in vitro*. However, the contribution of trastuzumab-mediated ErbB2 internalisation to the mechanism of action of the antibody has been repeatedly questioned.^{41,42} Therefore, we directly tested whether the CD44-hyaluronan pathway can influence the therapeutic efficacy of trastuzumab. Mice injected with JIMT-1 xenografts were treated with 4-MU (an inhibitor of hyaluronan synthase³³) or trastuzumab, or both starting immediately after tumour inoculation. Each treatment group contained

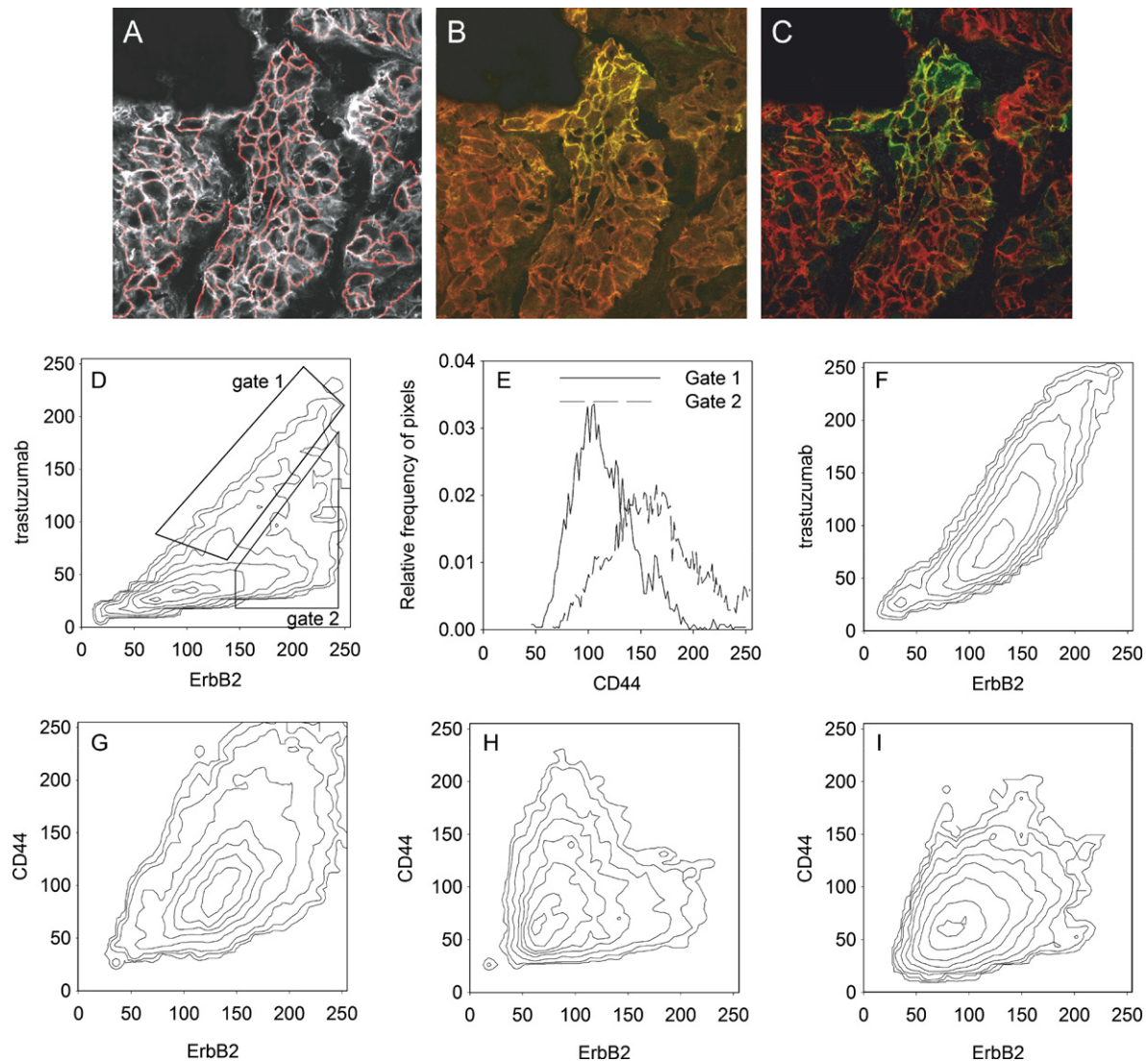


Fig. 2 – CD44 enhances trastuzumab internalization in JIMT-1 xenografts. (A–E) Immunofluorescent staining of JIMT-1 xenografts samples was carried out 6 weeks after finishing trastuzumab treatment. Sections were triple-stained with AlexaFluor488-ErbB2-76.5, Cy3-anti-human IgG (recognising trastuzumab) and AlexaFluor647-Hermes-3 (against CD44). The cell membrane identified by a manually-seeded watershed algorithm is shown in red (A). Dual colour images show the correlation of signals (B: red – ErbB2, green – trastuzumab; C: red – CD44, green – trastuzumab). Gate 1 and 2 identify regions in the trastuzumab-ErbB2 contour plot (D) corresponding to pixels with high and low trastuzumab-ErbB2 ratios, respectively. The CD44 intensity distributions corresponding to gate 1 and 2 are shown in part E. (F) Mice xenografted with JIMT-1 tumours were treated by trastuzumab for 15 weeks and tumour samples were stained with AlexaFluor488-ErbB2-76.5 and Cy3-anti-human IgG recognising trastuzumab. The contour plot shows a much stronger correlation between the ErbB2 and trastuzumab signals compared to that observed in part D. (G–I) Mice injected with JIMT-1 cells were treated with saline (G) or trastuzumab (H) for 15 weeks. In another group, mice were sacrificed 6 weeks after a 9-week long trastuzumab treatment was stopped (I), and the correlation between CD44 and ErbB2 expression levels was analysed in the three samples.

eight animals. The fact that trastuzumab treatment was started the same day as tumour injection made JIMT-1 cells (showing *in vitro* trastuzumab resistance) responsive to the treatment.²⁶ 4-MU combined with trastuzumab was significantly more effective in inhibiting the growth of JIMT-1 xenografts than trastuzumab alone (Fig. 4A). 4-MU did not have any effect on tumour growth on its own (Fig. 4A), although it reduced the amount of hyaluronan in JIMT-1 tumour tissue by ~40% (Fig. 4B). In addition, CD44 expression

was increased by 4-MU probably due to its decreased hyaluronan-mediated down-regulation.⁴³ The increased CD44 level of trastuzumab-treated cells is probably the consequence of reduced CD44 shedding in the presence of trastuzumab (our unpublished observation). 4-MU downregulated ErbB2 to the same extent as trastuzumab. We attribute this finding to the fact that 4-MU increased the CD44 level of cells which was shown to correlate with increased ErbB2 internalisation (Fig. 2). Not only did 4-MU synergise

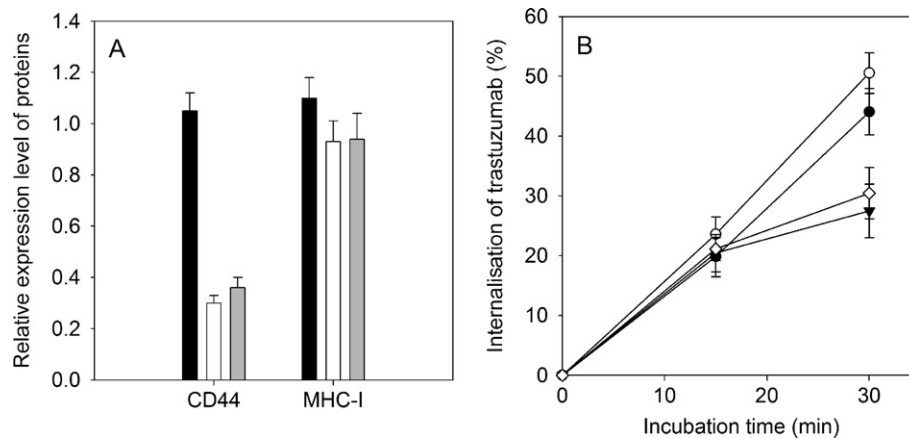


Fig. 3 – siRNA-mediated suppression of CD44 expression inhibits trastuzumab internalisation. (A) JIMT-1 cells were transfected with an irrelevant siRNA against GFP (black), CD44 siRNA-4 (white) and CD44 siRNA-5 (grey). The bars show the expression levels of CD44 and an irrelevant protein, MHC-I, calculated from flow cytometric histograms analysed 48 h after transfection. (B) Internalisation of trastuzumab was measured in mock-transfected cells (●), cells transfected with GFP siRNA (○), CD44 siRNA-4 (▼) or CD44 siRNA-5 (◇). The fraction of internalised trastuzumab (100% = amount of trastuzumab bound to the cell surface at 0 min) is plotted as a function of the duration of trastuzumab treatment. The 30 min values of both CD44 siRNA-transfected samples were statistically significantly different from the GFP siRNA-transfected one (Student's *t* test, *p* < 0.05). Error bars indicate the standard error of the mean.

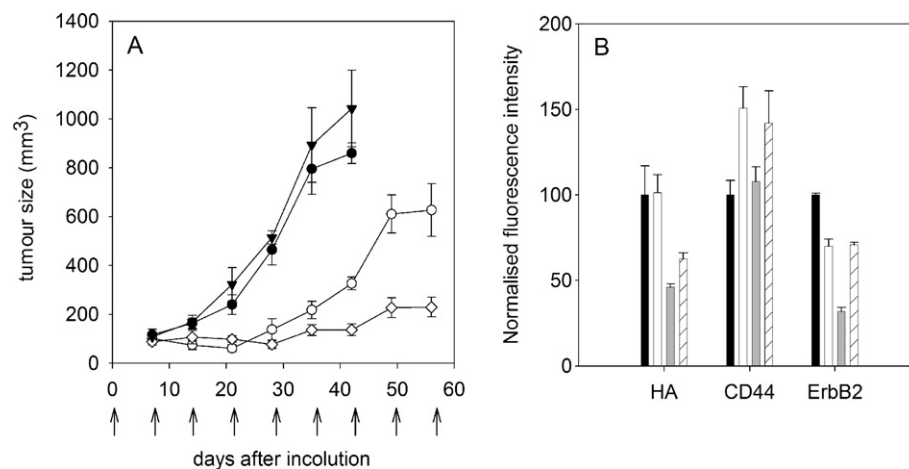


Fig. 4 – 4-methylumbelliferone acts synergistically with trastuzumab in inhibiting tumour growth and down-regulating ErbB2 expression. (A) Mice were xenografted with JIMT-1 cells, and treated with saline (●), trastuzumab (○), 4-methylumbelliferone (▼) or trastuzumab + 4-methylumbelliferone (◇). 4-methylumbelliferone treatment was carried out daily. At the times of weekly saline and trastuzumab treatments, indicated by the arrows, the size of tumours was measured, and it is displayed as a function of time. (B) Tissue sections were triple-labelled with biotin-HABC and fluorescein-avidin to visualise hyaluronan, and antibodies against CD44 and ErbB2. The cell membrane of JIMT-1 cells was identified by the manually-seeded watershed algorithm. The mean (±standard error of the mean) expression levels of hyaluronan, CD44 and ErbB2 measured around the cell membrane in trastuzumab (white), 4-methylumbelliferone (left-hatched) and trastuzumab + 4-methylumbelliferone (grey) treated animals were normalised to the saline-treated xenograft samples (black). The means were calculated from six images taken of tissue sections of three animals.

with trastuzumab in slowing tumour growth, but it also enhanced trastuzumab-mediated down-regulation of ErbB2 (Fig. 4B). Since we assumed that 4-MU may unmask the trastuzumab-binding epitope on ErbB2, it was necessary to show that hyaluronan colocalised with the plasma membrane. By staining tumour sections for hyaluronan and CD44 we demonstrated that hyaluronan is present near

the cell membrane, although it is also found in high density in areas which seem to be interstitial tissue (Fig. 5A–C). Next, we determined the trastuzumab/ErbB2 ratio in control and trastuzumab+4-MU-treated tumour samples and observed an obvious increase in the relative binding of trastuzumab to ErbB2 upon 4-MU treatment (Fig. 5D–E). Quantitative analysis of the images showed a shift on the

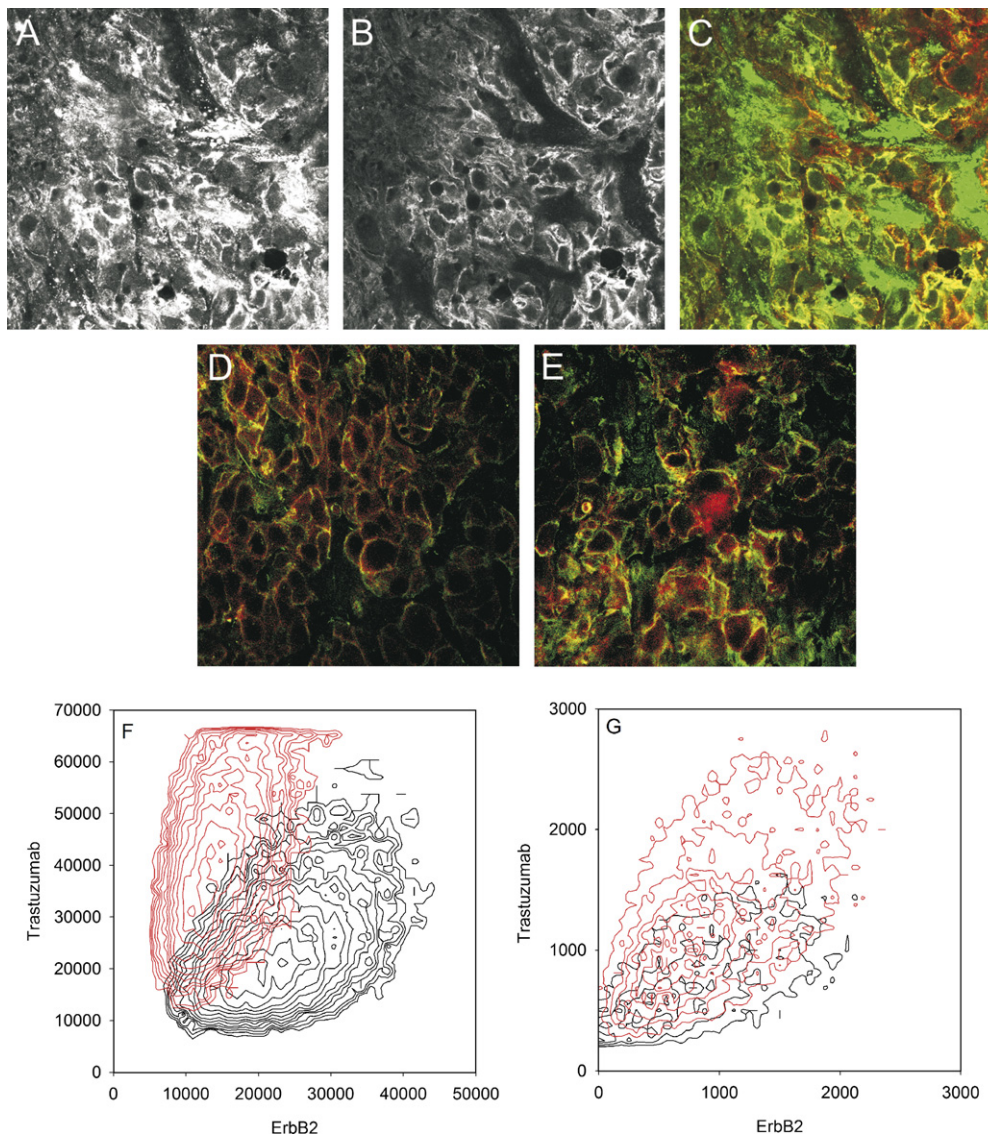


Fig. 5 – Inhibition of hyaluronan synthase increases binding of trastuzumab to ErbB2. (A–C) JIMT-1 tumour samples of saline-treated animals were stained for hyaluronan with biotin-HABC (A) and for CD44 (B). The colour composite image shows the overlap of the distribution of the hyaluronan (green) and CD44 (red) signals. (D–F) Tumour sections of mice treated with trastuzumab (D) or trastuzumab + 4-methylumbelliferone (E) were stained with anti-human IgG (recognising trastuzumab, green) and ErbB2-76.5 (red) antibodies. The contour plot (F) shows increased binding of trastuzumab to ErbB2 in samples taken from the trastuzumab + 4-methylumbelliferone-treated animals (red contours) compared to the trastuzumab-treated ones (black contours). The axes of the contour plot show fluorescence intensity values of trastuzumab and ErbB2-76.5 antibodies recorded in membrane pixels which were identified by manually-seeded watershed segmentation. (G) Cultured JIMT-1 cells were treated with 4-methylumbelliferone for 2 days. Both control (black contours) and 4-methylumbelliferone-treated (red contours) cells were stained with AlexaFluor488-trastuzumab and AlexaFluor647-2C4. The latter antibody measured the amount of ErbB2.

trastuzumab-ErbB2 contour plot towards higher trastuzumab binding upon 4-MU treatment (Fig. 5F). Next, we tested the effect of *in vitro* 4-MU treatment on trastuzumab binding. JIMT-1 cells were treated with 4-MU for 48 h, and subsequently labelled with AlexaFluor488-trastuzumab and AlexaFluor647-2C4. We found that 4-MU treatment enhanced the amount of bound trastuzumab relative to the intensity of ErbB2 (Fig. 5G). ErbB2 was not downregulated by trastuzumab in Fig. 5G, since cells were labelled with

trastuzumab for 30 min on ice, as opposed to Fig. 5F, where mice were treated with the antibody for several weeks. The trastuzumab epitope is masking sensitive⁴ due to its membrane proximal position.⁴⁴ Since the ErbB2-76.5 and 2C4 antibodies recognise an epitope distinct from and less masking sensitive than that of trastuzumab (our unpublished observation), we assumed that their intensities represented the total amount of ErbB2. The 4-MU-induced changes in the trastuzumab/ErbB2 ratio present convincing

evidence for the *in vitro* and *in vivo* masking effect of hyaluronan on the trastuzumab binding epitope.

3.5. CD44 is essential for the proliferation of JIMT-1 cells *in vitro*

Since CD44 is overexpressed by JIMT-1 cells and it is involved in regulating trastuzumab-mediated ErbB2 internalisation, we investigated whether the inhibition of CD44 expression alters the effect of trastuzumab *in vitro*. As previously shown JIMT-1 cells were resistant to trastuzumab *in vitro*,³⁰ but their proliferation was inhibited by siRNA-mediated suppression of CD44 expression (Fig. 6). However, no additive effect between trastuzumab and CD44 siRNA was detected. SKBR-3 cells responded to trastuzumab treatment, but in agreement with their low CD44 expression failed to show any response to siRNA-mediated suppression of CD44 expression (Fig. 6).

4. Discussion

CD44 is widely recognised as a key factor in lymphocyte homing and cancer progression.¹⁴ It has been shown to enhance the signalling potency of several growth factor receptors in general¹² and to interact with ErbB2 in particular,^{5,10,11} but the involvement of the hyaluronan-CD44 pathway in trastuzumab resistance has not been investigated. Since the mechanisms behind trastuzumab resistance are largely unknown, therapeutic failure during prolonged treatment is practically inevitable. In JIMT-1, a cell line showing *in vitro* and partial *in vivo* resistance to trastuzumab,^{4,30} we found an overexpression of CD44. High expression of CD44 is a property of breast cancer stem cells⁴⁵ raising the possibility that the JIMT-1 cell line has been initiated from a stem/progenitor cell. Immunoprecipitation, flow cytometric FRET and confocal

microscopic experiments showed that CD44 associates with ErbB2. The interaction between CD44 and ErbB2 hinted that the hyaluronan-CD44 pathway may be involved in trastuzumab internalisation and resistance.

siRNA-mediated suppression of CD44 expression inhibited trastuzumab internalisation *in vitro* (Fig. 3). Since CD44 is known to be linked to actin⁶ and endocytosis and post-endocytic sorting are coupled to the cytoskeleton in several ways,⁴⁶ it is possible that inhibition of the expression of CD44 interferes with the endocytic process. Alternatively, since CD44 plays a permissive role in the activation of ErbB2,¹¹ elimination of this constitutive regulator of ErbB2 may inhibit trastuzumab internalisation. The role of CD44 in trastuzumab internalisation and down-regulation was confirmed by studying the correlation between trastuzumab binding, ErbB2 and CD44 expressions in JIMT-1 xenografts. In control mice the correlation between CD44 and ErbB2 expressions was positive (Fig. 2G), which was converted to an inverse correlation by trastuzumab treatment (Fig. 2H). ErbB2 is down-regulated by trastuzumab (Fig. 4B), and we assume that this process is more efficient in cells with high CD44 expression, therefore CD44^{high} cells will end up being low expressers of ErbB2 upon trastuzumab treatment. The fact that CD44 expression correlates with trastuzumab-induced ErbB2 internalisation *in vivo* agrees with our *in vitro* data showing decreased internalisation of trastuzumab after knocking down CD44 expression (Fig. 3). We showed previously that after stopping trastuzumab injections ErbB2 expression returns to a level observed in untreated animals.²⁶ While trastuzumab is cleared from the circulation, resynthesis of ErbB2 takes place primarily in cells in which ErbB2 expression was down-regulated, i.e. the CD44^{high} subpopulation. Trastuzumab will probably not bind to ErbB2 synthesised 6 weeks after stopping trastuzumab treatment, since the antibody is eliminated from the circulation with a half-life of ~25 days,⁴⁷ and its concentration is probably lower in the tumour stroma than in the circulation. Even if trastuzumab had bound to newly synthesised ErbB2 6 weeks after stopping trastuzumab treatment, we would not have observed substantial cell-to-cell variation in the binding of trastuzumab to ErbB2 (Fig. 2D). Therefore, CD44^{high} cells in which ErbB2 has been down-regulated will display newly synthesised ErbB2 without trastuzumab on their surface, i.e. their trastuzumab/ErbB2 ratio will be lower than that of CD44^{low} cells in which ErbB2 has been down-regulated less efficiently (Fig. 2D,E). The trastuzumab/ErbB2 ratio of CD44^{low} cells was high because they still displayed ErbB2 in their membrane to which trastuzumab had bound during trastuzumab treatment. Although the role of trastuzumab-induced ErbB2 down-regulation in the therapeutic action of the antibody has been questioned,^{41,42} the debate is not yet settled. The finding that CD44 is involved in ErbB2 internalisation and down-regulation shows that it influences the distribution of ErbB2 among different cellular compartments, which may alter the therapeutic effects of trastuzumab. Additionally, if trastuzumab is used to target ErbB2 overexpressing cells with radioactive isotopes or chemotherapeutics, entry of the antibody-coupled drug into the cell will be affected by the level of CD44 expression.

In addition to its involvement in regulating endocytosis of ErbB2, CD44 can modify trastuzumab-induced effects by masking ErbB2 by its bulky ligand, hyaluronan. We have

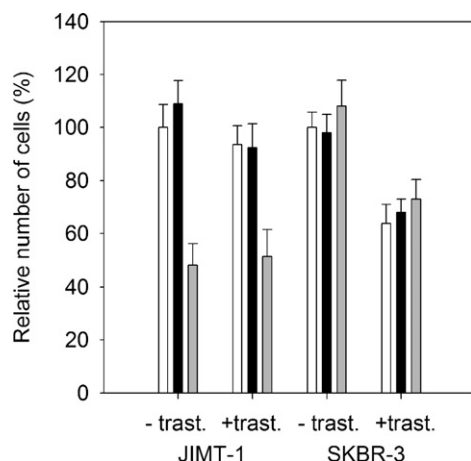


Fig. 6 – RNA interference-mediated suppression of CD44 expression inhibits proliferation of JIMT-1 cells. An equal number of mock-transfected cells (white bars) and cells transfected with GFP siRNA (black bars) or CD44 siRNA-4 (grey bars) were seeded into 6-well plates 24 h after transfection, and cultured in the absence (–trast) or presence (+trast) of 20 µg/ml trastuzumab for 72 h. Cell numbers are normalised to the number found in mock-transfected, trastuzumab-untreated cells 72 h after transfection.

previously described masking of ErbB2 by MUC-4, a cell surface mucin, also expressed by the JIMT-1 cell line.⁴ In this paper we established that hyaluronan also masks the trastuzumab binding epitope by showing that hyaluronan was present in the immediate vicinity of JIMT-1 cells (Fig. 5A–C), and 4-MU, an inhibitor of hyaluronan synthase,³³ substantially reduced its concentration accompanied by increased trastuzumab binding (Fig. 4B, 5F). The enhanced expression level of CD44 in 4-MU-treated xenografts (Fig. 4B) is probably attributable to a lower rate of hyaluronan-induced CD44 endocytosis.⁴³ Since CD44 seems to increase ErbB2 down-regulation (Fig. 3B), the higher CD44 level in 4-MU-treated samples may lead to the lower level of ErbB2 expression observed in these animals (Fig. 4B). Decreased concentration of hyaluronan around JIMT-1 cells resulted in increased binding of trastuzumab to (Fig. 5D–F) and more efficient down-regulation of ErbB2 (Fig. 4B). More importantly, the enhanced binding of trastuzumab to ErbB2 induced by 4-MU increased the efficiency of trastuzumab therapy *in vivo* (Fig. 4A). It has been shown previously that hyaluronan-cell interactions are important for cancer cell survival and metastasis.⁴⁸ The fact that 4-MU alone did not alter the rate of JIMT-1 tumour growth compared to saline-treated animals suggests that either hyaluronan is not necessary for the survival of JIMT-1 cells or the extent of suppression of hyaluronan synthesis was not sufficient for evoking an effect on tumour growth. Nevertheless, the suppression of hyaluronan synthesis induced by 4-MU treatment was large enough to decrease masking of ErbB2 and increase the binding and therapeutic effect of trastuzumab. It was reported that 4-MU inhibited the proliferation of keratinocytes more strongly than expected based on the level of inhibition of hyaluronan synthesis.⁴⁹ This phenomenon was attributed to direct antiproliferative effects of 4-MU unrelated to hyaluronan synthesis. The fact that 4-MU as a single agent was ineffective in our study argues against this possibility in JIMT-1 cells. Although 4-MU specifically inhibits the synthesis of hyaluronan,³³ we cannot rule out the possibility that the production of other glycosaminoglycans was also suppressed. However, the observed increase in trastuzumab binding after 4-MU treatment is a solid evidence for masking whatever molecules are behind it.

The extremely high overexpression of CD44 by JIMT-1 cells (~2.3 million/cell) must be maintained by a selective advantage conferred upon cells by CD44. Although the overexpression of ErbB2 is less high (0.1–0.5 million/cell^{3,4}) it is probably a key signalling molecule in JIMT-1. Therefore, we wanted to investigate whether the combination of therapies against two overexpressed proteins results in synergism, i.e. whether suppression of CD44 expression will turn trastuzumab resistance into responsiveness. siRNA-mediated knockdown of CD44 expression decreased the proliferation of JIMT-1, but cells remained trastuzumab resistant (Fig. 6). The failure to induce trastuzumab sensitivity in JIMT-1 cells by CD44 siRNA transfection may have been caused by a possible antagonism between CD44 siRNA and trastuzumab, since the former inhibits the internalisation of the latter (Fig. 3B). Alternatively, the CD44-hyaluronan pathway involved in *in vivo* trastuzumab resistance may have very limited role in the *in vitro* resistance of JIMT-1.⁴ It is also possible that although CD44 is involved in trastuzumab internalisation, it is unrelated to

trastuzumab resistance due to the dubious role of ErbB2 internalisation and downregulation in trastuzumab resistance.⁴¹

In conclusion, our results point to a role of CD44 in regulating trastuzumab internalisation and masking ErbB2 by serving as an anchor of the hyaluronan meshwork. These findings warrant further investigation of the correlation between trastuzumab resistance, masking of ErbB2, CD44 and hyaluronan expressions, and a deeper look at how CD44-expressing breast cancer stem cells are affected by trastuzumab therapy.

Conflict of interest statement

None declared.

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EGFR and ErbB2 are functionally coupled to CD44 and regulate shedding, internalization and motogenic effect of CD44

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Abstract

Activation of the ErbB family of receptor tyrosine kinases is involved in a range of human cancers. Transmembrane signaling mediated by ErbB proteins is stimulated by peptide growth factors and is blocked by monoclonal antibodies such as trastuzumab and pertuzumab. ErbB receptors exert their function in conjunction with non-ErbB proteins, e.g. CD44. Here we show that epidermal growth factor (EGF) and heregulin induce CD44 shedding in JIMT-1, an ErbB2-overexpressing cell line resistant to trastuzumab, accompanied by internalization and intramembrane proteolysis of CD44 and enhanced cellular motility. These effects of EGF and heregulin are blocked by pertuzumab. Trastuzumab inhibits the heregulin- and hyaluronan oligosaccharide-induced shedding and internalization of CD44 and their motogenic effect. Trastuzumab also blocks CD44 shedding from JIMT-1 xenograft tumors *in vivo*. At the same time the internalization rate of trastuzumab is increased by hyaluronan oligosaccharide treatment *in vitro*. Our experiments point to an unexpected, but potentially important mechanism of action of ErbB receptor-targeted monoclonal antibodies used in the treatment of cancer.

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1. Introduction

The ErbB family of transmembrane receptor tyrosine kinases (RTK) constitutes a diverse signal

transduction network involved in the development of several human cancers [1]. The family consists of ErbB1 (also known as epidermal growth factor receptor, EGFR) and ErbB2–4. Since they bind several peptide growth factors and activate multiple signal transduction pathways (including MAPK and PI3K), the ErbB system functions as a robust regulator and integrator of signaling leading to cell survival and proliferation [2]. ErbB1 is directly

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activated by EGF, ErbB3 and ErbB4 bind heregulins [3], but ErbB2 is an orphan receptor functioning as a co-receptor for other ErbB proteins [4]. Signaling mediated by ErbB receptors is further diversified by their interaction with non-ErbB proteins including G protein-coupled receptors [5], integrins [6], CD44 [7] and MUC-4 [8]. Due to their fundamental importance in cell signaling, ErbB proteins are both predictive markers and targets of cancer therapy [9]. Several ErbB receptor-targeted monoclonal antibodies are in different stages of clinical testing or use. Cetuximab (Erbix) competitively blocks binding of EGF to its receptor [10]. While trastuzumab (Herceptin), a humanized monoclonal antibody against ErbB2, is widely used in the treatment of ErbB2-overexpressing metastatic breast cancer [11], another anti-ErbB2 monoclonal antibody, pertuzumab (Omnitarg) is in clinical trials [12]. While the mechanism of action of pertuzumab has been unequivocally explained by its inhibitory effect on ErbB2 heterodimerization [13], mechanisms including antibody-mediated cellular cytotoxicity (ADCC) [14,15] and direct, trastuzumab-induced effects on ErbB2-mediated signal transduction, have been only putatively linked to the therapeutic action of trastuzumab [16]. Although trastuzumab significantly improves the overall survival rate of metastatic breast cancer patients, the development of trastuzumab resistance is an insurmountable problem in clinical practice. Among others impaired ADCC reaction [17], enhanced ErbB receptor-mediated signaling [18,19] and masking of ErbB2 [8,20] have been implied to cause resistance to trastuzumab, but the actual mechanisms in particular patients are usually unknown to clinicians and are probably patient dependent.

CD44 is an adhesion receptor mediating cell-matrix interactions by binding to hyaluronan [21]. Ligation of CD44 by endogenous large molecular weight hyaluronan leads to the activation of the PI3K pathway [22]. Hyaluronan oligosaccharides are regarded as inhibitors of CD44 by displacing endogenous hyaluronan and inhibiting CD44-mediated signaling [22]. CD44 engages in interactions with ErbB proteins: it forms an active signaling complex with ErbB2 [7] and anchors matrix metalloprotease-7 to the membrane which leads to the activation of ErbB4 by liberating heparin-binding EGF from its precursor [23]. CD44 takes part in cytoskeletal rearrangements by interacting with ezrin [7] and neural Wiskott–Aldrich syndrome protein [24]. Due to its involvement in locomotion, cell

survival and upregulation of its expression in pre-malignant lesions [25] CD44 is assumed to be important in cancer progression [26]. The accumulation of hyaluronan has been shown to be a predictor of poor prognosis in breast cancer [27], and inhibition of CD44-hyaluronan interactions by soluble CD44 inhibits growth of mammary carcinoma cells [28]. Besides providing a substratum for crawling [29] and activating CD44, hyaluronan has non-canonical ways of promoting tumor spreading. It has been suggested to mask ErbB2 and prevent trastuzumab binding [20]. In addition, low molecular weight hyaluronan fragments generated in certain tumors [30] induce neoangiogenesis [31] and activate macrophages [30] known to enhance metastasis formation [32].

The transmembrane form of CD44 is processed by consecutive cleavages by metalloproteases and γ -secretase generating a soluble extracellular domain and an intracellular domain, which is translocated to the nucleus and activates transcription [33]. CD44 shedding is stimulated by hyaluronan oligosaccharides [34] and EGF [35]. The soluble extracellular domain of CD44 has important roles in its own right by disrupting endogenous CD44-hyaluronan interactions [36]. The serum level of soluble CD44 has been shown to correlate with tumor burden [37] and indicate poor prognosis [38] and resistance to chemotherapy [39]. In addition to their involvement in CD44 processing both metalloproteases [40] and γ -secretase [41] participate in the cleavage of ErbB4. More importantly, the metalloprotease-mediated shedding of ErbB2 extracellular domain is inhibited by trastuzumab [42]. These findings imply an intimate relationship between CD44, ErbB proteins and membrane-associated proteolytic enzymes.

In the current paper, we investigated the influence of ErbB receptor-mediated signaling on CD44 shedding in JIMT-1, an ErbB2 and CD44 overexpressing cell line showing *in vitro* [43] and partial *in vivo* [14] resistance to trastuzumab. Our results suggest a mutual interaction between ErbB proteins and CD44, and a potential for anti-ErbB2 antibodies to inhibit CD44 shedding.

2. Materials and methods

2.1. Cells, antibodies and hyaluronan

JIMT-1 cells, available from the German Collection of Microorganisms and Cell Cultures (www.dsmz.de), were grown in F-12/DMEM (1:1) supplemented with 20%

FCS, 60 units/L insulin and antibiotics [43]. For microscopic experiments, cells were cultured on Lab-Tek II chambered coverglass (Nalge Nunc International, Rochester, NY). Trastuzumab was purchased from Roche Ltd. (Budapest, Hungary), pertuzumab and cetuximab were from Genentech (South San Francisco, CA) and Merck KGaA (Darmstadt, Germany), respectively. Polyclonal antibody against the cytoplasmic domain of CD44 (anti-CD44cyto) was produced by immunizing rabbits with a peptide corresponding to the intracellular domain of CD44 [44]. The monoclonal antibody, Hermes-3, against the extracellular domain of CD44 was produced from its hybridoma, HB-9480, obtained from the American Type Culture Collection (Rockville, MD) and purified using protein A affinity chromatography. Conjugation of primary antibodies with AlexaFluor (Molecular Probes, Eugene, OR) was carried out according to the manufacturer's specifications. Heregulin- β 1 and EGF were purchased from R&D systems (Minneapolis, MN). Purified hyaluronan decasaccharide was kindly provided by Seikagaku Corporation (Tokyo, Japan).

2.2. Xenograft tumors

The severe combined immunodeficiency (SCID) C.B-17 scid/scid mouse population originated from the laboratory of Fox Chase Cancer Center, Philadelphia, PA, and were housed in a pathogen-free environment. Only non-leaky mice with IgG levels below 100 ng/ml were used in this study. Seven-week old female SCID mice were given a single subcutaneous injection of 4×10^6 JIMT-1 cells suspended in 100 μ l Hank's buffer. Tumor volumes were calculated as the product of the length, width and height of the tumor measured once a week with a caliper. Trastuzumab was administered at a dose of 5 μ g/g by weekly intraperitoneal (i.p.) injection. Control mice received weekly i.p. injection of 100 μ l physiological saline. Blood samples were taken from trastuzumab-treated and control mice having tumors with similar size by retroorbital puncture. Animals were euthanized by CO₂ inhalation. The experiments were done with the approval of the ethical committee of the University of Debrecen.

2.3. Detection of CD44 ectodomain shedding by ELISA

JIMT-1 cells were grown to confluency in 24-well plates. The culture medium was removed, and the cells were washed twice with PBS followed by adding 200 μ l of Hank's buffer supplemented with 1 mg/ml BSA. After inducing CD44 ectodomain shedding by various treatments the supernatant was collected and centrifuged at 400g for 10 min to get rid of cells detached from the plate. The concentration of shed CD44 ectodomain was measured from undiluted supernatants with a commercially available Instant-ELISA kit (Bender MedSystems, Vienna, Austria) according to the manufacturer's speci-

cations. The limit of detection of soluble CD44 was reported to be 0.016 ng/ml by the company. Alternatively, undiluted serum samples collected from mice with JIMT-1 xenograft tumors were analyzed using the same kit. Statistical significance was tested by Student's *t* test using SigmaStat (Systat Inc., San Jose, CA).

2.4. Measurement of trastuzumab internalization

Cells were labeled with 20 μ g/ml AlexaFluor647-trastuzumab for 30 min on ice followed by incubation at 37 °C. The samples were treated with acid strip buffer (0.5 M NaCl, 0.1 M glycine, pH 2.5) for 3 min on ice followed by washing and resuspension in PBS. Cells were analyzed by flow cytometry (FacsCalibur, Becton Dickinson, Franklin Lakes, NJ), and the internalized fraction of trastuzumab was calculated by dividing the mean fluorescence intensity of the acid-stripped sample with that of the non-acid-treated control labeled with AlexaFluor647-trastuzumab only on ice. The statistical significance of the difference between control and trastuzumab-treated samples was tested by Student's *t* test using SigmaStat.

2.5. Immunofluorescence labeling and confocal microscopy

Cells were fixed in 4% formaldehyde for 10 min at room temperature followed by permeabilization by 0.1% Triton X-100 + 0.3% BSA for 5 min at room temperature. Fixed and permeabilized cells were labeled with anti-CD44cyto rabbit polyclonal antibody (dilution 1:100) and 10 μ g/ml AlexaFluor647-Hermes3 mouse monoclonal antibody against the extracellular domain of CD44 for 90 min at room temperature followed by washing and secondary labeling with AlexaFluor488-tagged anti-rabbit antibody against anti-CD44cyto.

A Zeiss LSM 510 confocal laser-scanning microscope (Carl Zeiss AG, Göttingen, Germany) was used to image samples. AlexaFluor488 was excited with the 488 nm line of an argon ion laser and its fluorescence was detected between 505 and 530 nm. AlexaFluor647 was excited with the 633 nm line of a red He-Ne laser, and its emission was measured over 650 nm. Fluorescence images were taken as 1- μ m optical sections using a 63 $\times$ (NA = 1.4) oil immersion objective.

2.6. Image analysis

Image processing was carried out with the DipImage toolbox (Delft University of Technology, Delft, The Netherlands) under Matlab (Mathworks Inc., Natick, MA). Segmentation of images into membrane and non-membrane pixels was carried out with the manually-seeded watershed algorithm using a custom-written Matlab program [20,45]. Circumferential pixels located 5–10 pixels from the membrane according to Euclidian distance transform were added to the membrane mask. The area inside

the membrane mask was considered to be the intracellular space (Fig. 2C). This algorithm ensured that membrane fluorescence erroneously appearing in the intracellular space due to membrane folding is not regarded as fluorescence of internalized membrane proteins. The background-corrected fluorescence intensity was summed in the membrane and intracellular masks, and intracellular fluorescence divided by membrane fluorescence was used as a measure of membrane protein internalization. Hermes-3 and anti-CD44cyto antibodies recognize the extra- and intracellular domains of CD44, respectively. Full length CD44 binds both of them, whereas the intracellular domain of CD44 generated during intramembrane proteolysis is recognized only by anti-CD44cyto. Therefore, the anti-CD44cyto/Hermes-3 fluorescence intensity ratio is proportional to intramembrane proteolysis or shedding of CD44.

2.7. *In vitro* scratch assay

JIMT-1 cells were cultured on chambered coverslips, and a cell-free stripe was generated by scratching a confluent cell layer with a micropipette tip [46]. A ~1 mm-wide cell-free scratch was reproducibly created. Cells were treated with different agents for 24 h, and they were imaged at the beginning and at the end of the experiment with a low magnification objective using the transmission channel of Zeiss LSM 510 microscope. In order to take images of

identical fields at the beginning and at the end of the experiment the x - y coordinates displayed by the microscope were used. The pixel width is saved in the image header by the microscope. The scratch width in these calibrated images was analyzed by ImageJ.

3. Results

3.1. Hyaluronan oligosaccharide, EGF and heregulin induce shedding and internalization of CD44

Hyaluronan fragments [34] and EGF [35] have been shown to enhance CD44 cleavage. We wanted to test whether these agents and heregulin are effective in the trastuzumab resistant JIMT-1 cell line. We found that CD44 shedding was significantly stimulated by hyaluronan oligosaccharide, EGF and heregulin (Fig. 1, $p < 0.05$). We also followed the fate of the intracellular domain of CD44 in confocal microscopic experiments. JIMT-1 cells were dual-stained with antibodies against the intracellular (anti-CD44cyto) and extracellular (Hermes-3) domains of CD44. Hyaluronan oligosaccharide, EGF and heregulin induced a striking increase in CD44 endocytosis as evidenced by the appearance of vesicles positive for CD44 (Fig. 2). We quantitated CD44 internalization by separately measuring the fluorescence intensities in the intracellular space and in the cell membrane. This analysis confirmed that all three of the agents significantly increased CD44 endocytosis (Fig. 3, $p < 0.05$). Intra-

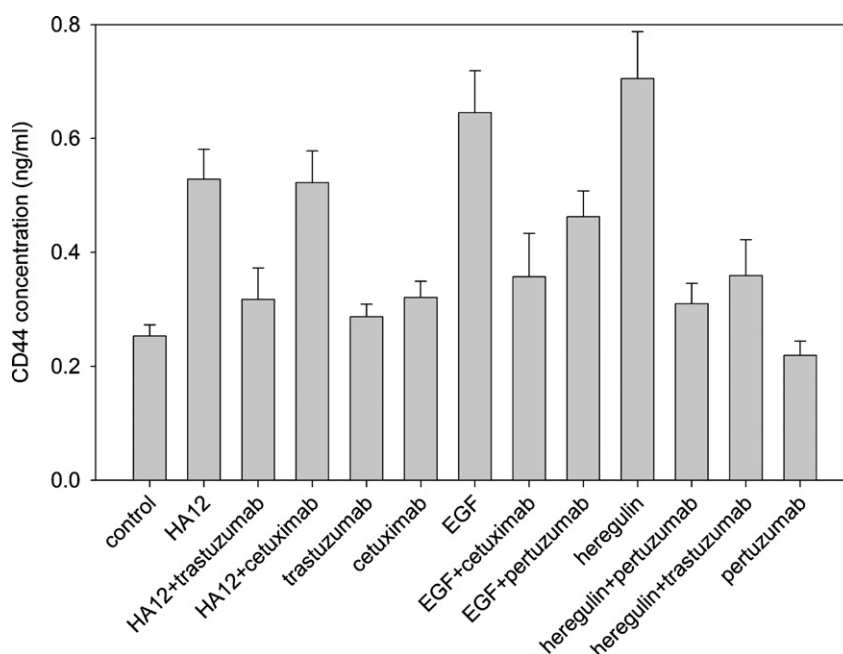


Fig. 1. The effect of trastuzumab, pertuzumab and cetuximab on hyaluronan-, EGF- and heregulin-induced CD44 shedding. JIMT-1 cells were pretreated with the monoclonal antibodies for 30 min on ice followed by incubation with 100 μ g/ml hyaluronan oligosaccharide, 10 nM EGF or 10 nM heregulin for 60 min at 37 $^{\circ}$ C. The supernatant was collected and cleared of cell debris and detached cells, and the concentration of CD44 ectodomain was determined by ELISA. The means ($\pm$ SD) of three measurements are shown.

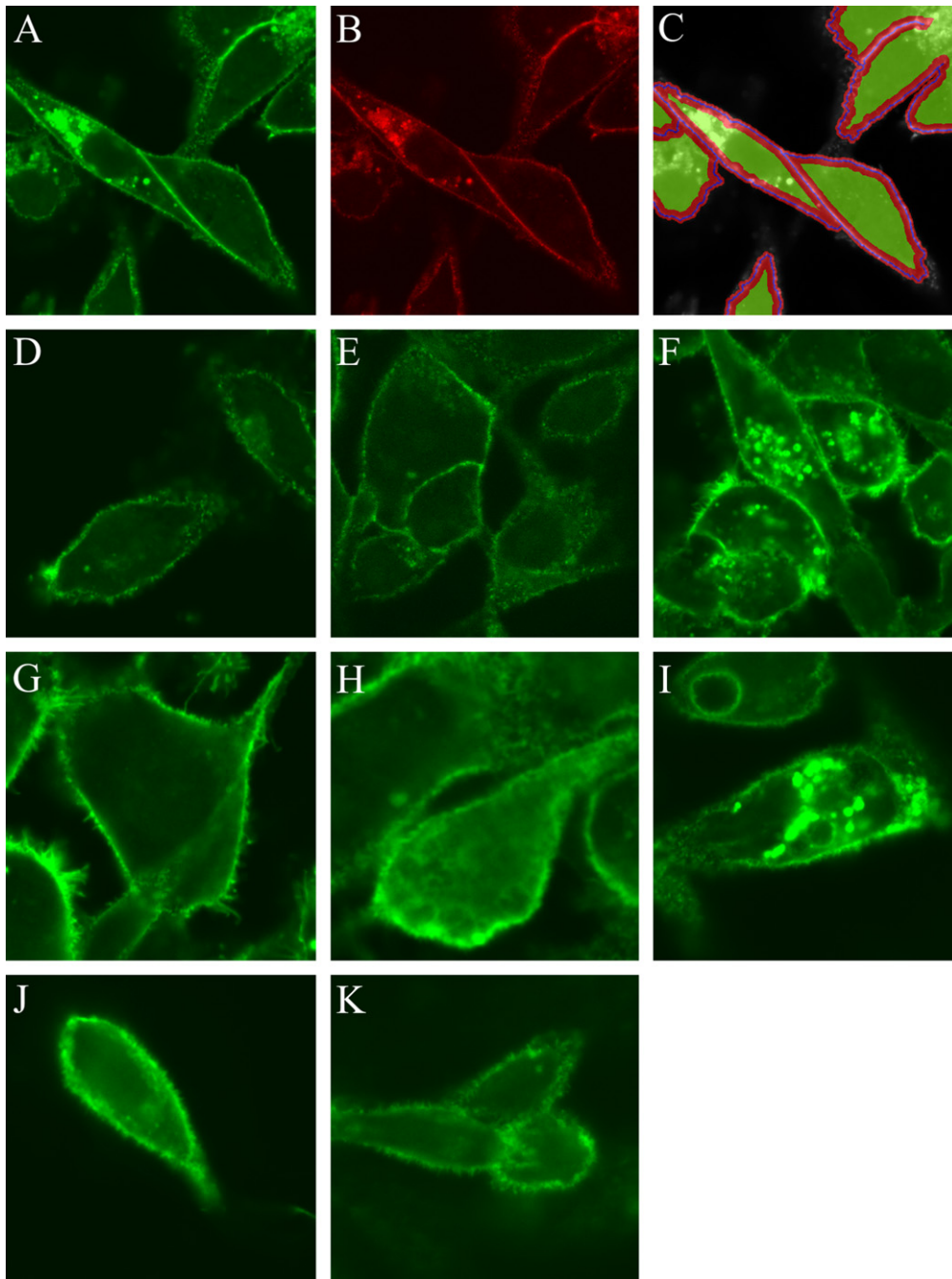


Fig. 2. Confocal microscopy confirms the effect of EGF-like growth factors, anti-ErbB1 and anti-ErbB2 antibodies on CD44 shedding. JIMT-1 cells were treated by peptide growth factors, hyaluronan and monoclonal antibodies in chambered coverslips overnight followed by fixation, permeabilization and staining with AlexaFluor647-Hermes3 (red image). The intracellular domain of CD44 was visualized by staining with anti-CD44cyto rabbit polyclonal antibody labeled by secondary AlexaFluor488-anti-rabbit IgG (green images). (A and B) hyaluronan oligosaccharide; (D) untreated cells; (E) trastuzumab + hyaluronan oligosaccharide; (F) EGF; (G) cetuximab + EGF; (H) pertuzumab + EGF; (I) heregulin; (J) trastuzumab + heregulin; (K) pertuzumab + heregulin. (C) Image segmentation. The cell membrane was identified by watershed segmentation (blue line). The width of the cell membrane was increased by adding circumferential pixels around the blue line to the membrane mask (red-shaded region). The area inside the membrane was considered to represent the intracellular space (green-shaded area).

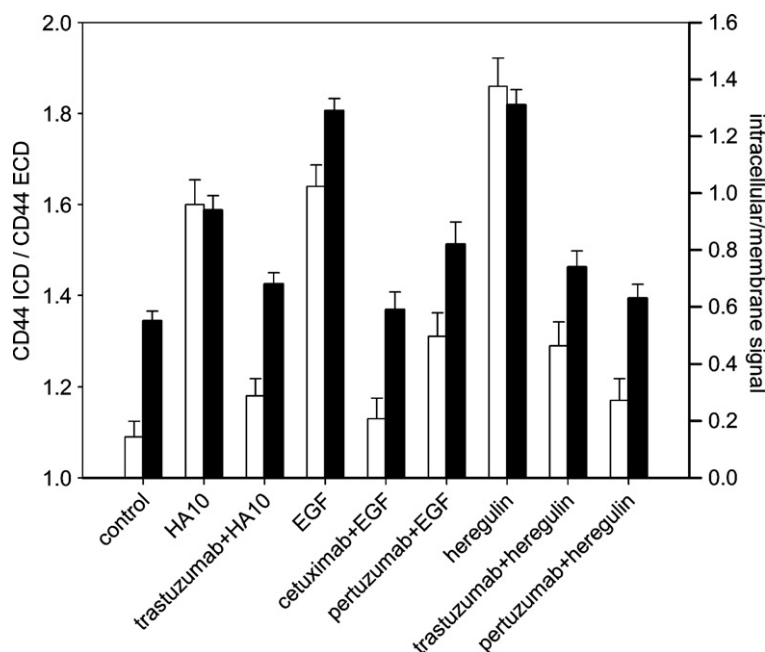


Fig. 3. Quantitative analysis of confocal microscopic images shows internalization and intramembrane proteolysis of CD44. The total background-corrected fluorescence intensities of the anti-CD44cyto antibody (CD44ICD) and Hermes-3 (against CD44 extracellular domain, CD44ECD) were divided by each other yielding the CD44ICD/CD44ECD parameter characterizing the generation of truncated CD44 (white bars, left scale). The background-corrected fluorescence intensity in the images corresponding to anti-CD44cyto staining was summed in the cytoplasmic and membrane masks. Their ratio (intracellular/membrane signal, black bars, right axis) is proportional to CD44 internalization.

membrane cleavage of CD44 generates a truncated protein binding only the anti-CD44cyto antibody. The fact that the ratio of anti-CD44cyto fluorescence to Hermes-3 fluorescence significantly increased upon hyaluronan oligosaccharide, EGF and heregulin treatment indicated the accumulation of CD44 intracellular domain in JIMT-1 cells (Fig. 3, $p < 0.05$). The fact that the anti-CD44cyto and Hermes-3 signals show considerable overlap implies that both full length and cleaved CD44 accumulate in the intracellular space.

3.2. The effect of pertuzumab and cetuximab on EGF- and heregulin-induced CD44 cleavage

Cetuximab and pertuzumab block EGF receptor and ErbB2-mediated signaling, respectively, by different mechanisms. We tested whether these receptor-blocking antibodies inhibit EGF- and heregulin-induced CD44 cleavage. ELISA experiments showed that pertuzumab significantly inhibited CD44 shedding (Fig. 1, $p < 0.05$), CD44 internalization and intramembrane cleavage induced by EGF and heregulin (Figs. 2 and 3, $p < 0.05$). Since cetuximab blocks the binding of EGF to its receptor, we only tested its effect on the EGF-mediated effects, which were completely abolished (Figs. 1–3, $p < 0.05$). The antibodies did not have any effect on CD44 cleavage on their own (Fig. 1).

3.3. The effect of trastuzumab on heregulin and hyaluronan oligosaccharide-induced CD44 cleavage in vitro

The mechanism of action of trastuzumab and reasons for trastuzumab resistance are multifactorial, and often determined by ErbB-related signaling pathways [47]. Here we examined whether trastuzumab exerts any effect on CD44 shedding which was shown to be regulated by ErbB family members (Fig. 1–3, and Ref. [35]). Heregulin-induced CD44 shedding, endocytosis and intramembrane cleavage were all inhibited by trastuzumab pretreatment (Figs. 1–3, $p < 0.05$). Somewhat surprisingly, trastuzumab almost completely abolished the hyaluronan oligosaccharide-evoked effect on CD44 shedding, internalization and cleavage (Figs. 1–3, $p < 0.05$) implying that ErbB2 is incorporated into the membrane protein complex in which CD44-mediated signaling and cleavage of CD44 take place.

3.4. The effect of trastuzumab treatment on CD44 shedding in JIMT-1 xenografts in vivo

Since *in vitro* experiments suggested that trastuzumab inhibited both heregulin- and hyaluronan-mediated CD44 cleavage, we examined whether it has any effect *in vivo* on CD44 shedding from JIMT-1 xenograft tumors. Mice were injected with 4×10^6 JIMT-1 subcutaneously. Trastuzumab-treated animals were given 5 $\mu\text{g/g}$ trastuzumab

weekly starting on the day of tumor inoculation, while control mice were treated with physiological saline. Since trastuzumab significantly retarded tumor growth [14,20], blood samples taken from control mice on week 5 were compared to blood samples of trastuzumab-treated animals collected on weeks 9–11, since the mean tumor size in both cases was $\sim 400 \text{ mm}^3$ (small tumors in Fig. 4). Trastuzumab significantly decreased the CD44 concentration in the blood ($p < 0.05$, Fig. 4). Since the applied ELISA assay is specific for human CD44, which could get to the mouse circulation only from JIMT-1 tumor cells, the results strongly suggest that trastuzumab inhibited CD44 shedding from the tumor cells. We also compared the effect of trastuzumab on large tumors with a mean volume of $\sim 850 \text{ mm}^3$. These blood samples were collected on weeks 7–9 and 13–14 from control and trastuzumab-treated mice, respectively. The effect of trastuzumab on CD44 shedding from large JIMT-1 xenografts was negligible.

3.5. The effects of anti-ErbB antibodies on hyaluronan oligosaccharide, EGF and heregulin-induced cell motility

Although CD44 shedding has been convincingly linked to cell motility, we wanted to examine if changes observed in CD44 cleavage correlate with cell locomotion in our experiments. *In vitro* scratch assays showed that migration of JIMT-1 cells was significantly increased by hyaluronan oligosaccharide, EGF and heregulin (Fig. 5). The motogenic effects of hyaluronan and heregulin were inhibited by trastuzumab. Similarly, the effect of EGF was sensitive to cetuximab, and pertuzumab blocked both EGF- and

heregulin-induced effects on cell motility. All effects proved to be statistically significant ($p < 0.05$). In summary, the effect of every treatment on CD44 shedding and internalization was perfectly mirrored by its effect on cell motility.

3.6. The influence of hyaluronan oligosaccharide on the rate of trastuzumab internalization

Since the results presented above strongly imply that ErbB receptors influence CD44 cleavage and internalization, we tested whether a reciprocal effect of CD44 on trastuzumab internalization is detectable. The rate of trastuzumab internalization was significantly higher in JIMT-1 cells pretreated with hyaluronan oligosaccharide than in control cells ($p < 0.05$, Fig. 6). We conclude that CD44 and ErbB receptors mutually affect each other's internalization rate in JIMT-1 cells.

4. Discussion

CD44 cleavage and subsequent shedding of the extracellular domain accompanied by nuclear translocation of the intracellular domain has been shown to regulate cell attachment and migration [29]. The ErbB receptor family also induces cell motility [48]. Since both CD44 and ErbB proteins are key factors in regulating cell locomotion known to be important in cancer metastasis formation, we tested whether these two signaling networks interact with each other at the level of endocytosis and intramembrane cleavage. We observed that hyaluronan oligosaccharide, EGF and heregulin induced CD44 shedding accompanied by increased cellular motility in JIMT-1, a cell line showing partial resistance to trastuzumab. CD44 shedding induced by any of these ligands was also accompanied by endocytosis of full length CD44, since the fluorescence signal of antibodies both against the extra- and intracellular domains of CD44 increased inside cells. It has been reported previously that hyaluronan is taken up by receptor-mediated endocytosis for endosomal/lysosomal degradation [49]. The fact that we observed a significant increase in the fluorescence signal corresponding to CD44 intracellular domain relative to that of the extracellular domain strongly suggested that proteolytically processed CD44 accumulated in the cytosol, since truncated CD44 only binds antibodies raised against the intracellular domain. We concluded that hyaluronan oligosaccharide, EGF and heregulin induce endocytosis of full length CD44 and intramembrane cleavage accompanied by CD44 shedding and

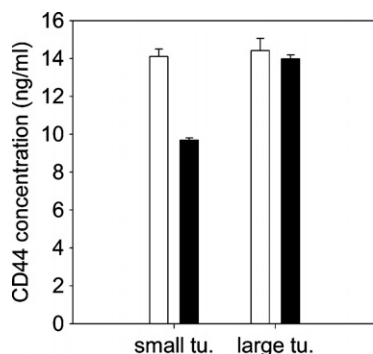


Fig. 4. Trastuzumab inhibits CD44 shedding in small JIMT-1 xenograft tumors. Blood from immunodeficient mice transplanted with JIMT-1 xenograft tumors was collected. Blood samples were assigned to two groups based on the size of the tumor at the time of blood collection. Small tumors had a mean volume of 393 mm^3 and 403 mm^3 in the control and trastuzumab-treated groups, respectively, large tumors were 848 mm^3 and 859 mm^3 in the control and trastuzumab-treated groups, respectively. The concentration of CD44 was determined in control (white bars) and trastuzumab-treated (black bars) mice and the means ($\pm$ SD) of at least four mouse samples are plotted.

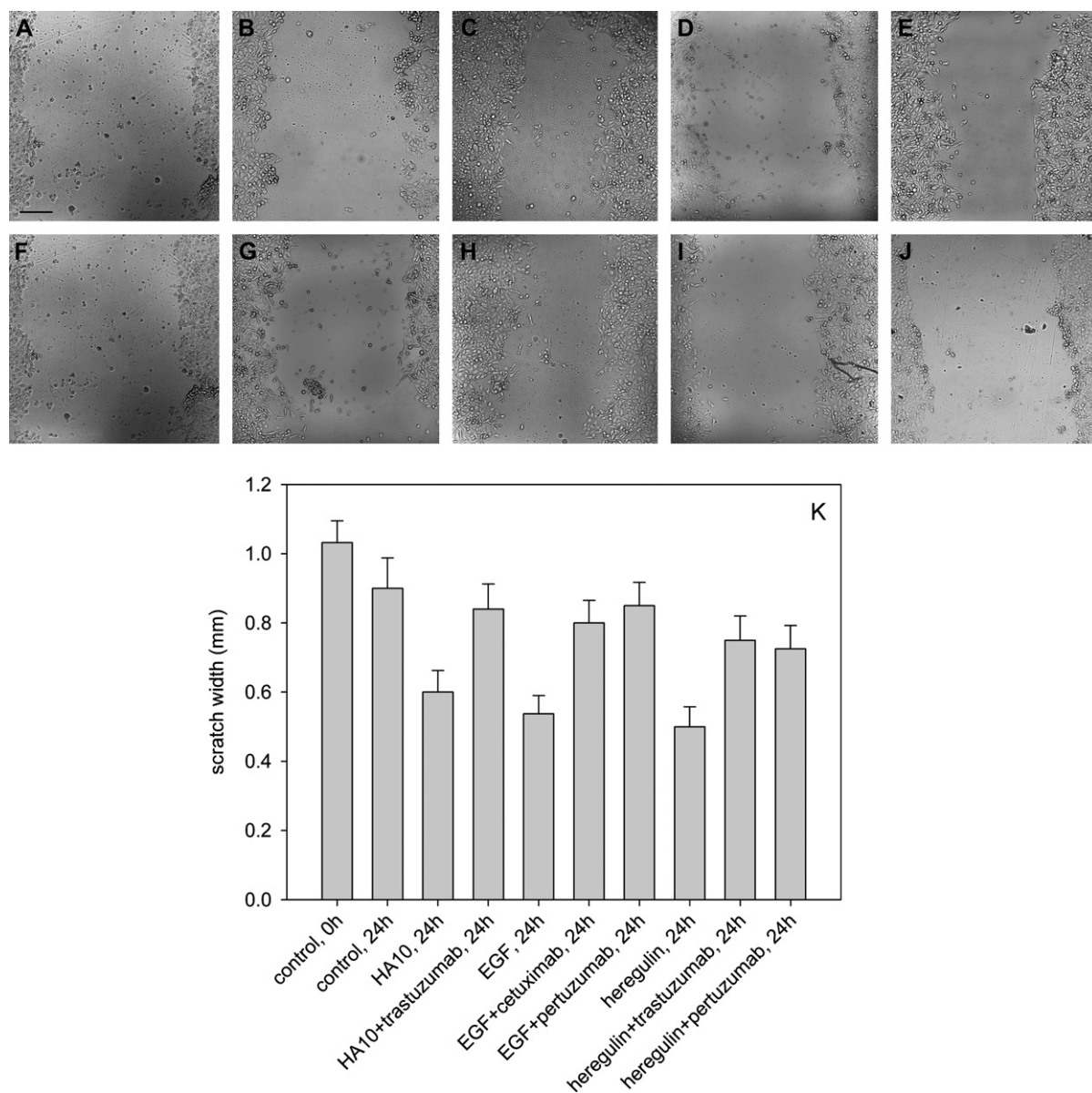


Fig. 5. The effect of trastuzumab, pertuzumab and cetuximab on hyaluronan-, EGF- and heregulin-induced motility. (A–J) Confluent cultures of JIMT-1 cells treated in different ways were scratched with a pipette tip to create a cell-free stripe, and images were taken at 0 h and 24 h. (A) control, 0 h; (B) control, 24 h; (C) hyaluronan oligosaccharide, 24 h; (D) hyaluronan oligosaccharide + trastuzumab, 24 h; (E) EGF, 24 h; (F) EGF + cetuximab, 24 h; (G) EGF + pertuzumab, 24 h; (H) heregulin, 24 h; (I) heregulin + trastuzumab, 24 h; (J) heregulin + pertuzumab, 24 h. The images were taken with identical magnification. The bar in A corresponds to 0.2 mm. (K) The width of scratches ($\pm$ SD) at 0 h and 24 h were determined. Since the scratch widths at 0 h did not differ significantly between different samples, only the control one is shown.

accumulation of its intracellular domain in the cytosol. Since ErbB-specific ligands (EGF binding to ErbB1 and heregulin binding to ErbB3) influence intramembrane processing of CD44, we assumed that antibodies inhibiting the function of ErbB1 and ErbB2 may alter the rate of CD44 endocytosis

and cleavage. Indeed, cetuximab, an antibody competitively blocking binding of EGF to ErbB1 [10], inhibited EGF-induced cleavage and internalization of CD44. The effects of both heregulin and EGF were inhibited by pertuzumab, a monoclonal antibody blocking heterodimerization of ErbB2 with

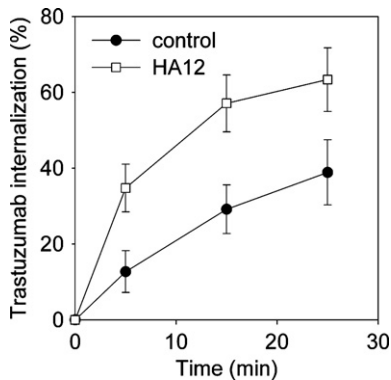


Fig. 6. Hyaluronan oligosaccharide enhances trastuzumab internalization. Control JIMT-1 cells or cells pretreated with 100 μ g/ml hyaluronan oligosaccharide for 15 min at room temperature were labeled with 20 μ g/ml AlexaFluor647-trastuzumab for 15 min on ice followed by incubation at 37 °C for the indicated amount of time. The fraction of internalized trastuzumab was determined as described in Materials and methods. Error bars indicate the standard deviation.

other ErbB proteins [13]. The pertuzumab-and cetuximab-mediated decrease in CD44 shedding is probably a consequence of the inhibition in the growth factor-induced activation of ErbB-mediated signaling caused by the antibodies. Pertuzumab inhibited the heregulin-induced effects more efficiently than the EGF-induced ones, because heterodimerization of ErbB2 with ErbB3, the only heregulin receptor expressed at sufficiently high levels in JIMT-1 [43], is obligatory for heregulin-induced signaling due to the kinase deficiency of ErbB3 [13]. In contrast, ErbB1 can efficiently transmit signals in homodimers as well [3], whose formation is not inhibited by pertuzumab [50]. Our results confirm that the sensitivity of certain cellular responses to different anti-ErbB antibodies is also influenced by the growth factor receptor driving the signaling process [51].

We compared changes in CD44 trafficking (shedding, endocytosis) with cell motility, and found a high degree of correlation between these parameters. These observations confirm previous findings showing that shedding of CD44 leads to cell migration [29,44] and also strengthen the validity and implications of our claims that anti-ErbB antibodies inhibit cell migration and possibly metastasis formation.

During the era of the clinical application of trastuzumab several models have been put forward to explain its clinical anti-tumor effect [16]. Although most reports attribute the tumor inhibitory effect

of trastuzumab to direct inhibition of ErbB2 signaling or ADCC-mediated immune responses, Molina et al. implicated the involvement of trastuzumab-mediated inhibition of ErbB2 ectodomain cleavage in the therapeutic action [42]. Here we showed that trastuzumab also inhibits CD44 cleavage and internalization induced by heregulin and hyaluronan oligosaccharide. Trastuzumab probably prevents the heregulin-induced activation of ErbB2 necessary for transactivation of CD44 proteolysis. We propose that proper, baseline activation of ErbB2 is necessary for the CD44-ErbB complex in which proteases must also be present. Trastuzumab may block hyaluronan oligosaccharide-induced CD44 shedding by inhibiting this constitutive activation of ErbB2. A similar relationship has been revealed between multidrug resistance, CD44 and ErbB2 expression, where ErbB2 has a permissive role in the hyaluronan-mediated induction of P-glycoprotein [52].

Our results imply that the activation of ErbB receptors induces cleavage and internalization of CD44 which has been implicated to play a role in cancer progression [38,39]. Furthermore, we showed that CD44 shedding and endocytosis are blocked by inhibitory antibodies against ErbB1 (cetuximab) and ErbB2 (trastuzumab, pertuzumab). The finding that trastuzumab decreased the concentration of the shed extracellular domain of CD44 in serum samples in mice with early stage JIMT-1 xenografts was even more interesting. This effect of trastuzumab is in accordance with our previous findings that it decreases the number of circulating and disseminated tumor cells in mice xenografted with JIMT-1 tumor [53]. These findings imply that trastuzumab-induced inhibition of CD44 shedding may be causally related to its anti-metastatic effect. The fact that each of the three anti-ErbB antibodies inhibited CD44 shedding *in vitro* raise the possibility that they retard metastasis formation in cancer patients. This intriguing supposition is supported by our findings that blocking CD44 shedding lead to decreased cellular motility in *in vitro* scratch assays. Since long-term survival in cancer is determined by the development of metastases, these anti-ErbB antibodies having an inhibitory effect on the primary tumor and metastatic spread can play an important role in the management of patients with ErbB-overexpressing tumors. Our results confirm that soluble CD44 can be used as a predictor of metastatic spread, and agents interfering with CD44 shedding, including but not limited to

anti-ErbB antibodies, are promising candidates for the treatment of advanced tumors.

We did not find any effect of trastuzumab on CD44 shedding from large, late stage tumors. We assume this is the consequence of these tumors being resistant to trastuzumab, since we have shown previously that resistance of JIMT-1 xenograft tumors to trastuzumab develops in ~10 weeks [14].

We found evidence for the mutual influence of CD44 and ErbB receptors on each others internalization, since not only did EGF and heregulin induce CD44 shedding and endocytosis, but hyaluronan oligosaccharide increased the endocytosis of trastuzumab bound to ErbB2 as well. We suspect that the rate of ErbB2 endocytosis was increased by hyaluronan oligosaccharide because ErbB2 co-internalized with CD44 whose endocytosis was induced by hyaluronan.

Our results suggest that ErbB1, ErbB2 and CD44 are clustered to form a functional unit in the cell membrane in which they mutually influence each other's cleavage and endocytosis rates. The interaction between them is not limited to the regulation of proteolysis and internalization, but involves potentiation of signaling processes as well. The motogenic, but not the mitogenic activity of EGF depends on the activation of CD44 [54]. Hyaluronan has been shown to be required for heart valve mesenchyme formation induced by ErbB2 and ErbB3 [55]. These observations also support our model in which the complex of CD44 and ErbB proteins are assumed to be involved in the regulation of cell motility. From the standpoint of a clinician the functional interaction between CD44 and ErbB proteins has the potential to modify the therapeutic efficacy of ErbB1- and ErbB2-directed therapies suggesting that the interaction, or merely the expression level of CD44 is taken into consideration when patients are selected for antibody therapy. Much work needs to be done before these experimental observations will make their way to clinical practice.

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Quantitative Characterization of the Large-Scale Association of ErbB1 and ErbB2 by Flow Cytometric Homo-FRET Measurements

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ABSTRACT The association of receptor tyrosine kinases is a key step in the initiation of growth factor-mediated signaling. Although the ligand-induced dimerization of inactive, monomeric receptors was the central dogma of receptor tyrosine kinase activation for decades, the existence of larger oligomers is now accepted. Both homoassociations and heteroassociations are of extreme importance in the epidermal growth factor (EGF) receptor family, leading to diverse and robust signaling. We present a statistically reliable, flow-cytometric homo-fluorescence resonance energy transfer method for the quantitative characterization of large-scale receptor clusters. We assumed that a fraction of a certain protein species is monomeric, whereas the rest are present in homoclusters of N -mers. We measured fluorescence anisotropy as a function of the saturation of fluorescent antibody binding, and fitted the model to the anisotropy data yielding the fraction of monomers and the cluster size. We found that ErbB2 formed larger homoclusters than ErbB1. Stimulation with EGF and heregulin led to a decrease in ErbB2 homocluster size, whereas ErbB1 homoclusters became larger after EGF stimulation. The activation level of ErbB2 was inversely proportional to its homocluster size. We conclude that homoclusters of ErbB1 and ErbB2 behave in a fundamentally different way. Whereas huge ErbB2 clusters serve as a reservoir of inactive coreceptors and dissociate upon stimulation, small ErbB1 homoclusters form higher-order oligomers after ligand binding.

INTRODUCTION

The dimerization of membrane proteins is believed to play a fundamental role in the activation of receptor tyrosine kinases (RTKs). The groundbreaking discovery of the importance of receptor oligomerization in the 1970s (1) was supported by the crystal structure of the epidermal growth factor (EGF) receptor (2). The ligand-induced dimerization of RTKs became textbook dogma, and because of the limited potential of classical molecular biological methods to detect clusters composed of more than two receptors, larger oligomers were neglected. Promiscuous homoassociation and heteroassociation are prominent features of the EGF receptor family (also known as the ErbB family) of RTKs consisting of four members, i.e., ErbB1 (EGF receptor) and ErbB2–4 (3–5). The prototypical RTK, ErbB1, is believed to undergo ligand-induced homodimerization and activation (2). The ligandless coreceptor, ErbB2, is activated either by heterodimerization with other ErbB proteins or by overexpression-driven constitutive homoassociation (6,7). The kinase-deficient ErbB3 receptor can only transmit signals by heterodimerization, primarily with ErbB2 (8). The simplistic view of inactive monomers and active dimers was complicated by discovering preformed clusters of inactive ErbB receptors (9–12). A further twist in the story involved the repeated identification of large receptor clusters in both quiescent and stimulated

cells. Fluorescence correlation microscopy revealed clusters containing 10 to 30 EGF receptors (13). Webb et al. also reported on 4 to 5 EGF receptors/cluster, based on the number of photobleaching steps in single-molecule fluorescence time traces (14). A combined fluorescence resonance energy transfer (FRET)-correlation microscopic study confirmed the existence of ErbB1 tetramers and large homoclusters containing 15 to 30 receptors (15). The EGF receptor was shown to undergo activation-induced dimer-tetramer transition (16), whereas ErbB3 was shown to behave differently. It forms homoclusters whose upper size limit is a dodecamer. Heregulin, the ligand of ErbB3, reverses the homoassociation of ErbB3 (17,18). The ErbB3 molecules that break free from the homoclusters most probably heteroassociate with ErbB2, because kinase-dead ErbB3 can only transmit signals by heteroassociating with other ErbB proteins. Scanning near-field optical microscopy revealed clusters containing ~1000 ErbB2 molecules in microdomains with a diameter of ~500 nm, which increased upon stimulation (19). These reports present convincing evidence for the existence and importance of large-scale protein associations.

Apart from biochemical methods, which are usually semiquantitative and are not appropriate for the investigation of living cells, several approaches are available for the quantitative characterization of large-scale receptor associations in intact cells. Fluorescence correlation spectroscopy (13,15), single-molecule imaging (14,20), and measurement of the rotational diffusion rate (21) were applied for this purpose. Probably the most widely used quantitative technique for

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the analysis of protein-clustering is FRET, in which energy is transferred in a nonradiative fashion from an excited donor molecule to a nearby acceptor. The rate of FRET varies inversely with the sixth power of the donor-acceptor separation, turning it into a useful distance-measuring tool (22). When the process takes place between a donor and a spectroscopically different acceptor, it is termed hetero-FRET. Although a careful analysis of hetero-FRET signals can reveal the stoichiometry of association, the physical interaction in hetero-FRET takes place between a single pair of donor and acceptor, limiting the potential of hetero-FRET measurements to analyze large protein clusters (16,23,24). In homo-FRET, the interaction takes place between a donor and a spectroscopically identical “acceptor” which can serve as the donor in the next homo-FRET step. This sequential aspect of the interaction is reflected in the term energy migration FRET (emFRET), used as a synonym for homo-FRET (10). Because energy is distributed among the interacting molecules, homo-FRET can be used for the quantitative analysis of large protein clusters. Because the only manifestation of homo-FRET is decreased fluorescence anisotropy, Runnels and Scarlata derived a formula for the steady-state anisotropy of clusters of N molecules (25). Steady-state anisotropy is ambiguously related to homo-FRET, because factors other than energy transfer can also change its value. Therefore, anisotropy is either measured in the time domain (23), or its dependence on fluorophore density is analyzed to enumerate the number of interacting fluorophores. The latter strategy was applied by measuring the increase in anisotropy as a function of fluorophore photobleaching (10,26,27). Alternatively, the loss of homo-FRET occurring at red-edge excitation was used to isolate the contribution of homo-FRET to the measured anisotropy in microscopy (28).

Microscopy suffers from low statistical accuracy because of the limited number of cells measured. Flow cytometry offers superior statistics, without the capability to quantitate photobleaching. Here we present a flow-cytometric method for the measurement of homo-FRET, based on varying the amount of saturation of antibody binding to cell-surface receptors. We derived a formula for the dependence of fluorescence anisotropy on the fraction of monomers and on the number of proteins in a single cluster, and fitted the model to anisotropy data measured on cells labeled with anti-ErbB1 or anti-ErbB2 antibodies. The data presented here provide significant new insights into the large-scale association properties of ErbB receptors.

MATERIALS AND METHODS

Cell lines

The human breast-cancer cell line SKBR-3 and the human epithelial carcinoma cell line A431 were obtained from the American Type Culture Collection (Rockville, MD), and were grown according to their specifications. For flow cytometry, cells were harvested by trypsinization.

Antibodies and growth factors

The anti-ErbB1 monoclonal antibody EGFR455, which does not block the binding of EGF, was produced by hybridoma 455 obtained from the European Collection of Cell Cultures (Wiltshire, UK). Mab528 against ErbB1, competing with EGF binding, was prepared from the supernatant of the HB-8509 hybridoma cell line obtained from American Type Culture Collection. Antibodies were purified from hybridoma supernatants, using protein A affinity chromatography. The anti-ErbB2 antibody, trastuzumab (Herceptin), was purchased from Roche (Budapest, Hungary). The conjugation of primary antibodies with AlexaFluor (Molecular Probes, Eugene, OR) dyes was performed according to the manufacturer's specifications. The OP15 monoclonal antibody against an intracellular epitope of ErbB2 was obtained from Calbiochem-Merck Biosciences (Schwalbach, Germany). Ab-18, recognizing the activated, tyrosine-phosphorylated form of ErbB2, was purchased from Lab Vision (Fremont, CA). Pertuzumab (Omnitarg) was a gift from Genentech (South San Francisco, CA). EGF and heregulin- β 1 were obtained from R&D Systems (Minneapolis, MN). The Alexa488-conjugated and Alexa546-conjugated F(ab')₂ fragments of goat anti-mouse immunoglobulin G (IgG) and the Cy3-labeled F(ab')₂ fragment of goat anti-human IgG were from Molecular Probes/Invitrogen (Eugene, OR).

Labeling of cells with antibodies

Freshly harvested cells were washed twice in ice-cold phosphate-buffered saline (PBS; pH 7.4), and the cell pellet containing ~ 1 million cells was suspended in 100 μ L of Hanks' buffer with 1 mg/mL bovine serum albumin (BSA). For homo-FRET measurements, ErbB1 or ErbB2 receptors were labeled with different ratios of unlabeled and Alexa488-labeled antibodies for 30 min on ice in the dark. The total concentration of unlabeled and labeled antibodies was 50 μ g/mL, and the fraction of labeled antibody varied from 0% to 100%, with increments of 10%. Labeled cells were washed twice with cold PBS and fixed with 1% formaldehyde in PBS. All samples were kept at 4°C before performing measurements, to prevent the modulation of antibody-induced receptor clustering.

To determine the limiting anisotropy of Alexa488-conjugated antibodies, ErbB1 or ErbB2 receptors were incubated with 50 μ g/mL donor-conjugated antibody (Alexa488-trastuzumab, Alexa488-Mab528, or Alexa488-EGFR455) for 30 min on ice in the dark. After washing, the samples were divided into five parts, which were labeled by different concentrations (0.5 to 9 μ g/mL) of acceptor-conjugated secondary antibody (Cy3-labeled F(ab')₂ fragments of goat anti-human IgG in the case of Alexa488-trastuzumab, or Alexa546-conjugated F(ab')₂ fragments of goat anti-mouse IgG in the case of Alexa488-Mab528 and Alexa488-EGFR455) for 30 min on ice in the dark. Labeled cells were washed and fixed with 1% formaldehyde in PBS.

For the measurement of ErbB2 phosphorylation, quiescent and stimulated cells were fixed with 3.7% formaldehyde in PBS for 30 min on ice, and washed twice with cold Tris-buffer (100 mM Tris + 100 mM NaCl; pH 7.4) to quench unreacted formaldehyde. Each sample was divided into two parts, which were separately labeled with OP15 (10 μ g/mL) and Ab-18 (10 μ g/mL) against ErbB2 and phosphorylated ErbB2 (p-ErbB2), respectively. Labeling was performed in PBS-BSA-TX permeabilization buffer (0.1% (v/v) Triton X-100, 1 mg/mL BSA in PBS, pH 7.4) for 30 min on ice. After washing with cold PBS-BSA-TX, cells were labeled with Alexa488-conjugated F(ab')₂ fragments of goat anti-mouse IgG for 30 min on ice in the dark. Labeled cells were washed twice, resuspended in 1% formaldehyde, and analyzed by flow cytometry.

Treatment of cells with EGF, heregulin, and pertuzumab

Cells were starved for 24 h before the experiment in medium containing 0.1% fetal calf serum (FCS). Freshly harvested cells were washed twice in cold PBS, and resuspended in 100 μ L Hanks' buffer supplemented with 1 mg/mL

BSA. Pretreatment with 20 $\mu\text{g/mL}$ pertuzumab was performed for 15 min at 37°C. Control and pertuzumab-pretreated cells were stimulated with 100 ng/mL EGF or 100 ng/mL heregulin for 15 min at 37°C, and subsequently used for homo-FRET measurements, as described above.

Flow cytometry and data analysis

Cell-by-cell fluorescence anisotropy measurements were performed in the L-format arrangement on a FACSVantage SE instrument with a DiVa option (Becton Dickinson, Franklin Lakes, NJ). Alexa488 was excited by a vertically polarized 488-nm beam, which was produced by letting the 488-nm line of an Ar ion laser pass through a half-wave plate retarder (model 481AS, Newport, Irvine, CA). The angle of the half-wave plate corresponding to vertical polarization was adjusted by light-scatter measurements. Emitted photons passed through a 530 ± 15 nm band-pass filter, followed by a broadband polarizing beam-splitter cube (model 10FC16PB.3, Newport) separating the vertically and horizontally polarized components. Two polarized fluorescence intensities, designated by I_{vv} and I_{vh} , were detected, where the first and second indices refer to the polarization directions of the exciting laser light and of fluorescence, respectively. For the determination of the G -factor, cells were excited by horizontally polarized light produced by rotating the half-wave plate by 45° relative to its vertical position. The two polarized fluorescence intensities recorded with horizontal excitation are labeled by I_{hv} and I_{hh} . After subtracting the intensity of unlabeled cells from each polarized intensity, the G -factor compensating for the different sensitivities of the detection system to vertically and horizontally polarized light, the total fluorescence intensity (I_{tot}), and the fluorescence anisotropy (r) were calculated as follows:

$$G = \frac{I_{hv}}{I_{hh}} \quad (1)$$

$$I_{\text{tot}} = I_{vv} + a \times G \times I_{vh} \quad (2)$$

$$r = \frac{I_{vv} - G \times I_{vh}}{I_{\text{tot}}} \quad (3)$$

The influence of high-aperture fluorescence collection on the determined anisotropy was compensated according to Jovin (constant a in Eq. 2) (29). Fifty thousand cells were recorded from each sample containing a certain fraction of labeled and unlabeled antibodies. Anisotropy was calculated on a cell-by-cell basis according to Eq. 3. Cells were gated on the forward scatter-side scatter dot plot and on the total fluorescence intensity-anisotropy dot plot, using ReFlex software (available at <http://www.freewebs.com/cytoflex>) (30); ~10,000 to 20,000 cells were gated out, leaving 30,000 to 40,000 cells for calculating the average fluorescence intensity and anisotropy values. Anisotropy was plotted against saturation, and the graph from a single experiment was fitted by Eq. 7 (see Theory, below). Fitting and Monte Carlo

simulation were performed using Mathematica (Wolfram Research, Champaign, IL). Cluster size and monomer percentage values reported in the text and in Table 1 were calculated by averaging the values obtained from the fitting of three separate experiments. The sequence of data collection and processing is summarized in Fig. 1.

Hetero-FRET efficiency was determined using a FACSVantage SE flow cytometer with a DiVa option, with dual-laser excitation at 488 and 532 nm. The donor (Alexa488) and FRET signals were excited at 488 nm and detected at 530 ± 15 nm and 585 ± 21 nm, respectively. Direct acceptor emission (Alexa546 or Cy3) was detected at 585 ± 21 nm upon 532-nm excitation with a diode-pumped, solid-state laser. The FRET efficiency was determined on a cell-by-cell basis, using ReFlex software as described previously (30,31).

Theory

Homo-FRET efficiency changes as a function of fluorophore density. Previously, photobleaching was used to create a range of fluorophore densities (10,27), but the same approach is not applicable in flow cytometry. Instead, we labeled cell-surface proteins with a mixture of fluorophore-labeled and unlabeled antibodies against the same epitope, so that the total antibody concentration saturated the available binding sites. In this way, the fraction of epitopes to which fluorophore-tagged antibody bound (saturation designated by s in the equations) was approximately equal to the fraction of labeled antibody in the mixture. The amount of saturation (s) was estimated by dividing the fluorescence intensities of each sample with that of the brightest one. We assumed that a fraction (mon) of the proteins is monomeric, and that the rest ($1-mon$) are present in homoclusters of N -mers. Essentially, any protein unable to undergo homo-FRET is regarded as monomeric in the calculations, e.g., a labeled protein in a heterodimeric complex, because it does not interact with another protein of the same kind. The probability that k out of N proteins is labeled by a fluorescent antibody in the N -mer when the fraction of labeled proteins is s is described by the binomial distribution:

$$P_{s,k,N} = \binom{N}{k} s^k (1-s)^{N-k} \quad (4)$$

The anisotropy of such a homocluster according to Runnels and Scarlata (25) is

$$r_k = r_1 \frac{(1+d^6)}{1+k d^6} + r_{\text{FRET}} \frac{(k-1)d^6}{1+k d^6}, \quad d=(R_0/R) \quad (5)$$

where r_1 and r_{FRET} are the anisotropies of an initially excited molecule and that of a molecule excited by homo-FRET, respectively, and d is the distance between the fluorophores in the clusters normalized to R_0 . The determination of r_1 will be explained in Results. We assumed a complete depolarization of fluorescence emitted by every fluorophore except for the initially excited

TABLE 1 Cluster sizes and monomer percentages of ErbB1 and ErbB2

		ErbB1		ErbB2	
		Cluster size	Monomer %	Cluster size	Monomer %
Starved	Control	4 $\pm$ 1	88 $\pm$ 4	111 $\pm$ 12	60 $\pm$ 5
	EGF	11 $\pm$ 2	71 $\pm$ 3	71 $\pm$ 6	61 $\pm$ 4
	Heregulin	—	—	32 $\pm$ 4	59 $\pm$ 5
	Pertuzumab	—	—	84 $\pm$ 7	70 $\pm$ 4
	Pertuzumab + heregulin	—	—	73 $\pm$ 6	58 $\pm$ 3
Nonstarved	Control	7 $\pm$ 1	75 $\pm$ 4	9 $\pm$ 2	53 $\pm$ 3
	Pertuzumab	—	—	30 $\pm$ 4	55 $\pm$ 3

ErbB1 and ErbB2 were labeled by Alexa488-EGFR455 and Alexa488-trastuzumab, respectively. The cluster size and monomer percentage are shown on cells pretreated and stimulated in different ways. Means ($\pm$ SE) were calculated from the fitted cluster size and monomer percentage values obtained from three independent experiments. Cluster size is the average number of ErbB1 or ErbB2 proteins in the cluster. Monomer percentage is the fraction of ErbB1 or ErbB2 present outside homoclusters (either as monomers or heterodimers with other proteins).

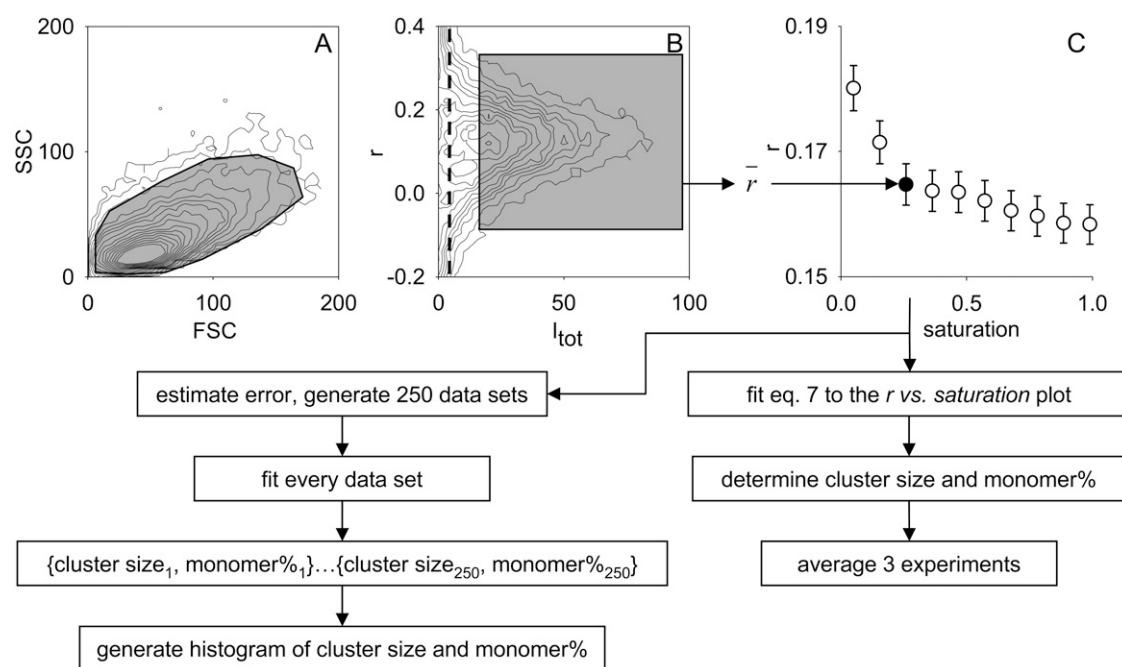


FIGURE 1 Data collection, gating, and processing strategy to calculate cluster size and monomer percentage from flow-cytometric anisotropy measurements; 50,000 cells, labeled by a mixture of labeled and unlabeled antibodies, were measured. Cells were first gated on the forward scatter-side scatter (FSC-SSC) dot plot (A), followed by gating on the anisotropy versus total fluorescence intensity dot plot (B). Data were recorded with 18-bit resolution, but were rescaled for the dot plots. The thick dashed line in B indicates the fluorescence intensity of unlabeled cells. Gates are represented by shaded polygons. The position of the gates was identical for every sample with a different concentration of the labeled antibody measured on the same day. The lower threshold of total fluorescence intensity was visually adjusted to gate out dim cells whose anisotropy had a large variation, and the threshold was kept constant for all samples. The average anisotropy was calculated, yielding a total intensity-anisotropy data point on the graph showing the dependence of anisotropy on the concentration of labeled antibody (saturation, in C). Equation 7 was fitted to the anisotropy-saturation plot, yielding the estimated cluster size and monomer percentage. The results of three independent experiments were averaged. Anisotropy was assumed to be normally distributed, and its error was estimated. The mean and error values were used to generate 250 random data sets, similar to the one shown in C. Every data set was fitted, yielding 250 pairs of cluster sizes and monomer percentages from which frequency histograms were generated.

one, and therefore, $r_{\text{FRET}} = 0$ (25). Although membrane proteins are ordered in the plane of the membrane and their rotation is slow on the timescale of fluorescence, bending of the antibody around the epitope, segmental motions of the Fab arms around the Fc portion, and rapid rotation of the antibody-bound fluorophore make the system practically randomly oriented. If the assumption of $r_{\text{FRET}} = 0$ is incorrect, it leads to an underestimation of cluster

where k/N is a correction factor normalizing the sum of the fluorescence intensity of N -mers and monomers to unity. Multiplication by k is necessary to weigh the anisotropy values according to the fluorescence intensity. Without dividing by N , the fluorescence intensity of clusters would be overweighted by a factor of N . By combining Eqs. 4–6, the total anisotropy is given by the following formula:

$$r_{s,k,N} = \frac{(1 - \text{mon}) \sum_{k=0}^N \left[\binom{N}{k} s^k (1-s)^{N-k} \frac{k}{N} \left(r_1 \frac{(1+d^6)}{1+k d^6} + r_{\text{FRET}} \frac{(k-1)d^6}{1+k d^6} \right) \right] + s \times \text{mon} \times r_1}{(1 - \text{mon}) \sum_{k=0}^N \binom{N}{k} s^k (1-s)^{N-k} \frac{k}{N} + s \times \text{mon}} \quad (7)$$

size or an overestimation of monomer percentage. The resultant anisotropy of the mixture of monomers and N -mers is the intensity-weighted average of the individual anisotropies (32):

$$r_{s,k,N} = \frac{(1 - \text{mon}) \sum_{k=0}^N P_{s,k,N} \frac{k}{N} r_k + s \times \text{mon} \times r_1}{(1 - \text{mon}) \sum_{k=0}^N P_{s,k,N} \frac{k}{N} + s \times \text{mon}} \quad (6)$$

Equation 7 predicts that the total anisotropy is constant, if only monomers are present, and that the anisotropy is a linear function of saturation if homoclusters are dimers. The higher the number of proteins in a homocluster, the steeper the anisotropy declines at low values of saturation (Fig. 2). Equation 7 was fitted to the measured anisotropy-saturation plot, with mon and N as the fitting parameters (Fig. 3). We then calculated the standard deviation of the anisotropy values and generated 250 random data sets, with mean and standard deviation values equal to the measured data. These random data sets were also fitted by Eq. 7,

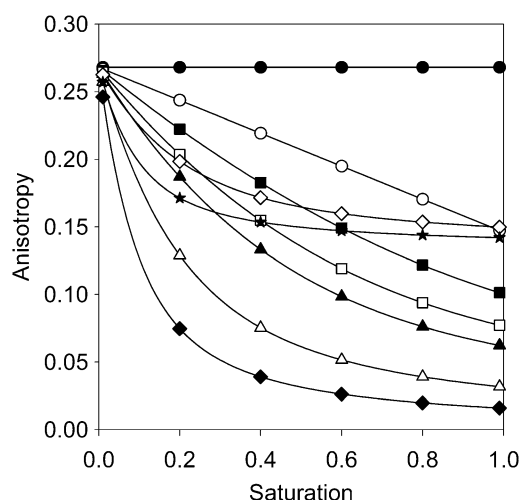


FIGURE 2 Theoretical curves show the dependence of fluorescence anisotropy on the saturation of binding sites as a function of cluster size and monomer percentage. The anisotropy of fluorescence antibodies as a function of the saturation of binding sites was determined according to Eq. 7, assuming a limiting anisotropy of 0.27. The calculation was performed for several cluster sizes, with no monomers present (numbers indicate number of proteins in the homocluster: ●, 1; ○, 2; ■, 3; □, 4; ▲, 5; △, 10; ◆, 20), and for the case when half of the proteins are monomers (◇, 10; ★, 20).

yielding 250 values for cluster size (N) and monomer percentage (mon). The distribution of both parameters was calculated, and the graphs for ErbB1 and ErbB2 under different conditions are given in Figs. 5 and 6.

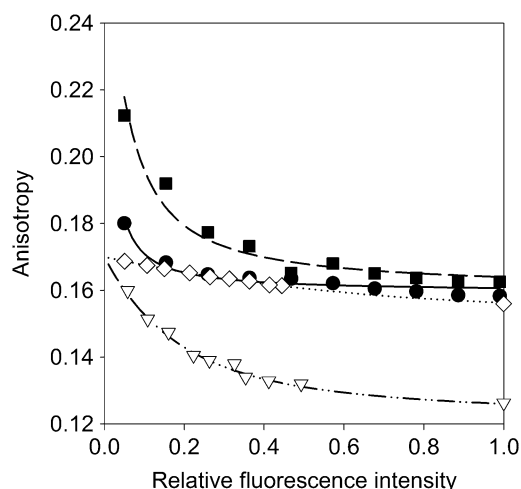


FIGURE 3 Fitting of the anisotropy model to ErbB1 and ErbB2 anisotropy data. Starved (●) and heregulin-stimulated (■) SKRB-3 cells were labeled with a mixture of unlabeled and Alexa488-labeled trastuzumab. Starved (◇) and EGF-stimulated (▽) A431 cells were labeled with a mixture of unlabeled and Alexa488-labeled EGFR455. The anisotropy of samples was determined and plotted as a function of saturation of binding sites. The lines show the results of fitting according to Eq. 7.

RESULTS

Determination of the limiting anisotropy of antibody-bound fluorophores

We approximated the anisotropy of the initially excited fluorophore (r_1) by the limiting anisotropy of the antibody-bound fluorophore (r_0). The emission anisotropy of a fluorophore (r) changes as a function of its rotational correlation time and fluorescence lifetime, according to the Perrin equation:

$$\frac{1}{r} = \frac{1}{r_0} \left(1 + \frac{\tau_{fl}}{\tau_{rot}} \right), \quad (8)$$

where τ_{fl} is the fluorescence lifetime and τ_{rot} is the apparent rotational correlation time of the fluorophore, taking into account all depolarizing processes taking place during the excited-state lifetime (rotational diffusion of the antibody, and segmental motion of parts of the antibody and that of the fluorophore). The apparent limiting anisotropy of the fluorophore is given as r_0 . Because hetero-FRET decreases the fluorescence lifetime, a Perrin plot can be constructed by varying the hetero-FRET efficiency (33):

$$\tau_{fl} = (1 - E)\tau_D \Rightarrow \frac{1}{r} = \frac{1}{r_0} \left(1 + \frac{(1 - E)\tau_D}{\tau_{rot}} \right), \quad (9)$$

where τ_D is the lifetime of the fluorophore ("donor") in the absence of FRET. The y-intercept of a line fitted on the $1/r$ versus $1 - E$ plot is the reciprocal of the limiting anisotropy. To create a range of donor fluorescence lifetimes, i.e., FRET efficiencies, cells labeled with saturating concentrations of the donor-tagged antibody were labeled by different concentrations of an acceptor-tagged secondary antibody. Both the hetero-FRET efficiencies and the fluorescence anisotropies of the donor were measured and displayed on a Perrin plot (Fig. 4), and the limiting anisotropies were calculated for each of the IgGs and Fab fragments ($r_{0,A488-trastuzumab} = 0.268$; $r_{0,A488-trastuzumab\ Fab} = 0.289$; $r_{0,A488-Mab528} = 0.226$; and $r_{0,A488-Mab\ EGFR455} = 0.170$).

Calculation of the separation distance between two antibodies bound to a homocluster

The distance between two antibodies bound to homoclusters has to be known in units of R_0 . We first determined R_0 for the Alexa488 homo-FRET case, according to the following formula in units of nm:

$$R_0 = 8.8 \times 10^{-18} (J \times \Psi_D \times n^{-4} \times \kappa^2)^{1/6}. \quad (10)$$

The overlap integral (J) was calculated from spectra available from the Invitrogen web site, according to the following formula:

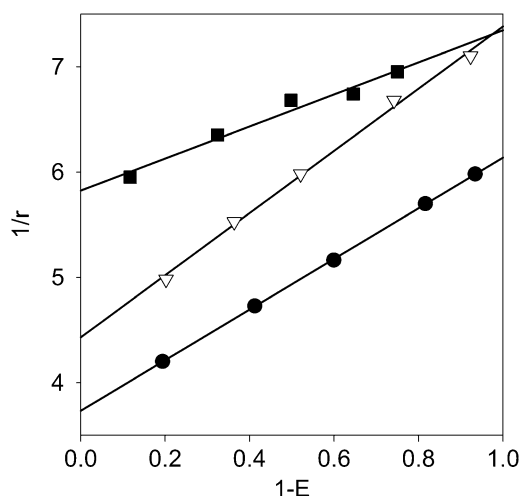


FIGURE 4 Determination of limiting anisotropy. Cells were labeled with saturating concentration of the primary antibody (Alexa488-tagged trastuzumab, ●; EGFR455, ■; or Mab528, ▽) followed by secondary labeling by Cy3-anti-human F(ab')₂ or Alexa546-anti-mouse F(ab')₂. The fluorescence anisotropy and the hetero-FRET efficiency were calculated for each sample, and the y-intercept of the line fitted on the Perrin plot yielded the reciprocal of the limiting anisotropy.

$$J = \int_0^\infty f_D(\lambda) \varepsilon_A(\lambda) \lambda^4 d\lambda, \quad (11)$$

where f_D and ε_A are the normalized fluorescence spectrum and the molar absorption coefficient of Alexa488, respectively. The quantum yield (Ψ_D) of Alexa488 was previously reported to be 0.6 (34). We assumed a value of 1.4 and 2/3 for the index of refraction (n) and the orientation factor (κ^2), respectively, yielding 4.8 nm for the R_0 between two Alexa488 molecules undergoing homo-FRET. The hetero-FRET efficiency for ErbB2 homodimerization, measured by Cy3-trastuzumab and Cy5-trastuzumab, was found to be 20% (35). Using 5 nm as the R_0 between Cy3 and Cy5 (35), the above FRET efficiency corresponds to an average separation distance of 6.29 nm, according to the formula:

$$R = R_0 \sqrt[6]{\frac{1-E}{E}}, \quad (12)$$

where E is the FRET efficiency. Therefore, the average separation between two trastuzumab molecules (d in Eq. 7) in units of R_0 is 6.29 nm/4.8 nm = $1.3 \times R_0$. Using the same approach, the average distance between two EGFR455 antibodies and between two Mab528 antibodies was found to be $1.1 \times R_0$ and $1.4 \times R_0$, respectively.

Stimulation of ErbB2 leads to a decrease in its cluster size

The homo-FRET model described above was used for evaluation of the homocluster formation of ErbB1 and ErbB2 in quiescent and stimulated cells. SKBR-3 cells expressing $\sim 8 \times 10^5$ ErbB2 proteins (36) were starved in low-serum

medium (0.1% FCS) for 24 h, and the percentage of monomeric ErbB2 and the cluster size of homoassociated ErbB2 were determined; $\sim 60\%$ of ErbB2 molecules were monomeric, but the rest formed huge clusters composed of ~ 110 ErbB2 proteins (Fig. 5, A and B; Table 1). Stimulation of starved cells with EGF or heregulin did not change the percentage of monomers significantly, but led to a substantial decrease in the size of ErbB2 homoclusters. The effect of heregulin was more pronounced than that of EGF. Pretreatment of cells with pertuzumab led to a slight increase in the percentage of ErbB2 monomers and to a significant decrease in cluster size (Fig. 5, C and D; Table 1). Pertuzumab pretreatment practically abolished the effect of heregulin on ErbB2 cluster size, although the effect of heregulin on ErbB2 monomer percentage was unaffected. If cells were not starved before the experiment, the percentage of monomers was lower than in starved cells, and the size of ErbB2 homoclusters was even smaller than in heregulin-stimulated or EGF-stimulated cells. To examine the possible effect of labeling with bivalent antibodies on ErbB2 clustering, non-starved SKBR3 cells (cultured in the presence of 10% FCS) were labeled with a mixture of Alexa488-labeled and unlabeled Fab fragments of trastuzumab. The observation that both the cluster size and monomer percentage were identical in cells labeled with trastuzumab IgG and Fab implies that labeling with bivalent IgG does not modify the clustering properties of ErbB2 (Fig. 5, E and F). Treatment of non-starved cells with pertuzumab significantly increased the size of ErbB2 homoclusters, without an effect on monomer percentage (Fig. 5, E and F; Table 1). We concluded that ErbB2 is homoclustered to different degrees in quiescent and stimulated cells, and its cluster size changes as a function of its activation state.

Stimulation of ErbB1 induces an increase in its cluster size

Next, we compared the homoclustering properties of ErbB1 to those of ErbB2. The fluorescence anisotropy of Alexa488-EGFR455 antibody was evaluated in A431 cells starved in low-serum medium for 24 h. The A431 cell line expresses ~ 2 million copies of ErbB1 per cell (37); $\sim 90\%$ of ErbB1 was monomeric, and the remaining $\sim 10\%$ formed homoclusters containing ~ 4 ErbB1 molecules (Fig. 6; Table 1). Stimulation of cells with EGF reduced the monomer percentage of ErbB1 to $\sim 70\%$, and increased its homocluster size to ~ 10 receptors/homocluster. In cells cultured under normal serum conditions (10% FCS), the homocluster size of ErbB1 was larger, and the monomer percentage was lower, than in starved cells. The experiments were repeated with A431 cells labeled with Mab528 against ErbB1. Because Mab528 sterically blocks the EGF binding site, the labeling of EGF-stimulated cells by Mab528 would have been unpredictable. Therefore, only cells cultured in the presence of 0.1% and 10% FCS were measured. Both the ErbB1 monomer per-

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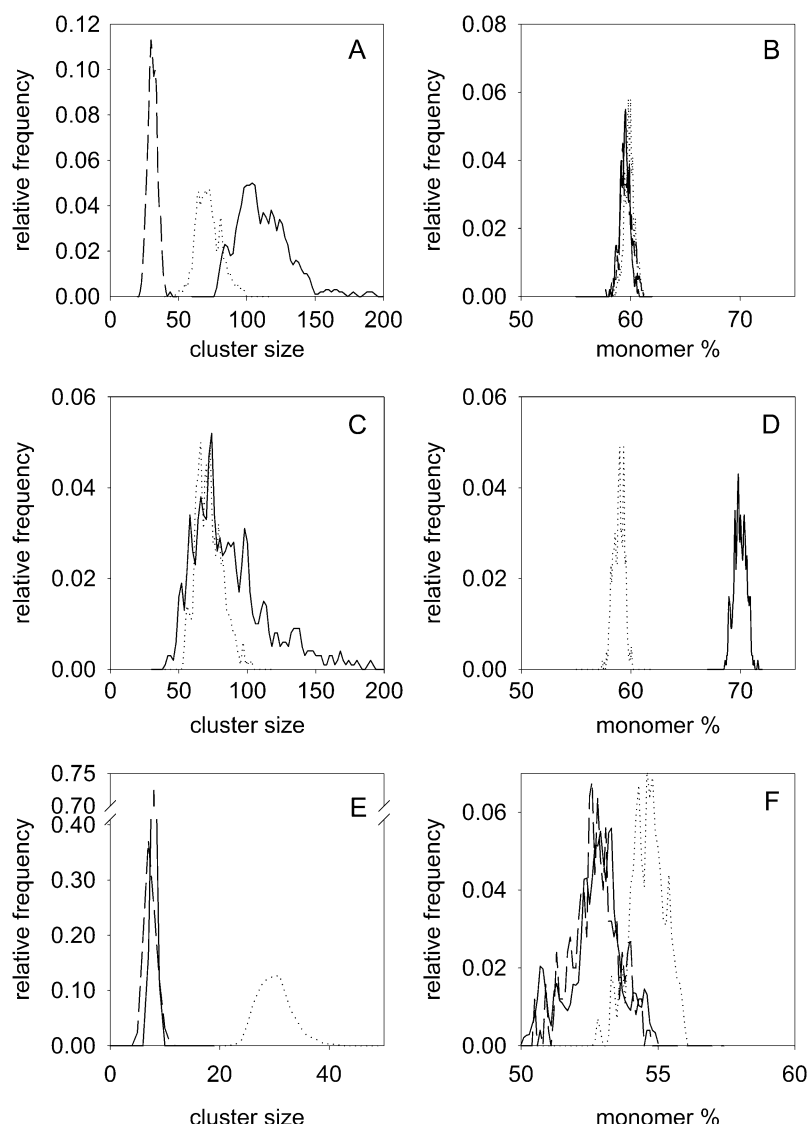


FIGURE 5 Monomer percentage and cluster size of ErbB2 in quiescent and stimulated SKBR-3 cells (A and B) SKBR-3 cells starved in the presence of 0.1% FCS for 24 h (solid line) were stimulated with heregulin (dashed line) or EGF (dotted line). The distributions of cluster size (A) and monomer percentage (B) were determined by Monte Carlo simulation, based on the anisotropy data. (C and D) Starved SKBR-3 cells were pretreated with pertuzumab (solid line) and stimulated with heregulin (dotted line). (E, F) SKBR-3 cells cultured under normal serum conditions (10% FCS, solid line) were treated with pertuzumab (dotted line) and labeled with a mixture of Alexa488-labeled and unlabeled trastuzumab IgG. SKBR-3 cells cultured in the presence of 10% FCS were also labeled with a mixture of Alexa488-labeled and unlabeled trastuzumab Fab (dashed line).

centages and the cluster sizes determined by Mab528 labeling were practically identical to those obtained by EGFR455 (cluster size, 4 and 10 in the presence of 0.1% and 10% FCS, respectively; monomer percentage, 90% and 78% in the presence of 0.1% and 10% FCS, respectively). We concluded that ErbB1 forms substantially smaller homoclusters than ErbB2, and that its cluster size increases in response to activation.

ErbB2 activation state and cluster size are inversely related to each other

Factors known to induce ErbB2 activation (EGF, heregulin, and serum) decreased its homocluster size (Fig. 5; Table 1). To analyze the correlation between ErbB2 cluster size and activation, the amount of phosphorylated and total ErbB2 were determined in parallel with its cluster size. We calculated the level of ErbB2 tyrosine phosphorylation by normalizing the intensity of the antibody against phosphorylated ErbB2 to the intensity characterizing the total amount of

ErbB2. A plot of ErbB2 tyrosine phosphorylation against ErbB2 homocluster size shows an inverse correlation between the two parameters, both in starved and nonstarved SKBR-3 cells (Fig. 7 A).

DISCUSSION

The potential of widely used approaches to analyze the composition of large protein aggregates is limited. Therefore, the unique capability of homo-FRET for measuring the size of homoclusters makes it a valuable tool in cell biological research (10,27,38). The fact that the relationship between anisotropy and homocluster size is ambiguous increases the complexity of the equations and necessitates the incorporation of simplifying assumptions into the model. Here, we present a flow-cytometric method for the quantitative analysis of protein homoclustering. The model relies on the correlation between fluorescence anisotropy and the local

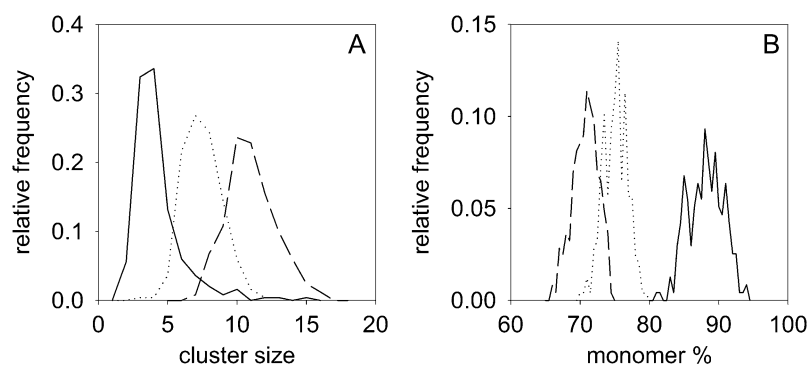


FIGURE 6 Monomer percentage and cluster size of ErbB1 in quiescent and stimulated A431 cells. A431 cells were either cultured under normal serum conditions (10% FCS, *dotted line*) or starved in the presence of 0.1% FCS for 24 h (*solid line*). Starved cells were stimulated with EGF (*dashed line*). The distributions of cluster size (A) and monomer percentage (B) were determined by Monte Carlo simulation.

density of fluorophores, and in this sense, it is similar to the fluorescence microscopic approach of Yeow and Clayton (27). We assumed that a fraction of the investigated protein species is monomeric, whereas other proteins of the same species form homoclusters with a constant size. This is obviously not true, but given the low number of data points and the measurement error achievable by flow-cytometric anisotropy measurements, this simplification was required to make fitting of the model to measurement data possible. The theoretical curves derived from the model show that the shape of the anisotropy curves and the anisotropy values at low fluorophore density (low saturation) are substantially different between different cluster sizes (Fig. 2). Therefore, the distinct cluster sizes of ErbB1 and ErbB2, and their changes, were reliably determined.

Emitted fluorescence is split into horizontally and vertically polarized components in anisotropy measurements. In addition, the method described here involves the gradual increase in concentration of the unlabeled antibody, at the expense of the labeled one, leading to a decrease in fluorescence intensity. These circumstances set a relatively high fluorescence limit for the reliable detection of cluster size by our method. Based on extrapolation of the gradual increase of

detection error at low fluorescence intensities, we estimate that the expression level of the investigated protein has to be $\sim 10^5$ /cell for accurate cluster-size determination. However, the shape of the anisotropy versus saturation curve strongly depends on the cluster size in the case of small clusters, and the curve is significantly different for large ($N > 30$) and small clusters. These observations allow us to conclude that a rough estimation of cluster size is possible even at lower protein expression levels. In addition, the accuracy of measurements at low fluorescence intensities can be increased by increasing the number of cells measured, which is easily achievable in flow cytometry. The fact that the total fluorescence intensity of a single cell is measured in flow cytometry, whereas it is distributed to several pixels in microscopy, increases the signal/noise ratio in flow cytometry compared with microscopy. This circumstance makes the measurement of dim samples more accurate in flow cytometry. However, one must not ignore the fact that subcellular resolution is not possible in flow-cytometric measurements.

Labeling of cells was usually performed using bivalent antibodies. To minimize the possibility that antibodies cross-link their target, labeling was performed on ice. Although antibodies are expected to bind monovalently in the saturation

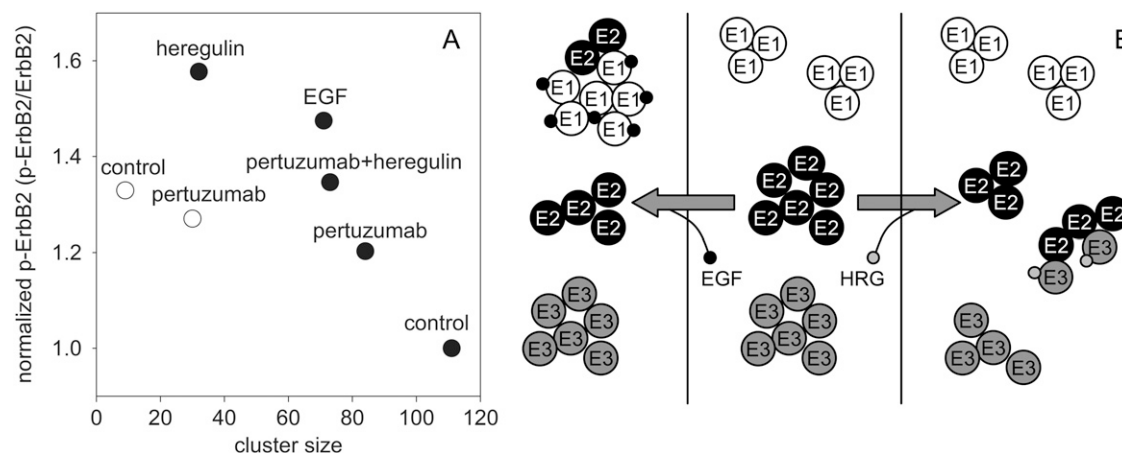


FIGURE 7 Relationship between the homocustering of ErbB proteins and the tyrosine phosphorylation of ErbB2. (A) The normalized tyrosine phosphorylation level of ErbB2 in starved (●) and nonstarved (○) SKBR-3 cells was determined under different conditions, and plotted against the cluster size of ErbB2. (B) The clustering of ErbB1 (E1), ErbB2 (E2), and ErbB3 (E3) in quiescent (*middle*), EGF-stimulated (*left*), and heregulin (HRG)-stimulated (*right*) cells.

concentration range used throughout the study, we performed control experiments in which ErbB2 clusters in SKBR-3 cells labeled with trastuzumab IgG or Fab were compared. The curves obtained using the two labeling protocols showed remarkable overlap, indicating that labeling with bivalent IgG did not alter the clustering properties of membrane proteins under the labeling conditions used in the experiments.

The anisotropy of the initially excited fluorophore (r_1) had to be determined for the model calculations. This anisotropy (r_1) corresponds to the anisotropy of a single, isolated, fluorophore-tagged antibody, which is decreased by homo-FRET between antibodies bound to different proteins in the cluster. We assumed that the limiting anisotropy of the antibody-bound fluorophore determined by Perrin plots is a good approximation of r_1 (Fig. 4). The data could be accurately fitted by a linear equation, indicating that the limiting anisotropies of the antibody-bound fluorophore could be reliably determined. The anisotropy of a single, isolated, fluorophore-tagged antibody is smaller than the limiting anisotropy of the fluorophore because of fast hindered rotations. These rotations are most likely missed by the Perrin plots, because they are expected to cause a curvature of the leftmost part of the graph, corresponding to very short lifetimes (FRET efficiencies close to 100%), which were not represented on the plots. Therefore, the determined r_1 values are smaller than the canonical limiting anisotropy of the fluorophore. However, the anisotropy of a single fluorescent antibody (emitting fluorescence depolarized by these fast rotations), and not the canonical limiting anisotropy, is needed for Eq. 7; therefore, the fact that r_1 most likely underestimates r_0 is actually a benefit. We note that homo-FRET taking place between fluorophores bound to the same antibody can be neglected, because the number of fluorophore/antibody was ~ 1 . We conclude that the anisotropy of the initially excited fluorophore needed as an input parameter for Eq. 7 could be accurately measured.

We found in unstimulated cells that homoclusters of ErbB2 are larger than those of ErbB1, and a lower fraction of ErbB2 is monomeric than of ErbB1. These results imply that ErbB2 has a higher homoclustering tendency than ErbB1, in accordance with previous findings obtained by classical biochemical (7,39) and FRET methods (9,40). Two different anti-ErbB1 antibodies yielded identical results within experimental error, giving credence to our conclusion. Stimulation of A431 cells with EGF or serum induced an increase in ErbB1 homocluster size and a decrease in the percentage of monomers (Fig. 6). Although textbook dogma supported by crystallographic data suggests that ErbB1 undergoes a monomer-dimer transition upon ligand binding (2), several studies using FRET, correlation spectroscopic, and single molecule techniques reported higher-order ErbB1 oligomers in unstimulated cells, and a ligand-induced increase in ErbB1 cluster size (13–16). The cluster sizes of 4 and 11 that we found in quiescent and stimulated cells, respectively, are in agreement with what was reported previously.

Ligand-induced changes in ErbB1 cluster size always have to be interpreted in the context of the expression levels of all ErbB proteins. Because ErbB2 is the preferred heterodimerization partner of all other ErbB proteins (6), it competes with them for association partners. However, A431 cells express a huge excess of ErbB1 over other ErbBs (37) (also according to our unpublished observations). Therefore, the ligand-induced homodimerization and homoclustering of ErbB1 are not hindered by ErbB2.

In contrast to ErbB1, ErbB2 formed huge homoclusters containing ~ 100 ErbB2 proteins in unstimulated cells, which became smaller upon stimulation with serum, EGF, or heregulin (Fig. 5).

Very large clusters of ErbB2 containing 50 to 80 receptors were observed by immunoelectron microscopy (41). Because serum, EGF, and heregulin stimulation leads to ErbB2 activation, the above results imply that activated ErbB2 forms smaller homoclusters. This assumption is corroborated by the negative correlation between the level of ErbB2 tyrosine phosphorylation and its cluster size (Fig. 7 A). Because ErbB2 is a ligandless coreceptor (6), its ligand-induced activation can only be achieved by heterodimerization with ErbB1 or ErbB3, whose ligands are EGF and heregulin, respectively, because ErbB4 is not expressed by SKBR-3 cells. The EGF-induced or heregulin-induced coclustering of ErbB2 with ErbB1 or ErbB3, respectively, were reported previously (41). We assume that ligand-binding to ErbB1 and ErbB3 recruits ErbB2 molecules from large ErbB2 homoclusters, leading to diminished ErbB2 homocluster size upon stimulation. The fact that neither EGF nor heregulin induced any change in the monomer percentage of ErbB2 needs explanation. The ErbB2 removed from large homoclusters can redistribute to smaller clusters in which several ErbB2 proteins heteroassociate with ErbB1 or ErbB3, i.e., ErbB2 will not become monomeric. Alternatively, if ErbB2 proteins expelled from large ErbB2 homoclusters form heterodimers with ErbB1 or ErbB3 (regarded as monomers by our method detecting only homoclusters), these heterocomplexes can be removed from the membrane by the low rate of endocytosis of ErbB2-containing heterodimers (42). If the rate of formation of ErbB2 heterodimers is comparable with their endocytosis rate, the fraction of ErbB2 monomers will remain constant.

Pertuzumab, an antibody sterically blocking the heterodimerization of ErbB2 (43), inhibited the heregulin-induced decrease in ErbB2 cluster size and the increase in tyrosine phosphorylation, and slightly decreased the effect of serum on tyrosine phosphorylation and cluster size of ErbB2 in SKBR-3 cells. These observations follow from the inability of ErbB2 to heterodimerize with ErbB1 or ErbB3. Consequently, ErbB2 remains in large homoclusters.

Pertuzumab slightly increased the tyrosine phosphorylation of ErbB2 in starved SKBR-3 cells, accompanied by a decrease in ErbB2 cluster size. The reciprocal change in tyrosine phosphorylation and cluster size of ErbB2 was

expected, based on the negative correlation between them. However, the increased state of activation of ErbB2 was unexpected. Our previous unpublished results from hetero-FRET experiments showed that pertuzumab slightly blocks ErbB2 homoassociation. In starved cells, the majority of ErbB2 is inactive and present in large homoclusters, which are slightly disrupted by pertuzumab. Assuming that large ErbB2 homoclusters harbor inactive proteins, the ErbB2 expelled from these homoclusters will heterodimerize with other membrane proteins, somehow leading to its heteroactivation.

Although the higher-order oligomers of ErbB1 and ErbB2 described here were found in cells overexpressing these proteins, previous experimental evidence strongly indicates that the formation of such large-scale clusters (i.e., oligomers larger than dimers) is an inherent property of membrane proteins (15,19,41,44,45). We believe that proteins with both low and high expression levels form large-scale clusters, but the number of proteins/cluster and the cluster diameter are influenced by the expression level.

The model in Fig. 7 *B* integrates all these findings. The ErbB2 homoclusters are assumed to be depots of inactive proteins, which can be recruited by ligand-activated ErbB1 or ErbB3 to heterodimeric complexes, explaining the observed decrease in ErbB2 homocluster size upon activation. Because ErbB1 is a full-featured receptor tyrosine kinase with ligand-binding and tyrosine kinase activities, EGF not only induces ErbB1-ErbB2 heterodimers, but also ErbB1 homodimers, explaining the observed increase in ErbB1 homocluster size upon EGF stimulation. However, neither ErbB2 nor ErbB3 is a full-featured receptor tyrosine kinase, because the former has no ligand-binding activity, whereas the latter has no kinase activity. Therefore, they were likened to “the deaf and the dumb” (46). It was shown that unliganded ErbB3 forms homoclusters, which are disassembled by heregulin (18). Integrating this observation with ours, we can envisage that both ErbB2 and ErbB3 form large, inactive, and separate homoclusters in unstimulated cells. Heregulin stimulation removes ErbB3 molecules from ErbB3 homoclusters. Ligand-activated ErbB3 recruits ErbB2 proteins from inactive ErbB2 homoclusters to ErbB2-ErbB3 heterodimers. The fact that heregulin stimulation leads to the exclusive formation of ErbB2-ErbB3 heterodimers, whereas EGF induces both ErbB1 homodimers and ErbB1-ErbB2 heterodimers, explains why heregulin causes a much larger decrease in the size of ErbB2 homoclusters. Flow-cytometric homo-FRET measurements have the potential to shed light on the largely unrecognized role of higher-order oligomers in transmembrane signaling, which will significantly advance our understanding of the fine interactions governing the first steps of the activation cascade of receptor tyrosine kinases.

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Correction

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2008. Quantitative characterization of the large-scale association of ErbB1 and ErbB2 by flow cytometric homo-FRET measurements. *Biophys. J.* 95:2086–2096.

The interpretation of variable d in Eq. 5 appeared incorrectly in the article. d is correctly R_0/R , i.e. the reciprocal of the distance (normalized to R_0) between the fluorophores.

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Coclustering of ErbB1 and ErbB2 Revealed by FRET-Sensitized Acceptor Bleaching

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ABSTRACT Classical theory states that ligand binding induces the dimerization of ErbB proteins, leading to their activation. Although we and other investigators have shown the existence of preformed homoclusters of ErbB receptors and analyzed their composition, the stoichiometry of their heteroclusters has not been quantitatively described. Here, we report the development of the fluorescence resonance energy transfer (FRET)-sensitized acceptor bleaching (FSAB) technique to quantitate the ratio of ErbB1 and ErbB2 in their heteroclusters. In FSAB, photolabile acceptors within FRET distance from photostable donors are excited and photobleached by FRET, and the fraction of acceptors that are participating in FRET is determined. In quiescent SKBR-3 breast cancer cells, ~35% of ErbB1 and ~10% of ErbB2 have been found in heteroclusters. Epidermal growth factor (ligand of ErbB1) increased the fraction of ErbB2 heteroclustering with ErbB1, whereas the ratio of heteroclustered ErbB1 did not change significantly. The fractions of heteroclustered ErbB1 and ErbB2 were independent of their expression levels, indicating that the formation of these clusters is not driven by the law of mass action. In contrast, the FRET efficiency depended on the donor/acceptor ratio as expected. We present a model in which preformed receptor clusters are rearranged upon ligand stimulation, and report that the composition of these clusters can be quantitatively described by the FSAB technique.

INTRODUCTION

The fact that the association of cell surface receptors and their activation are linked was established a long time ago, and it has become a paradigm of receptor biology (1). ErbB proteins are the best characterized of the receptor tyrosine kinases (RTK), but most published results describe their associations in a qualitative way. Four ErbB proteins (ErbB1–4) have been characterized and shown to form an extensive network of homo- and heteroassociations (2). Except for ErbB2, the extracellular domains of ErbB proteins are thought to be in a tethered, inactive conformation that is rearranged upon ligand binding, leading to exposure of the dimerization arm. These events culminate in the formation of ligand-induced homo- and heterodimers and receptor activation (1,3). Dimerization of the intracellular kinase domain is directly involved in its activation, leading to the phosphorylation of several tyrosine residues in the C-terminus and recruitment of SH2 domain-containing proteins (2–4). ErbB2 is at the heart of the association pattern because its extracellular domain constantly assumes a conformation that is capable of forming heterodimers (5). The signaling potency of such heterodimers is significantly enhanced compared to that of homodimers (6). Although information derived from crystal structures of the ErbB2 extracellular domain does not support the formation of ErbB2 homodimers, the existence of overexpression-driven, ligand independent ErbB2 homoassociations is supported by strong experimental evidence (7).

Molecular clusters can be characterized by a multitude of techniques, including crystallography, several molecular biological approaches, the proximity ligation assay, and the VeraTag assay (8,9). Because of its quantitative nature and relative ease of implementation, fluorescence resonance energy transfer (FRET) has become the technique of choice in quantitative studies of receptor clustering (10–12). In hetero-FRET, an excited donor passes its energy to a spectroscopically different acceptor. The fact that the energy transfer process in hetero-FRET is unidirectional limits its sensitivity for distinguishing between clusters with more than two subunits. Although dimers are unquestionably the best-characterized type of receptor clusters, they are obviously not the only kind. Tetramers and even larger clusters of ErbB1 have been detected by fluorescence correlation spectroscopy (13,14). Very large clusters of ErbB2 and ErbB3 involving tens or hundreds of proteins on a scale of tens or hundreds of nanometers have been detected by electron (15) and near-field optical microscopy (16). Homo-FRET involving the interaction between spectroscopically identical fluorophores can also be used to detect the formation of large-scale homoclusters (17). Using flow cytometric homo-FRET measurements, we have shown that the number of monomers in an ErbB1 homocluster increases from ~4 to ~10 upon epidermal growth factor (EGF) stimulation. ErbB2 was found to behave in the opposite way, in that it formed extensive, large-scale clusters (~50–100 proteins/cluster) in the absence of stimulation, which decreased in size after treatment with EGF or neuregulin (18). Although the stoichiometry of large-scale heteroclusters could not be measured, we hypothesized that ligand-bound ErbB1 and ErbB3 recruits ErbB2 to heterodimers, leading to the

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observed decrease in the cluster size of ErbB2. By using number and brightness (N&B) measurements and two-color fluorescence correlation, investigators may be able to determine the stoichiometry of heteroclusters by measuring the comobility of different receptors (19–21).

In this study, we developed and used a technique originally proposed by Mekler et al. (22,23) to characterize the composition of heteroclusters of ErbB1 and ErbB2 in a quantitative way. Briefly, FRET from a photostable donor to a photolabile acceptor induces acceptor photobleaching. Optimally, only acceptors within FRET distance from donors will undergo photobleaching. Using this approach, which we call FRET-sensitized acceptor bleaching (FSAB), the fraction of acceptors within FRET distance from donors can be quantitatively determined. We demonstrate that FSAB can principally be used to determine the fraction of acceptors within FRET distance of donors by showing that the efficiency of FRET drops to zero long before all acceptor molecules are bleached, using Alexa-Fluor546-Cy5 as a donor-acceptor pair. In quiescent SKBR-3 breast tumor cells, only ~10% of ErbB2 heteroassociates with ErbB1, and this fraction is doubled by EGF stimulation. Although a higher fraction of ErbB1 forms heteroclusters with ErbB2, this fraction is not significantly changed by EGF treatment. The FSAB technique is suitable for measuring the heteroclustering of proteins in a quantitative way, and has the potential to provide new insight into the behavior of receptor tyrosine kinases upon activation.

MATERIALS AND METHODS

Cells and reagents

SKBR-3 breast cancer and A431 epithelial carcinoma cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and grown according to their specifications. For microscopic experiments, cells were grown in two- or eight-well chambered coverglasses (Nalge Nunc International, Rochester, NY). ErbB1 and ErbB2 were labeled by Mab528 and trastuzumab, respectively. Mab528 was purified from the supernatant of the HB-8509 hybridoma cell line (ATCC) by protein A affinity chromatography. Trastuzumab (Herceptin) was purchased from Roche Ltd. (Budapest, Hungary). Conjugation of antibodies with AlexaFluor (Molecular Probes, Eugene, OR), Cy5 (GE HealthCare, Freiburg, Germany), or Qdot605 (Invitrogen, Karlsruhe, Germany) was carried out according to the manufacturers' specifications. The number of fluorophores on a single antibody was determined by spectrophotometry and was always kept between 1–2 to reduce the possibility of interactions between neighboring Cy5 molecules (24). EGF and CBr₄ were purchased from R&D Systems (Minneapolis, MN) and Sigma-Aldrich (Schnelldorf, Germany), respectively.

Stimulation and labeling of cells

SKBR-3 cells grown in chambered coverglass were starved in medium containing 0.1% fetal calf serum for 24 h before experiments were conducted, and stimulated by 100 nM EGF in Hank's buffer supplemented with 1 mg/mL bovine serum albumin for 15 min at 37°C. Control and stimulated cells were labeled by a saturating concentration (10–20 µg/mL) of fluorescent Mab528 and/or trastuzumab in Hank's buffer containing 1 mg/mL

bovine serum albumin for 30 min on ice. Unbound antibodies were removed by washing twice in phosphate-buffered saline, and cells were fixed in 1% formaldehyde.

Confocal microscopy and photobleaching

A Zeiss LSM 510 confocal laser scanning microscope (Carl Zeiss AG, Jena, Germany) was used to image the samples. AlexaFluor546 was excited at 543 nm and its emission was detected at 560–615 nm. The fluorescence of Cy5 was excited at 633 nm and detected above 650 nm. When FRET between Alexa546 and Cy5 was measured, a third fluorescence image (FRET channel), excited at 543 nm and recorded above 650 nm, was also measured in addition to the donor (AlexaFluor546) and acceptor (Cy5) channels. Qdot605 was excited at 488 nm and its emission was recorded at 585–615 nm. Fluorescence images were taken as single optical sections using a 63x (NA = 1.4) oil immersion objective focused to the middle of the cell along the Z axis. The pinhole size was adjusted to 2 Airy units. The image size was 512×512 pixels, and the pixel size in the X and Y directions was 400 nm. To induce FRET-sensitized bleaching of Cy5, the sample was illuminated at 543 nm (bleaching beam). Bleaching illumination was interrupted approximately every 30 s, and donor, FRET, and acceptor images were recorded with an attenuated laser beam. The power of the 543 nm laser line was set to 5% and 100% for the imaging and bleaching illuminations, respectively. Photobleaching was carried out in the presence of 8×10^{-4} M CBr₄.

Image analysis

Image processing was carried out with the DipImage toolbox (Delft University of Technology, Delft, The Netherlands) under MATLAB (The MathWorks Inc., Natick, MA). Segmentation of images into membrane and nonmembrane pixels was carried out with the manually seeded watershed algorithm using a custom-written MATLAB program (25,26). Image stacks acquired during photobleaching were corrected for shift using the *correctshift* command of DipImage. FRET efficiency, unquenched donor and direct acceptor intensities corrected for spectral overspill were calculated in the photobleaching stack in the membrane of selected cells double-labeled by donor- and acceptor-tagged antibody as described previously (27), and the fraction of bleached acceptors ($F_{bleached}$ in Eq. 5) was determined at the time when the FRET efficiency dropped to zero. The fraction of directly bleached acceptors excited at the excitation wavelength of the donor (BCF in Eq. 5) was determined using a sample labeled with acceptor only.

To calibrate the donor and acceptor fluorescence intensities in terms of the number of molecules, two samples of SKBR-3 cells were separately labeled by either the donor- or acceptor-tagged antibody against the same epitope. The means of the background-corrected fluorescence intensities in the membrane of ~100 cells were determined separately in the donor-only and acceptor-only labeled samples ($\langle I_d \rangle$ and $\langle I_a \rangle$, respectively), and corrected for labeling ratio, i.e., the number of fluorophores per antibody. Since the cells in the two samples contained on average the same number of antibodies per cell, the ratio of the mean fluorescence intensities is the ratio of the fluorescence intensities generated by the same number of fluorophores in the donor and acceptor channels:

$$R_{a/d} = \frac{\langle I_a \rangle L_d}{\langle I_d \rangle L_a} \quad (1)$$

where L_d and L_a are the number of fluorophores on the donor- and acceptor-labeled antibodies, respectively. The fluorescence intensity in the donor image of double-labeled cells was multiplied by $R_{a/d}$ to ensure that the donor and acceptor fluorescence intensities would be on the same scale. To calibrate these fluorescence intensities in terms of receptor numbers, the number of binding sites of the respective antibody was determined by flow cytometry using Qifikit (DAKO, Hamburg, Germany). Briefly, a series of beads with calibrated numbers of bound primary mouse monoclonal antibodies

were labeled with a fluorescent anti-mouse IgG. After correcting for the fluorescence intensity of blank beads, a calibration curve was constructed from which the slope and y-intercept were determined. The cells were labeled by primary mouse monoclonal antibodies against the antigen of interest, followed by secondary labeling by the fluorescent secondary antibody used to label the beads, and flow cytometric determination of their fluorescence intensity. After background correction was performed, the number of antigens on the investigated cell line was determined by linear regression.

The number of molecules was then calculated for the microscopic images according to the following equation:

$$N_i = \frac{I_i}{\langle I \rangle} N_{Qifit} \quad (2)$$

where N_i and N_{Qifit} are the number of molecules in a given cell or pixel and the mean number of molecules in the population of cells determined by Qifit, respectively, and I_i and $\langle I \rangle$ are the fluorescence intensity in a given cell or pixel and the mean fluorescence intensity of the population of cells corrected by R_{ads} , respectively.

THEORY

Determination of the fraction of bound acceptors

Given a photostable FRET donor and an acceptor that is sensitive to a photochemical reaction, one can determine the FRET efficiency according to the enhanced rate of the photochemical reaction of the acceptor excited at the wavelength of the donor (22). Mekler et al. (23) claimed that even small FRET efficiencies can be accurately determined using this approach, which explains why the technique is called photochemical enhancement of sensitivity (PES). If the photochemical reaction of the acceptor is irreversible photobleaching, then FRET results in the accelerated rate of acceptor photobleaching. To emphasize that our approach is based on the enhanced rate of acceptor photobleaching as a result of FRET, we coined the term “FRET-sensitized acceptor bleaching” (FSAB). Since acceptors that take part in FRET are preferentially bleached, it is possible to calculate the relative contribution of the bleached acceptor subpopulation to the total FRET efficiency by analyzing the relationship between the decays of FRET efficiency and sensitized acceptor emission (23). We were interested in the theoretical endpoint of FSAB, i.e., the point at which all acceptors within FRET distance from donors are bleached. We assumed that two classes of acceptors exist with regard to their association with donors: 1) acceptors bound to donors, i.e., within FRET distance from donors (A_{bound}); and 2) free acceptors (A_{free}). When all of the bound acceptors are bleached, the FRET efficiency has to drop to zero. By comparing the acceptor intensity (proportional to the number of acceptors) at the time point when FRET drops to zero with the total acceptor intensity before the FSAB process is undertaken, one can determine the fraction of bound acceptors (A_{bound}/A_0). Because acceptors are also excited directly at the wavelength of the donor, some free acceptor molecules outside the range of FRET are also bleached. The bleached fraction of A_{free} (at the time of complete bleaching of A_{bound} , i.e., when FRET decreases to zero) is designated the bleaching correction factor (BCF). Therefore, the fraction of bleached

acceptors ($F_{bleached}$) and A_{bound} can be calculated according to the following equations:

$$F_{bleached} = \frac{A_{free} BCF + A_{bound}}{A_{free} + A_{bound}} \quad (3)$$

$$A_{bound} + A_{free} = A_0 \quad (4)$$

Solving for A_{bound} yields

$$A_{bound} = A_0 \frac{F_{bleached} - BCF}{1 - BCF} \quad (5)$$

Since BCF was found to vary between different antibodies, it had to be determined for every individual labeled batch of any acceptor-conjugated antibody used in the FSAB experiments. To reduce the effects of the possible intensity dependence of Cy5 photobleaching arising from the interactions between neighboring Cy5 molecules (24), BCF was determined on cells with fluorescence intensities similar to those of double-labeled cells used for the FSAB experiments. It is assumed in Eqs. 3–5 that 100% of the bound acceptor population is bleached. However, as discussed later, the bleaching of Cy5, the acceptor chosen for FSAB measurements, may not always be complete. If the bleached fraction of the bound acceptor population is designated by BCF_{FRET} , the fraction of bound acceptors can be calculated according to the following equation:

$$F_{bleached} = \frac{A_{free} BCF + A_{bound} BCF_{FRET}}{A_{free} + A_{bound}} \Rightarrow$$

$$A_{bound} = A_0 \frac{F_{bleached} - BCF}{BCF_{FRET} - BCF} \quad (6)$$

BCF_{FRET} can be determined with a sample labeled with Cy5-conjugated primary antibody followed by labeling with an AlexaFluor546-tagged secondary antibody.

RESULTS

Selection of an appropriate donor-acceptor pair for FSAB experiments

The prerequisite for successful application of the FSAB technique is a donor-acceptor FRET pair in which the donor is photostable and the acceptor is photolabile. Although Cy5 is known to be relatively photolabile in confocal microscopy, the time required for its complete bleaching is prohibitively long. In accordance with the results of Mekler et al. (22,23), we found that CBr_4 significantly accelerated the rate of photobleaching of Cy5 (see Fig. S1 in the Supporting Material). A donor that would be photostable in the presence of CBr_4 had to be found. Quantum dots were obvious candidates because of their known photostability, but CBr_4 significantly enhanced the rate of their photobleaching (Fig. S1).

AlexaFluor546, on the other hand, was photostable in both the absence and presence of CBR_4 , and therefore we used it as a donor for Cy5 in the FSAB experiments.

We measured the kinetics of Cy5 photobleaching at 633 nm (the excitation wavelength for Cy5) and at 543 nm (the excitation wavelength for AlexaFluor546). Photobleaching of Cy5 at 543 nm was incomplete, i.e., a residual fluorescence intensity amounting to 20–40% of the initial intensity was resistant to bleaching when the intensity was measured at 543 or 633 nm (Fig. S2 A). On the other hand, the photobleaching of Cy5 at 633 nm was complete independent of whether the images were recorded at an excitation wavelength of 543 or 633 nm (Fig. S2 A).

Proof of concept: the FSAB technique reliably detects receptor clusters

We first established theoretically the reliability of the FSAB approach using AlexaFluor546 and Cy5 dyes. We show in the [Supporting Material](#) that although the acceptor is photobleached both directly and via the donor, the fraction of bound acceptors can be reliably determined. In addition, donor bleaching does not have a significant effect on the value for the fraction of bound acceptors calculated by FSAB (Fig. S3). Next, we tested the FSAB approach using a positive and a negative control. It was previously shown that the majority of ErbB1 is monomeric, with a minority forming small aggregates in nonstimulated cells. On the other hand, the majority of ErbB2 forms large clusters in quiescent cells (18). We compared the photobleaching kinetics of Cy5 in SKBR-3 cells labeled with a 1:1 mixture of Cy5-conjugated

and unlabeled antibodies against ErbB2 or with a 1:1 mixture of AlexaFluor546-tagged and Cy5-tagged anti-ErbB2 antibodies. Unlabeled antibodies were used to reduce the binding of the Cy5-conjugated antibody to levels comparable to those found in donor-acceptor double-labeled samples, to prevent artifacts arising from the possible influence of interactions between Cy5 molecules on the photobleaching kinetics (24). In accordance with the results shown in the previous section, Cy5 was incompletely bleached in cells labeled only with Cy5-tagged antibodies (Fig. 1 A). However, its bleaching was practically complete by the second bleaching step, when the FRET donor was present, and the FRET efficiency dropped to zero at this time point (data not shown). For quantitative evaluation of the fraction of homoclustered ErbB2, we used the fluorescence intensities measured after two bleaching steps because the FRET efficiency dropped to zero at this time point. Substitution into Eq. 5 yielded 83% for the fraction of homoclustered ErbB2 (Fig. 2 A). Since the expression level of ErbB1 is much higher in A431 cells than in SKBR-3 cells, and previous experiments by our group (18) and others (14,28) to measure the homoassociation of ErbB1 were carried out in A431 cells, we used this cell line to compare the results of our previous homo-FRET experiments with those obtained using the FSAB approach. The photobleaching kinetics of Cy5 in cells labeled with a 1:1 mixture of Cy5-conjugated and unlabeled monoclonal antibodies against ErbB1 was comparable to that measured in cells labeled with a 1:1 mixture of AlexaFluor546-tagged and Cy5-tagged anti-ErbB1 antibodies (Fig. 1 A). This observation implies that the majority of ErbB1 is nonclustered. A quantitative comparison of the two curves reveals that

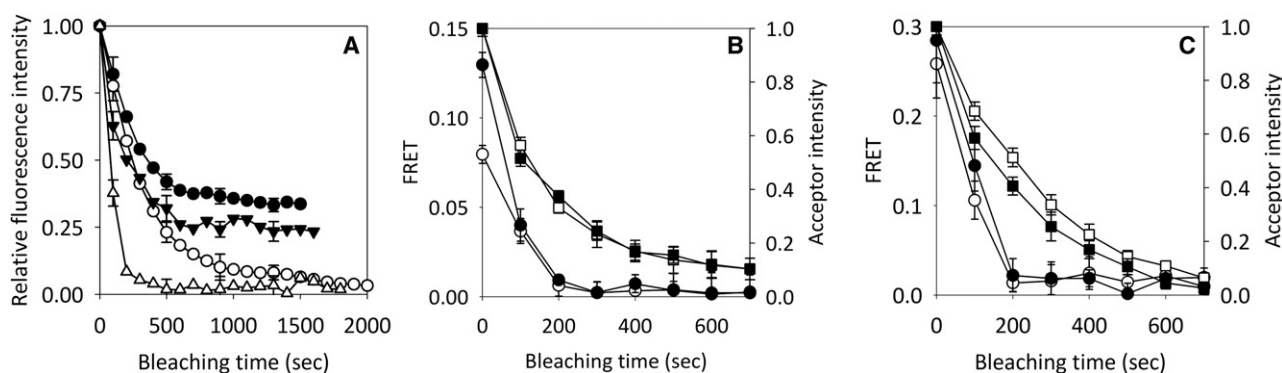


FIGURE 1 Decrease of FRET efficiency and acceptor fluorescence during FSAB. (A) Determination of the fraction of homoclustered ErbB1 and ErbB2 using FSAB. A431 cells were labeled with Cy5-Mab528 (●) or with a mixture of Cy5-Mab528 and AlexaFluor546-Mab528 (○). SKBR-3 cells were labeled with Cy5-trastuzumab (▼) or with a mixture of Cy5-trastuzumab and AlexaFluor546-trastuzumab (△), and the samples were photobleached in the presence of CBR_4 at 543 nm. The directly excited acceptor fluorescence intensities are shown in the graph. Error bars indicating the mean $\pm$ standard error (SE) are shown for every third data point for clarity. (B) Determination of the fraction of heteroclustered ErbB1 in the absence and presence of EGF stimulation. Quiescent and EGF-stimulated SKBR-3 cells were labeled with a mixture of AlexaFluor546-trastuzumab and Cy5-Mab528 against ErbB2 and ErbB1, respectively. Nonsensitized, directly excited acceptor fluorescence and the FRET efficiency were calculated by recording fluorescence images in the donor, FRET, and acceptor channels when the bleaching illumination was interrupted at the excitation wavelength of the donor (□, directly excited acceptor intensity of nonstimulated cells; ○, FRET efficiency of nonstimulated cells; ■, directly excited acceptor intensity of EGF-stimulated cells; ●, FRET efficiency of EGF-stimulated cells). Photobleaching was carried out in the presence of CBR_4 . Error bars indicate the mean $\pm$ SE of ~ 100 cells. (C) Determination of the fraction of heteroclustered ErbB2 in the absence and presence of EGF stimulation. SKBR-3 cells were labeled with a mixture of AlexaFluor546-Mab528 and Cy5-trastuzumab. Otherwise, the experimental conditions and symbol assignments are the same as in B.

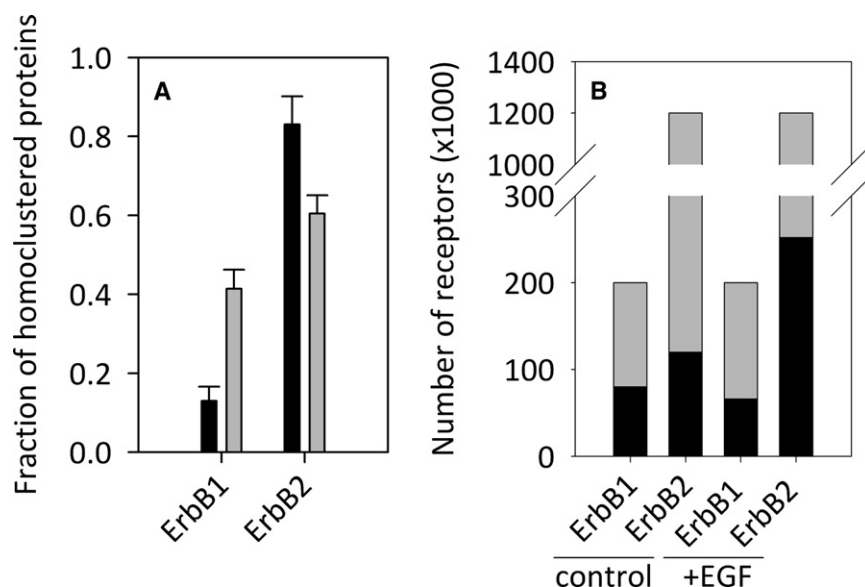


FIGURE 2 The fraction of homo- and heteroclustered ErbB1 and ErbB2 in quiescent and EGF-stimulated SKBR-3 cells determined by FSAB. (A) Determination of the fraction of homoclustered ErbB1 and ErbB2 in quiescent and stimulated SKBR-3 cells. Starved (*black columns*) and EGF-stimulated (*gray columns*) A431 cells were labeled with a mixture of Cy5-tagged and AlexaFluor546-tagged Mab528 (to measure the homoclustering of ErbB1). Starved (*black columns*) and EGF-stimulated (*gray columns*) SKBR-3 cells were labeled with Cy5-conjugated and AlexaFluor546-conjugated trastuzumab (to measure the homoclustering of ErbB2), and the fraction of homoclustered acceptor was determined using FSAB. Error bars indicate the mean $\pm$ SE of ~ 100 cells. (B) Determination of the fraction of heteroclustered ErbB1 and ErbB2 in starved and EGF-stimulated SKBR-3 cells. The fraction of ErbB1 molecules forming heteroclusters with ErbB2 was calculated by labeling SKBR-3 cells with a mixture of AlexaFluor546-trastuzumab and Cy5-Mab528 against ErbB2 and ErbB1, respectively. The fraction of ErbB2 hetero-

associating with ErbB1 was determined by labeling cells with AlexaFluor546-Mab528 and Cy5-trastuzumab. The fractions of free/homo- and heteroclustered molecules were converted to absolute numbers after determining the expression levels of ErbB1 and ErbB2. The numbers of heteroclustered and free/homo-clustered molecules are shown by the black and gray parts of the bars, respectively, in quiescent and EGF-stimulated cells. The FSAB approach only characterizes whether an acceptor is heteroassociated with a donor; therefore, a free acceptor may indeed be a free (i.e., monomeric) molecule or form homoclusters or heteroclusters with other molecules.

$\sim 13\%$ of ErbB1 forms homoclusters (Fig. 2 A). The measured fraction of homoclustered ErbB1 in EGF-stimulated A431 cells was ~ 3 -fold higher than in starved cells (Fig. 2 A). However, the fraction of **homo**clustered ErbB2 decreased upon EGF stimulation in SKBR-3 cells (Fig. 2 A). The fractions of homoclustered ErbB1 and ErbB2 in starved and EGF-stimulated cells determined by the FSAB technique are in agreement with previous results obtained by homo-FRET (18).

Since the duration of bleaching is long in FSAB experiments (~ 200 s), we tested whether protein mobility would affect the results of our experiments. As shown in Fig. S4, the lateral mobility of ErbB1 is abolished in formaldehyde-fixed cells. Therefore, we concluded that only proteins associated with each other at the time of fixation are detected by the FSAB approach, i.e., those acceptors that would transiently associate with donors (if they were free to diffuse) are not counted as bound. Since heteroclusters cannot be analyzed by homo-FRET measurements, we set out to characterize the heteroassociation of ErbB1 and ErbB2 using the FSAB approach. Since SKBR-3 cells express both ErbB1 and ErbB2 at moderate to high levels ($\sim 2 \times 10^5$ ErbB1 and $\sim 10^6$ ErbB2; values determined by Qifikit), and ErbB2 is only slightly expressed by A431 cells ($\sim 2 \times 10^6$ ErbB1 and $\sim 2 \times 10^4$ ErbB2), we chose to use SKBR-3 cells for these experiments. Cells were labeled with AlexaFluor546-tagged antibody against ErbB2 and Cy5-tagged antibody against ErbB1, and the directly excited fluorescence intensity of the acceptor and the FRET efficiency were calculated after each bleaching step (Fig. 1 B). In accordance with the afore-

mentioned results, the FRET efficiency dropped to zero after the second bleaching step, indicating that all acceptors within FRET distance of the donors were bleached (Figs. 1 B, 2 B, and 3). However, the fluorescence intensity of the acceptor was only reduced to $\sim 35\%$ of the initial value, indicating that a significant fraction of the acceptor was not within FRET distance of the donors (Figs. 1 B and 2 B). The labeling ratio (number of fluorophores per antibody) is expected to influence the initial value of FRET, but as long as the starting FRET efficiency is not too small, acceptors within FRET distance from the donors will be bleached. The extent of bleaching of donor fluorescence was much smaller than that of the acceptor or the fractional decrease in the FRET efficiency (Fig. 3). A quantitative evaluation according to Eq. 5 revealed that $\sim 40\%$ of the acceptor (ErbB1) was heteroclustered with the donor (ErbB2). To obtain reproducible results, we only analyzed cells with bright fluorescence in which FRET decreased monotonously with bleaching and could be reliably determined, and in which the fraction of bleachable acceptors in the double-labeled sample was at least 90%. We concluded that the fraction of heteroclustered proteins can be reliably determined by the FSAB technique, and started a systematic investigation of ErbB1 and ErbB2 in quiescent and stimulated cells.

Quantitative analysis of heteroclusters of ErbB1 and ErbB2 in quiescent and EGF-stimulated cells

SKBR-3 breast cancer cells were starved in the presence of 0.1% fetal calf serum for 24 h and labeled with

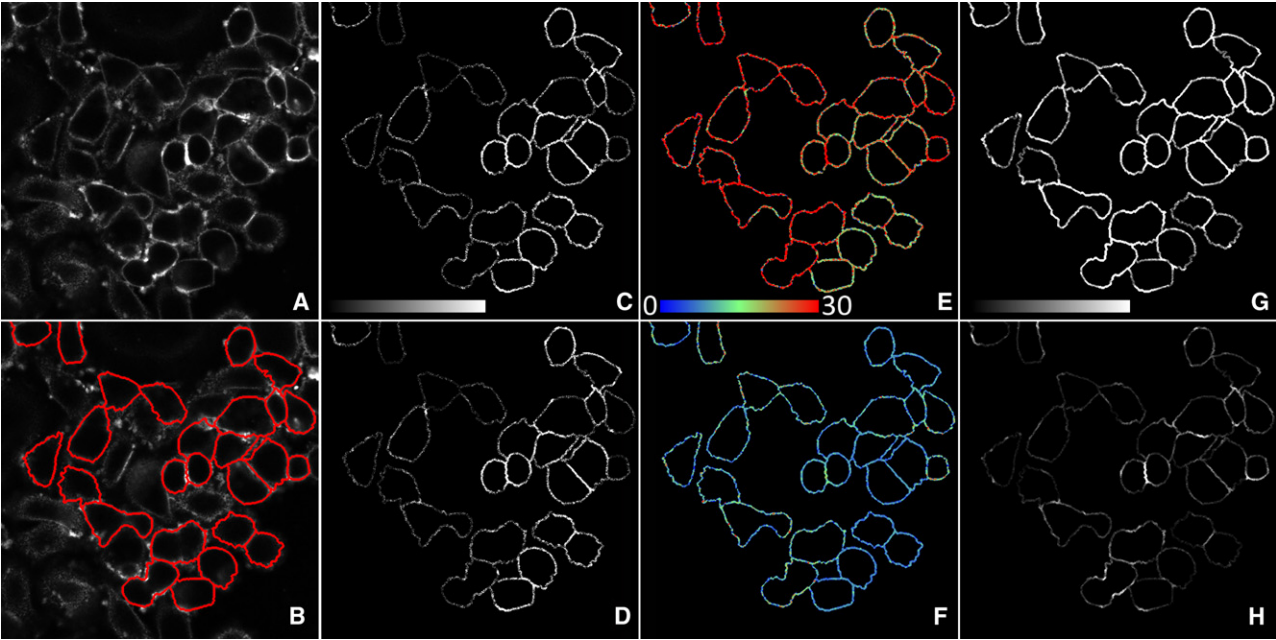


FIGURE 3 Representative images for calculating the fraction of bound acceptors using the FSAB technique. SKBR-3 cells were labeled with Alexa546-Mab528 and Cy5-trastuzumab, and the fraction of bound acceptors was analyzed by the FSAB approach. (A) The image recorded in the acceptor channel to which the manually seeded watershed segmentation algorithm was applied to segment the image into membrane and nonmembrane pixels. (B) The membrane mask (in red) overlaid on the fluorescence image shown in A. (C and D) Unquenched donor fluorescence intensity calculated for membrane pixels before the beginning of bleaching (C) and after the second bleaching step, corresponding to ~200 s of photobleaching (D). (E and F) The FRET efficiency calculated before bleaching (E) and after the second bleaching step (F). Pixels are color-coded according to the FRET efficiency, which ranges between 0 and 30%. (G and H) Nonsensitized acceptor emission calculated before bleaching (G) and after the second bleaching step (H). The fluorescence images (C, D, G, and H) were contrast-stretched, and therefore the donor (C and D) and acceptor fluorescence intensities (G and H) are not comparable.

AlexaFluor546-tagged antibody against ErbB1 and Cy5-tagged antibody against ErbB2. The fraction of ErbB2 heteroclustering with ErbB1 in these nonstimulated cells was found to be 10%. After EGF stimulation, ErbB2 was recruited into heteroclusters with ErbB1, which was reflected by an ~2-fold increase in the fraction of ErbB2 heteroassociating with ErbB1 (Table 1, Figs. 1 C and 2 B). To determine the fraction of ErbB1 that formed heteroclusters with ErbB2, we labeled the former with acceptor-tagged antibodies and the latter with donor-tagged antibodies. The fraction of heteroclustered ErbB1 in quiescent and EGF stimulated cells was 40% and 33%, respectively (Table 1, Figs. 1 B and 2 B). The absolute number of free and bound (i.e., clustered) ErbB1 and ErbB2 was also calculated using the expression

levels of the proteins determined by flow cytometry (Table 1 and Table S1). The term “free acceptor” means that the acceptor is not heteroassociated with the donor, but they may form homoclusters or associate with other molecules in the membrane.

The fraction of heterocluster-forming ErbB2 was also analyzed on a cell-by-cell basis, and was found to be independent of the expression levels of ErbB1 and ErbB2 (Fig. S5 A). To the contrary, the FRET efficiency was proportional to the acceptor/donor ratio (Fig. S5 B). If free and bound ErbB2 were in equilibrium according to the law of mass action, the fraction of heteroassociating ErbB2 would depend on the expression levels of ErbB1 and ErbB2 (Fig. S5 C). The fact that the fraction of bound ErbB2 was

TABLE 1 The numbers and fractions of free and heteroclustered ErbB1 and ErbB2 in control and EGF-stimulated SKBR-3 cells

		Quiescent ($\times 10^3$)	EGF stimulation ($\times 10^3$)
ErbB1	Free or homoclustered	120 $\pm$ 8 (60 $\pm$ 4%)	134 $\pm$ 10 (67 $\pm$ 5%)
	in complex with ErbB2	80 $\pm$ 8 (40 $\pm$ 4%)	66 $\pm$ 10 (33 $\pm$ 5%)
ErbB2	Free or homoclustered	1080 $\pm$ 24 (90 $\pm$ 2%)	948 $\pm$ 36 (79 $\pm$ 3%)
	in complex with ErbB1	120 $\pm$ 24 (10 $\pm$ 2%)	252 $\pm$ 36 (21 $\pm$ 3%)

The numbers and fractions of free (or homoclustered) and heteroclustered proteins were determined by FSAB as described in the legend to Fig. 2 B. The standard errors of the percentage of heteroclustered molecules determined from ~100 cells in three independent experiments are also shown. These errors are equal to those of free/homoclustered molecules since the latter was calculated by subtracting the percentage of heteroclustered molecules from 100%.

independent of the expression levels of ErbB1 and ErbB2 implies that the formation of these heteroclusters does not follow the law of mass action.

DISCUSSION

In this work, we report the successful implementation of the FSAB technique to measure the heteroclustering of membrane proteins in a quantitative way. The approach is based on preferential, FRET-induced bleaching of photolabile acceptors in the molecular proximity of photostable donors. Although both donor bleaching and direct bleaching of the acceptor take place, they do not compromise the reliability of the approach, and we concluded that AlexaFluor546 and Cy5 are a suitable donor-acceptor pair, since bound acceptors are indeed preferentially bleached (Fig. S3). The photophysics of Cy5 is complicated and involves *cis-trans* isomerization, reversible photoinduced transition between dark and bright states, triplet and higher excited states, and interaction between neighboring fluorophores (24,29–31). In the absence of oxygen and CBr_4 , Cy5 emits 100,000–200,000 photons before irreversible photobleaching occurs (30,32). The presence of oxygen, the short lifetime and large molar absorption coefficient, and the consequent rapid cycling between the ground and excited states deteriorates the photostability of Cy5 in confocal microscopy (33). CBr_4 substantially decreases the photostability of Cy5 by photoinduced electron transfer (22) without significantly accelerating the rate of AlexaFluor546 bleaching (Fig. S1). Because of their photostability, quantum dots would have been the best choice for the photostable donor, but the rate of their bleaching was significantly increased by CBr_4 (Fig. S1). We also considered AlexaFluor555 as a potential donor for Cy5 in the FSAB experiments. Based on its photobleaching quantum yield, AlexaFluor555 is ~10 times more photostable than AlexaFluor546 (34). In certain cases, AlexaFluor555 may be a suitable dye for FSAB experiments, but its low fluorescence quantum yield and the small R_0 for the Alexa-Fluor555-Cy5 pair ($R_{0,\text{A555-Cy5}} = 4.9$ nm, $R_{0,\text{A546-Cy5}} = 6.8$ nm) limit its applicability.

Transient, photoinduced dark states of Cy5 can invalidate the interpretation of FRET experiments involving Cy5 as an acceptor (31). However, the bleaching curves of Cy5 reported here represent irreversible photobleaching, since illumination at 543 nm did not recover the fluorescence intensity (data not shown). Contrary to the findings of Eggeling et al. (29), who reported accelerated photobleaching of Cy5 with excitation in the short-wavelength range of the absorption spectrum, we observed partial bleaching of Cy5 with illumination at 543 nm (Fig. S2 A). We do not know the reason for this observation. The ratios of fluorescence intensities excited at 543 and 633 nm were identical before and after photobleaching at 543 nm. In addition, the emission spectra of Cy5 bleached at 543 nm and that of unbleached Cy5 were identical (Fig. S2 B). These findings exclude the possibility that molecules that differed in their ground-state excitation

spectrum led to the observation. Either the photobleaching illumination at 543 nm reacts differently compared to the 633 nm light with the homogeneous population of molecules (and does not bleach them completely), or the subpopulation of molecules that display different photosensitivities at 543 and 633 nm have identical excitation spectra. Interaction between Cy5 molecules conjugated to the same IgG is unlikely to be behind the observations, since such interactions were reported to take place only if the number of Cy5 labels per IgG is $>2-3$ (24). Heterogeneous photobleaching of Cy5 was detected previously and assumed to be caused by heterogeneity of Cy5 generated during protein labeling or by impurities in the dye (30). Although photobleaching of Cy5 carried out at 543 nm was partial, FRET-induced bleaching of Cy5 was complete (Fig. 1). The reason for this finding is also obscure. If the FRET-induced bleaching had been incomplete, it should have been taken into consideration by Eq. 6.

Heteroclusters can be analyzed by several biophysical methods. Fluorescence correlation spectroscopy has been applied in confocal microscopy to study the dynamics of molecules (35). Fluorescence cross-correlation spectroscopy (19,36), cross-correlation raster image spectroscopy (21), and two-color N&B analysis (20) report the stoichiometry of stable molecular heteroassociations. Although homo-FRET is sensitive to the size of protein clusters, it can only be used for the analysis of homoassociations (17,18). Although conventional hetero-FRET measurements do not usually provide information about the stoichiometry of clusters or the fraction of clustered molecules, several FRET-based methods have been put forward to obtain these kinds of information. A systematic analysis of the dependence of FRET efficiency on the local density of acceptors allowed investigators to determine an upper bound for the percentage of clustered GPI-anchored proteins (37). A lower-bound value for the fraction of clustered donor molecules was obtained by comparing the photobleaching kinetics of the donor in the absence and presence of an acceptor (28,38). Dual measurements of the kinetics of donor bleaching in the donor and FRET channels enabled Clayton et al. (38) to calculate the fraction of donors undergoing FRET. Hoppe et al. (39) reported that the stoichiometry of CFP-citrine complexes can be determined provided that the FRET efficiency of a bound donor-acceptor pair is known. FSAB presents an alternative method for determining the fraction of heteroclustered acceptors without requiring sophisticated technologies or complex image processing.

The methods used to characterize molecular associations have different sensitivities for detecting clusters that differ in size and stability (40). Since the FSAB approach is based on hetero-FRET, it has all the inherent advantages and limitations of the hetero-FRET principle. Alterations in FRET efficiency can be caused by changes in the orientation or distance between the donor and the acceptor (27). Due to the flexibility of the chemical linkage between the

antibodies and the fluorophores, dynamic averaging of the orientation factor takes place, eliminating the dependence of FRET on orientation. It was also recently shown that although fixation is expected to restrict the rotational mobility of proteins, intermolecular FRET efficiencies are not significantly affected by fixation (41). The sensitivity of the FSAB approach for detecting clusters with different sizes and stabilities also has to be discussed. Steady-state FRET measurements cannot discriminate between dimers (two molecules bound to each other by specific molecular interactions) and the proximity of two molecules as a result of accidental apposition (random association due to high density) or partitioning into the same microdomain (co-confinement). Such discrimination would require an analysis of the dependence of FRET on the donor/acceptor ratio or the acceptor density (42). Consequently, FSAB measurements simply reveal the fraction of acceptors within FRET distance of the donors. In addition to dimers and small-scale clusters, aggregation of proteins on a much larger scale has been identified (16,18). Some acceptors in such clusters may be beyond the FRET distance from donors, especially if they outnumber the donor-labeled protein. Although these large-scale clusters are dynamic (43), because of the restricted lateral mobility of proteins in fixed cells, these distant acceptors are not reached by donors. Consequently, the contribution of large-scale clusters to the fraction of bleached acceptors is underestimated in FSAB measurements.

We applied the FSAB technique to investigate heteroclustering of ErbB1 and ErbB2. Almost half of ErbB1 is heteroclustered with ErbB2 in quiescent cells, whereas only ~10% of ErbB2 is in heteroclusters with ErbB1 (Table 1 and Table S1). The fact that the majority of ErbB2 is not heteroclustered with ErbB1 reflects the much greater numbers of ErbB2 expressed by SKBR-3 cells and the strong tendency of ErbB2 to form homoclusters (Fig. 4 B) (18). After EGF treatment, the fraction of heteroclustered ErbB1 did not change significantly, because EGF induces the formation of both ErbB1 homodimers and ErbB1-2 heterodimers. ErbB2 behaved in a different way. EGF induced an increase in the fraction of ErbB2 in heteroclusters with ErbB1. We previously reported that the size of ErbB2 homoclusters decreases after EGF stimulation (18). We assumed that this phenomenon is caused by ErbB2 being removed from its homoclusters due to recruitment into ErbB1-2 heteroclusters. Our current findings corroborate this hypothesis, as summarized in Fig. 4 B. According to the model, large-scale homoclusters of ErbB2 are partially disrupted after EGF stimulation. ErbB2 homoclusters contain inactive ErbB2, providing a pool for recruitment into heteroclusters with other ErbB proteins. Xiao et al. (44) reached a similar conclusion in a recent study. They found that the lateral diffusion coefficient and the size of the confinement zone of ErbB2 increased upon heregulin stimulation. Although they concluded that the observed

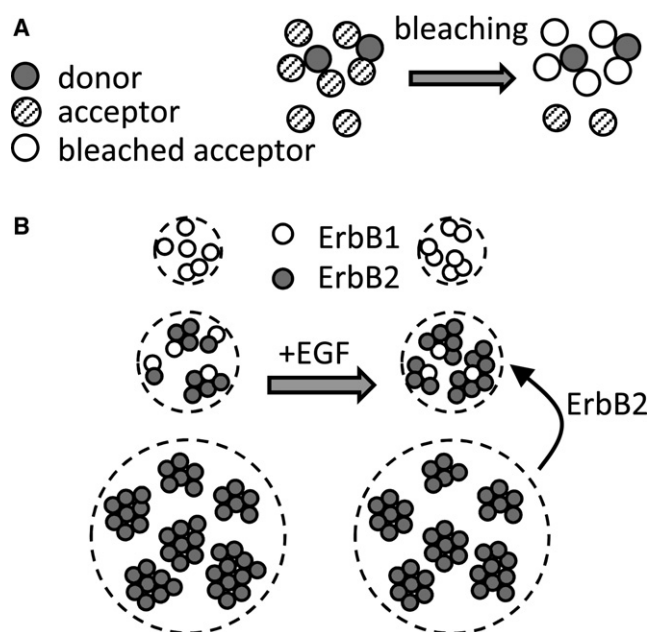


FIGURE 4 Clustering of membrane proteins revealed by FSAB. (A) Heteroclusters contain a mixture of donor- and acceptor-tagged antibodies. Multiple acceptors surrounding a donor molecule take part in FSAB interactions. All such acceptors in the immediate vicinity of donors will be photo-bleached. (B) A mixture of homo- and heteroclusters and free (unclustered) receptors exists in unstimulated SKBR-3 cells. The majority of ErbB1 is monomeric. The overwhelming majority of ErbB2 is outside ErbB1-2 heteroclusters and forms large-scale homoclusters (18). Upon EGF stimulation, ErbB2 is recruited from ErbB2 homoclusters to form heteroclusters with ErbB1, leading to a decrease in the homocluster size of ErbB2 and an increase in the fraction of heteroclustered ErbB2. At the same time, the size of the ErbB1 homoclusters increases because EGF induces the formation of ErbB1 homoaggregates in addition to ErbB1-2 heteroclusters. Due to the opposing effects of homo- and heterodimerization, the fraction of heteroclustered ErbB1 does not substantially change upon EGF stimulation. The percentages of homo- and heteroclustered ErbB proteins are expected to be cell-type-dependent, but the general tendency depicted in the figure is likely to be valid for cells overexpressing ErbB2 with a moderate expression level of ErbB1.

changes were caused by cytoskeletal interactions, a decrease in the size of ErbB2 homoclusters is also expected to lead to the same effects.

We found the fraction of heteroclustered ErbB2 to be independent of the expression levels of ErbB1 and ErbB2 when analyzed at the level of single cells (Fig. S5). This observation implies that the formation of clusters detected by FSAB is not governed by the law of mass action. We believe that the composition of these clusters is established during their export by the vesicular transport system to the cell membrane, and that the density of proteins in the membrane of these vesicles is more or less constant and independent of the number of proteins expressed in the cell membrane.

In conclusion, we have demonstrated that the FSAB technique can be applied to determine the fraction of heteroclustered molecules in a quantitative way, and to investigate the

composition of ErbB1-2 heteroclusters. Our interpretation of the experimental findings is in agreement with our previous results and suggests that the size of large-scale ErbB2 clusters decreases upon EGF stimulation. FSAB and other quantitative methods are required to shed light on the intricate details of the first steps of activation of the ErbB receptor tyrosine kinases.

SUPPORTING MATERIAL

One table and five figures are available at [http://www.biophysj.org/biophysj/supplemental/S0006-3495\(10\)00428-5](http://www.biophysj.org/biophysj/supplemental/S0006-3495(10)00428-5).

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Supporting Material

Coclustering of ErbB1 and ErbB2 revealed by FRET-sensitized acceptor bleaching

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Supplementary material

Determination of the effect of donor photobleaching

The process of FSAB was analyzed according to the scheme in Fig. S3A. For the sake of simplicity, but without the loss of generality, donor-acceptor complexes were assumed to be dimers. Four different molecular species were assumed to exist: DA , D_bA , DA_b , D_bA_b , where A and D are acceptor and donor, respectively, and subscript b denotes bleached molecules. Three different types of transition interconvert the species to each other: donor bleaching and acceptor bleaching induced by direct acceptor excitation and by FRET (FSAB). If the rate of donor photobleaching is not negligible compared to acceptor bleaching, then acceptor molecules present in complexes in which the donor is bleached before the acceptor cannot be bleached by FSAB. The scheme presented in Fig. S3A is described by the following set of differential equations:

$$\frac{dDA(t)}{dt} = -DA(t) \left[S_{1A,eq} k_{b,A} + S_{1D,eq} (1 - FRET) k_{b,D} + S_{1A,eqFRET} k_{b,A} \right] \quad (S1)$$

$$\frac{dDA_b(t)}{dt} = DA(t) (S_{1A,eq} k_{b,A} + S_{1A,eqFRET} k_{b,A}) - DA_b(t) S_{1D,eq} k_{b,D} \quad (S2)$$

$$\frac{dD_bA(t)}{dt} = DA(t) S_{1D,eq} (1 - FRET) k_{b,D} - D_bA(t) S_{1A,eq} k_{b,A} \quad (S3)$$

$$\frac{dD_bA_b(t)}{dt} = DA_b(t) S_{1D,eq} k_{b,D} + D_bA(t) S_{1A,eq} k_{b,A} \quad (S4)$$

where $FRET$ is the efficiency of FRET, $k_{b,D}$ and $k_{b,A}$ are the molecular bleaching rate constants of the donor and the acceptor, respectively, and $S_{1D,eq}$, $S_{1A,eq}$ and $S_{1A,eqFRET}$ are the equilibrium population probabilities of the S_1 state of the donor in the absence of FRET, the equilibrium population probability of directly excited acceptors and that of acceptors excited by FRET. Equations S1-S4 hold if the FRET efficiency is low, therefore the fractions of directly excited acceptors and those excited by FRET are additive. If the rate of excitation of a molecule is low, the equilibrium population probability is reasonably well approximated by the following formula:

$$S_{1,eq} = \frac{k_{exc}}{k_0} \quad (S5)$$

where k_{exc} and k_0 are the summed rate constants of excitation and relaxation, respectively. In the case of acceptors excited by FRET equation S5 takes the following form:

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$$S_{1A,eqFRET} = \frac{S_{1D,eq} (1-FRET) k_{FRET}}{k_{0A}} = S_{1D,eq} FRET \frac{\tau_A}{\tau_D} \quad (S6)$$

where τ_A and τ_D are the fluorescence lifetimes of the acceptor and donor, respectively.

In the case of a 3-level energy system (S_0 , S_1 and T_1 levels) the apparent photobleaching rate constant ($k_{b,app}$) is defined as follows (1):

$$k_{b,app} = k_b S_{1,eq} \quad (S7)$$

$k_{b,app}$ can be determined by fitting a mono-exponential function to the intensity vs. time plot:

$$I(t) = I_0 e^{-k_{b,app} t} \quad (S8)$$

k_b can be calculated according to the following equation (1):

$$k_b = \frac{\Phi_b}{\tau} \quad (S9)$$

Using equations S6, S7 and S9 $S_{1A,eqFRET} \times k_{b,A}$ can be expressed as follows :

$$S_{1A,eqFRET} k_{b,A} = k_{b,app,D} FRET \frac{\Phi_{b,A}}{\Phi_{b,D}} \quad (S10)$$

where $\Phi_{b,D}$ and $\Phi_{b,A}$ are the photobleaching quantum yields of the donor and acceptor, respectively.

If donor bleaching is neglected, only two kinds of species exist (DA , DA_b), and the differential equation describing the process is significantly simpler:

$$\frac{dDA_b(t)}{dt} = DA(t) (S_{1A,eq} k_{b,A} + S_{1A,eqFRET} FRET k_{b,A}) \quad (S11)$$

Solving differential equation S11 and the set of differential equations S1-S4 (after substituting equations S7 and S10) by Mathematica (Wolfram Research, Champaign, IL) acceptor bleaching was compared in the presence and absence of donor bleaching for the AlexaFluor546-Cy5 pair. The apparent photobleaching rate constants of AlexaFluor546- and Cy5-conjugated antibodies bound to cells were determined experimentally by fitting the fluorescence decay curves to equation S8 ($k_{b,app,D}=0.0367$, $k_{b,app,A}=0.16$). The photobleaching quantum yields of AlexaFluor546 and Cy5 were assumed to be 8.9×10^{-5} and 3.6×10^{-5} , respectively, in the absence of CBr_4 (2, 3). Since CBr_4 differentially changes the photobleaching of the two dyes, its effect on the photobleaching quantum yields has to be considered. The photobleaching quantum yield is the reciprocal of the mean number of

survived absorption cycles, which is proportional to the total number of emitted photons before the dye bleaches (1). Since the total number of emitted photons is the area under the curve (AUC) of the fluorescence decay curve, the CBr_4 induced change in the photobleaching quantum yield can be calculated according to the following equation:

$$\frac{\Phi_{b,\text{CBr}_4}}{\Phi_{b,0}} = \frac{\text{AUC}_0}{\text{AUC}_{\text{CBr}_4}} \quad (\text{S12})$$

We found that the photobleaching quantum yields of AlexaFluor546 and Cy5 were increased 2-fold and 95-fold, respectively, by CBr_4 when exciting the dyes at the donor excitation wavelength.

Using the aforementioned values, a FRET efficiency of 40% and assuming that half of the acceptors are bound to donors we found that bleaching of the donor negligible influences the rate of acceptor photobleaching, since the accumulation of the D_bA species is negligible and transient (Fig. S3B). In addition, the calculations showed that approximately 50% of free acceptors are bleached by the time all of the bound acceptors are photobleached. This finding is in accordance with the experimentally observed value of the bleaching correction factor (BCF) of ~40-50% and shows that the fraction of donor-bound acceptors can be reliably determined due to their preferential bleaching compared to free acceptors.

Determination of the effect of fixation on the lateral mobility of ErbB1***Cells and plasmids***

The plasmid for ErbB1 was the kind gift of Yosef Yarden (Weizmann Institute of Science, Rehovot, Israel). Generation of the plasmid coding for ErbB1-eGFP has been described elsewhere (4). A4erbB1 cells have been established by stably transfecting subclone E3 of the A431 cell line (obtained from Ernst Helmreich, University of Würzburg) with ErbB1-eGFP (5). A4erbB1 expresses $\sim 2 \times 10^6$ endogenous ErbB1 and $\sim 10^6$ ErbB1-eGFP as determined by flow cytometry using Qifikit (Dako-Cytomation, DAKO, Hamburg, Germany). Cells were cultured in 8-well chambered coverglass (Nalge Nunc International, Rochester, NY) for FRAP experiments.

Fluorescence recovery after photobleaching (FRAP)

A Zeiss LSM 510 confocal laser scanning microscope (Carl Zeiss AG, Jena, Germany) was used for FRAP experiments. The power of the 488 nm line of an Ar ion laser was set to 5% for imaging eGFP. Fluorescence of eGFP was recorded using a 505LP filter. The power of the 488 line was adjusted to 100% when bleaching rectangular areas ($\sim 2 \times 2 \mu\text{m}$) in the cell membrane, and the recovery of fluorescence was monitored by taking images every second for ~ 150 sec using excitation at 488 nm attenuated to 5%. Image processing was carried out with the DiImage toolbox (Delft University of Technology, Delft, The Netherlands) under Matlab (Mathworks Inc., Natick, MA). Image registration was carried out using the 'correctshift' command of DiImage followed by calculating the mean fluorescence intensity in the bleached region. Fluorescence recovery was fitted to equation S13 in Matlab:

$$a + (1 - a - b) \left(1 - e^{-\frac{t}{\tau}} \right) \quad (\text{S13})$$

where τ is the empirical recovery time constant. The fraction of immobile fluorescent molecules was calculated according to equation S14:

$$\frac{b}{1 - a} \quad (\text{S14})$$

Results and discussion

The lateral mobility of ErbB1 was investigated by FRAP in A4erbB1 cells. The recovery of fluorescence in non-fixed cells revealed an apparent recovery time constant of ~ 10 sec and

an immobile fraction of ~55% (Fig. S3). Fixation in 1% formaldehyde under conditions similar to FSAB experiments almost completely inhibited lateral mobility of ErbB1-eGFP since the immobile fraction was increased to >90%. Addition of CBr₄ to fixed cells did not significantly modify the mobility of ErbB1-eGFP. Although several previous publications showed that the lateral mobility of transmembrane proteins is only marginally inhibited by mild (~1%) formaldehyde fixation (6, 7), our FRAP experiments conclusively showed that the fixation protocol used for FSAB experiments essentially completely prevents the diffusion of ErbB1 in the plane of the membrane during the photobleaching. Therefore, only acceptors in the immediate vicinity of a donor are expected to get photobleached by FSAB.

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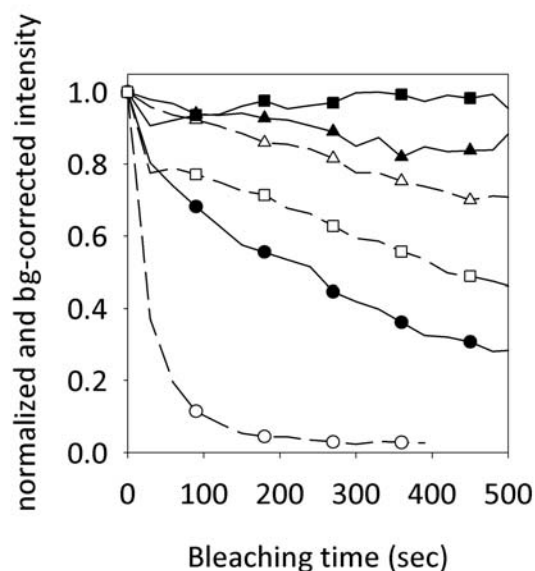
Supplementary table

Table S1. The composition of ErbB1 and ErbB2 homoclusters and their heteroclusters in quiescent and EGF-stimulated SKBR-3 cells.

	free/homoclustered ErbB1 ($\times 10^3$)	ErbB1-ErbB2 complex ($\times 10^3$)	free/homoclustered ErbB2 ($\times 10^3$)
quiescent	120 $\pm$ 8	80 $\pm$ 8 ErbB1 120 $\pm$ 24 ErbB2	1080 $\pm$ 24
EGF-stimulated	134 $\pm$ 10	66 $\pm$ 10 ErbB1 252 $\pm$ 36 ErbB2	948 $\pm$ 36

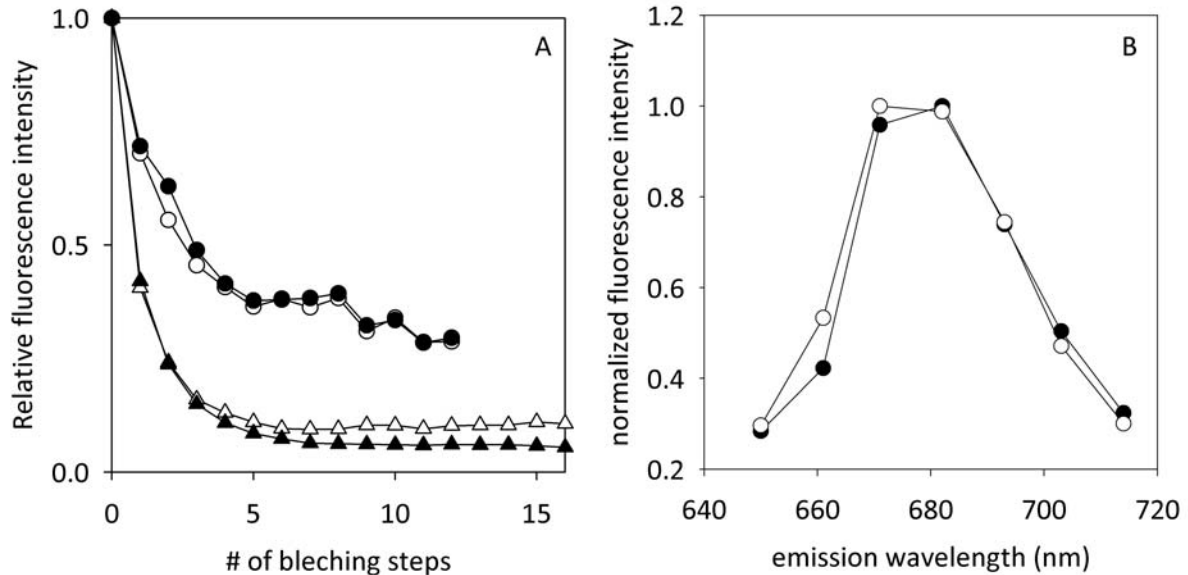
The numbers of free (or homoclustered) and heteroclustered ErbB proteins ($\pm$ standard error of the mean) determined by FSAB are rearranged compared to Table 1 in order to emphasize the composition of ErbB1-2 heteroclusters.

Supplementary figures

Figure S1. The effect of CBr_4 on the photobleaching rate of potential donors and acceptors.

SKBR-3 cells labeled by trastuzumab tagged with AlexaFluor546 (▲,△), Qdot605 (■,□) or Cy5 (●,○) were photobleached by illuminating the samples at 543 nm (AlexaFluor546, Qdot605) or 633 nm (Cy5). Images were recorded by interrupting the bleaching illumination every ~30 sec. Photobleaching was carried out in the absence (filled symbols) or presence (empty symbols) of CBr_4 . Symbols are shown for every third measurement point for easier discernability.

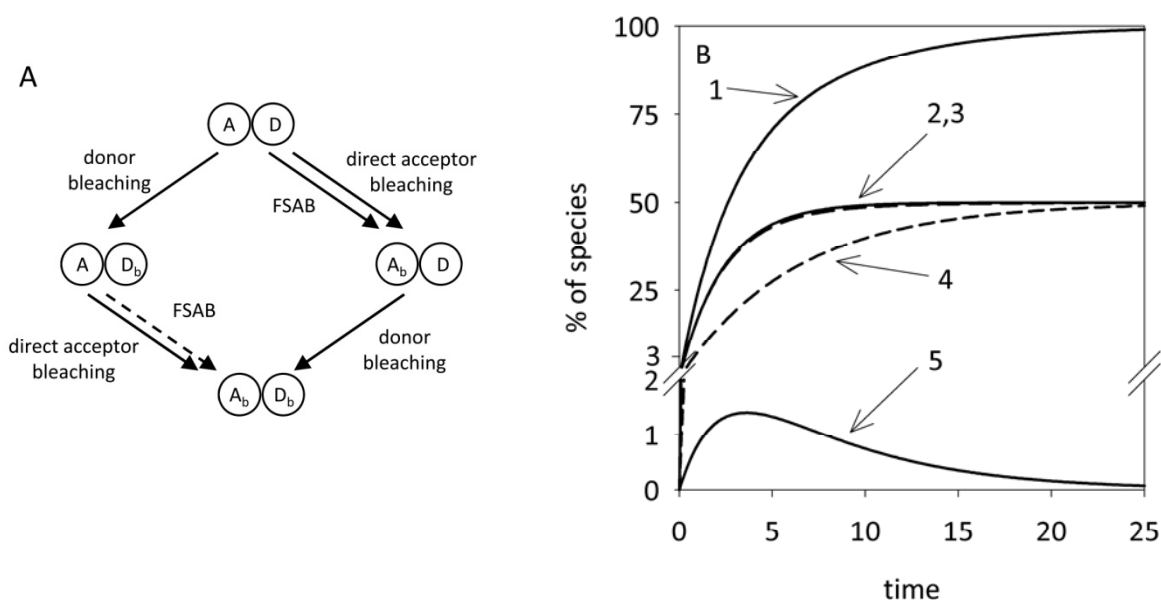
Figure S2. Wavelength-dependent photobleaching of Cy5.



A. SKBR-3 cells labeled by Cy5-trastuzumab were photobleached at 543 nm and the fluorescence intensity of Cy5 was measured at an excitation wavelength of 543 nm (○) or 633 nm (●) using an attenuated laser beam when interrupting the bleaching illumination. Cy5-trastuzumab-labeled SKBR-3 cells were photobleached by illuminating them at 633 nm, and their fluorescence intensity was recorded at an excitation wavelength of 543 nm (△) or 633 nm (▲). The emission of Cy5 was measured using a 650 nm long-pass filter.

B. The emission spectra of Cy5 excited at 633 nm were recorded on an unbleached sample (●) and on a sample bleached at 543 nm (○). The measurement was carried out at a resolution of 10 nm on cells attached to glass coverslips using the Meta unit of a Zeiss LSM510 confocal microscope.

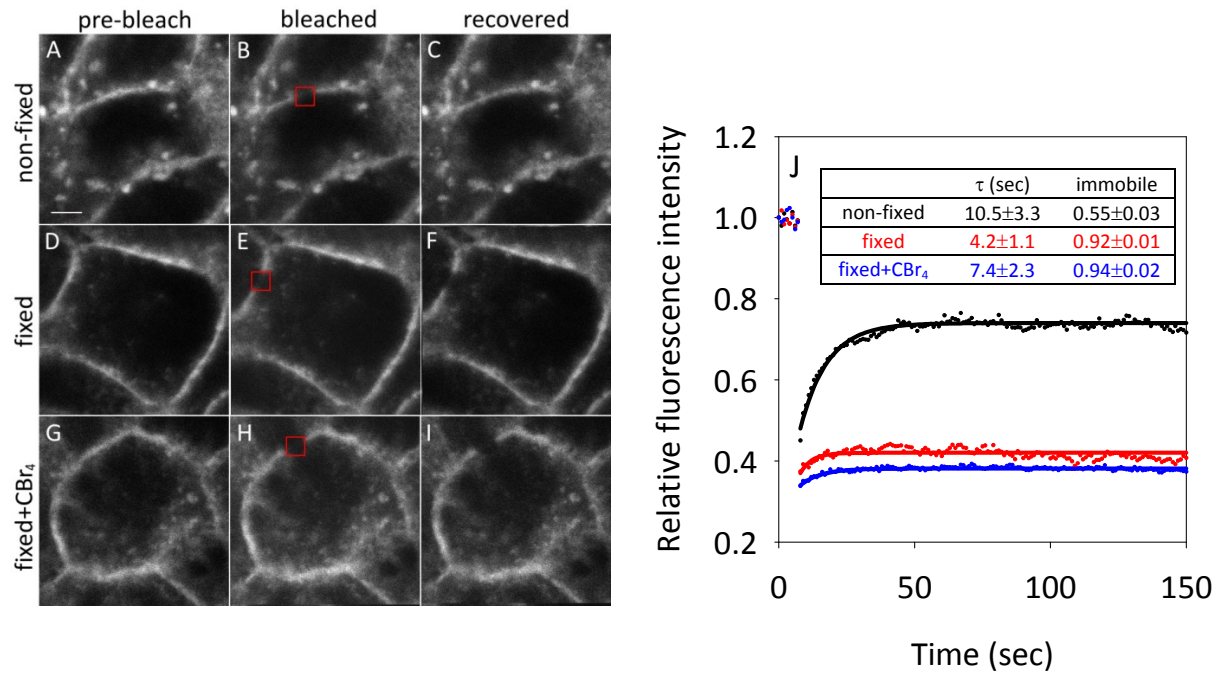
Figure S3. Bleaching of the donor has negligible effect on the rate of FRET-sensitized acceptor bleaching.



A. Reaction scheme of a donor-acceptor dimer undergoing FRET-sensitized acceptor bleaching. Letters *A* and *D* stand for the acceptor and donor, respectively. Subscript *b* indicates a bleached molecule. If a donor is bleached before the acceptor in a heterodimer, that acceptor cannot be bleached by FSAB indicated by the dashed arrow.

B. The sets of differential equations describing direct and FRET-sensitized bleaching of the acceptor with (equations S1-S4) and without (equation S11) donor bleaching were solved with the parameters described in the Theory section. A 50-50% mixture of free and donor-bound acceptors was assumed. The percentages of bleached acceptors (free+bound, curve 1), bound and bleached acceptors in the presence (curve 2) and absence (curve 3) of donor bleaching, free and bleached acceptors (curve 4) and the transient accumulation of the *D_bA* species (complex of a bleached donor and an unbleached acceptor, curve 5) are shown in the figure.

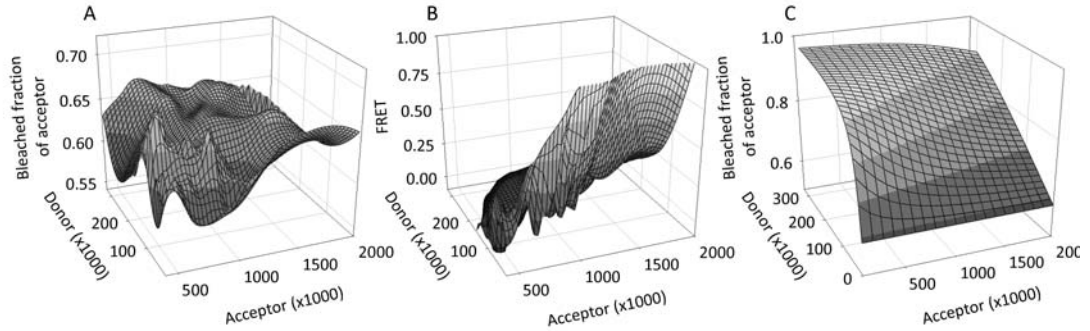
Figure S4. Formaldehyde fixation immobilizes ErbB1 expressed in the cell membrane.



A-I. Rectangular areas in the membrane of A4erbB1 cells were photobleached and the recovery of fluorescence was monitored by confocal microscopy. The experiment was carried out with non-fixed cells (A-C, black curve in J), cells fixed in 1% formaldehyde (D-F, red curve in J) and with fixed cells treated with CBr₄ (G-H, blue curve in J). “Pre-bleach”, “bleached” and “recovered” images were taken before and immediately after the bleaching and at the end of the observation period (150 sec), respectively. The bleached regions are marked by the red rectangles. The scale bar in A is 5 μ m.

J. Representative FRAP curves for non-fixed (black), fixed (red) and CBr₄-treated fixed cells (blue). The dotted lines display the measured fluorescence intensity values, and the continuous ones are curves fitted to equation S13. The inserted table shows the means ($\pm$ standard error of the mean) of the recovery time constants and the fraction of immobile ErbB1-eGFP calculated from five independent measurements.

Figure S5. Measured and calculated dependence of the FRET efficiency and clustered acceptors on the expression levels of the donor and acceptor.



A-B. SKBR-3 cells were labeled by a mixture of AlexaFluor546-Mab528 and Cy5-trastuzumab against ErbB1 and ErbB2, respectively. The fraction of ErbB2 heteroclustering with ErbB1 (bleached fraction of acceptor) determined by FSAB (A) and the FRET efficiency (B) are plotted as a function of the expression levels of the donor and acceptor in single cells. The graphs contain the data of ~100 cells.

C. The expected fraction of bleached (clustered) acceptors calculated according to the law of mass action assuming that six acceptors bind a single donor, a K_d of 10^5 and a bleaching correction factor (*BCF*) of 0.6.

Distribution of resting and ligand-bound ErbB1 and ErbB2 receptor tyrosine kinases in living cells using number and brightness analysis

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Ligand-driven dimerizations of ErbB receptor subunits fulfill a fundamental role in their activation. We have used the number and brightness analysis technique to investigate the existence of preformed ligand-independent dimers and clusters and to characterize the initial steps in the activation of ErbB1 and ErbB2. In cells expressing 50,000–200,000 receptors, ErbB1 was monomeric in the absence of ligand stimulation, whereas in CHO cells with receptor levels >500,000 as much as 30% of ErbB1 was present as preformed dimers. EGF induced the formation of ErbB1 dimers as well as larger clusters (up to pentamers) that colocalized with clathrin-coated pits. The distribution of unstimulated ErbB2 in cells expressing $3 \cdot 10^5 - 10^6$ receptors was fundamentally different, in that this receptor was present in preformed homoassociated aggregates containing 5–10 molecules. These constitutive ErbB2 homoclusters colocalized with caveolae, increased in size at subphysiological temperatures, but decreased in size upon EGF stimulation. We conclude that these ErbB2 clusters are promoted primarily by membrane-mediated interactions and are dispersed upon ligand stimulation.

EGFR | epidermal growth factor | ErbB proteins | receptor clusters | signal transduction

ErbB proteins (ErbB1–4, HER1–4) constitute the best characterized family of receptor tyrosine kinases (1). Biochemical analysis has demonstrated that ErbB1, the prototypical member of the family (also known as the epidermal growth factor receptor, EGFR or HER1) undergoes ligand-induced homodimerization as a key step in its activation (2). More recent crystallographic studies reveal that ligand binding induces a transition from a closed conformation of ErbB1 to an extended configuration with the capacity to dimerize via intermolecular interactions mediated by domain II (3, 4). The ultrastructural data also confirm that dimerization of ErbB1 plays a fundamental role in activating the kinase domain by a mechanism resembling that of cyclin dependent kinases (5, 6). The coreceptor ErbB2 has no known ligand but upon transactivation expresses the most potent kinase activity of the ErbB family, thereby increasing the efficiency of signaling mediated by ErbB2-containing heterodimers (7). ErbB2 constitutively adopts an extended conformation potentiating the formation of heterodimers (8, 9) that can be inhibited by pertuzumab, a monoclonal antibody sterically blocking the heterodimerization arm of ErbB2 (10). Although the extracellular domain of ErbB2 has failed to form crystallographic homodimers, molecular biological and fluorescence resonance energy transfer (FRET) experiments have shown that full-length ErbB2 exists in dimers or higher-order aggregates in the plasma membrane (11, 12). The implication is that the transmembrane, juxtamembrane and other intracellular domains (5, 13, 14) act in conjunction with the membrane environment (15) to mediate the dimerization and, thereby, functional states of ErbB proteins.

Many investigators have sought to determine the distribution of ErbB1 in the plasma membrane in an attempt to explain the

molecular basis for the apparent high- and low-affinity ligand binding. The findings suggest the existence of preformed (i.e., ligand-independent, ErbB1 receptor dimers) (16, 17) or larger aggregates (18) in addition to the classical dimers formed upon ligand-induced activation of receptor monomers. However, the different techniques, cell types, and conditions of the experiments have resulted in conflicting and contradictory results. Several studies using fixed cells and/or low temperature incubations based on hetero- or homo-FRET have indicated that <10% to as much as 50% of ErbB1 may exist as preformed dimers or oligomers (17, 19, 20). Inhibition of the tyrosine kinase activity of the receptor led to disaggregation of the transient dimers and clusters to monomers (18), suggesting that some measurements may have been made on nonstarved cells with preactivated receptors. On the other hand, fluorescence correlation spectroscopy (FCS) measurements on living, starved cells were consistent with earlier evidence for low levels of preformed dimers and very few oligomers (21). Recently, flow cytometric homo-FRET data demonstrated that homoclustering of ErbB2 was significantly stronger than ErbB1 and that homoaggregates of ErbB2, containing inactive proteins, were recruited into ErbB1- or ErbB3 containing heterodimers upon ligand binding (22).

We have utilized a new correlation method introduced by the Gratton lab (23) that directly assesses the molecular size of diffusing macromolecules in living cells by number and brightness (N&B) determinations based on the analysis of sequential confocal microscopy images acquired by calibrated single photon detection. The fluctuation of fluorescence intensities in single pixels provides a measure of molecular brightness (i.e., the number of detected photons emitted by a single diffusing unit of a given fluorescent species during the pixel dwell time). We found that ErbB1 is monomeric at low expression levels, but forms ligand-independent dimers in an expression-level and cell-type dependent manner. Binding of EGF induces a practically complete dimerization of ErbB1, with higher-order clusters primarily associated with clathrin-coated pits. On the contrary, ErbB2 forms homoclusters containing 5–10 proteins in the absence of stimulation, and these clusters are reduced in size after stimulation by EGF. In the absence of ligand the ErbB2 clusters increase in size at subphysiological temperatures. Our results resolve many of the published contradictions regarding the existence of pre-

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The authors declare no conflict of interest.

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formed ErbB clusters and their rearrangement after ligand stimulation and show that expression levels as well as temperature-dependent membrane rearrangements lead to significantly different receptor distributions.

Results

Preassociated ErbB1 Is Induced to Dimerize in Cells Expressing the Receptor at a High Level. The F1-4 line (transfected CHO cells stably expressing ~600,000 ErbB1-eGFP/cell (24) was used as a model system for cells overexpressing the ErbB1 receptor. Clustering of ErbB1 in starved cells was measured using the N&B approach. The distribution of brightness values was homogeneous indicating that clustering of ErbB1 did not show a steep dependence on the local density of the protein within the limited concentration range sampled on a single cell (Figs. 1*A* and 2*A*). The molecular brightness values of ErbB1-eGFP were ~30% higher than those of monomeric eGFP implying that a fraction of ErbB1 was present in preformed receptor clusters (Table 1). The existence of preformed receptor dimers in F1-4 cells was confirmed by photobleaching ~80% of the fluorescence after which the molecular brightness of ErbB1-eGFP approximated that of soluble, monomeric eGFP (Fig. 1*B* and Table 1). Stimulation of F1-4 cells by 100 nM EGF for 3 min at room temperature increased the cluster size of ErbB1 significantly (Figs. 1*A* and 2*B*) to a value that was ~80% higher than monomeric eGFP (Table 1). The brightness histogram shifted homogeneously to higher values upon EGF treatment (Fig. 1*A*), but the dependence of the brightness on the fluorescence intensity was weak (Fig. 2*B* and Fig. S1*E*). These observations indicate that the growth factor induced an almost complete dimerization of the receptor population.

Preformed Clusters of ErbB1 Are Absent in Cells Expressing the Receptor at Low Levels. The observation of preformed ErbB1 clusters in

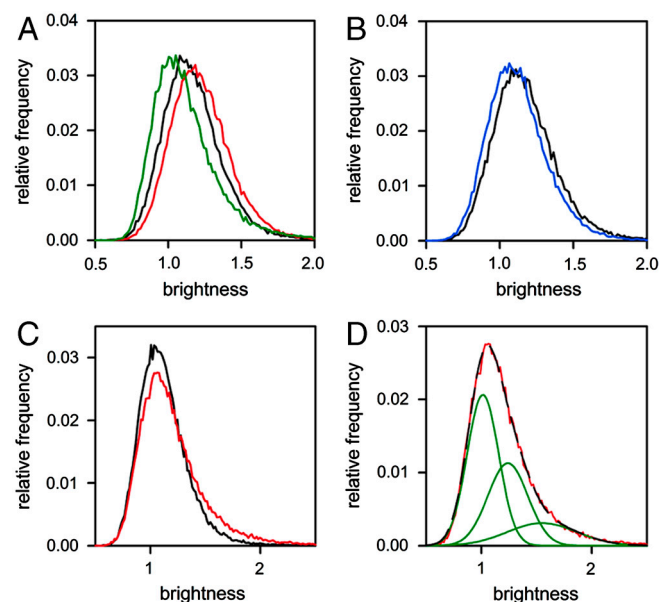


Fig. 1. Clustering of ErbB1 in quiescent and EGF-stimulated cells. (A). Starved F1-4 cells were measured before and after stimulation with EGF for 3 min. The brightness histograms are plotted for quiescent (black) and the EGF-stimulated cells (red). The brightness histogram of soluble monomeric eGFP is shown by the green line. (B). Starved F1-4 cells were photobleached until their fluorescence intensity reached ~20% of the initial value and the brightness histograms of the control (black) and the bleached cells (blue) are shown. (C). Brightness histograms of starved (black) and EGF-stimulated (red) F1-10 cells. (D). The brightness histogram of EGF-stimulated F1-10 cells (red) was fitted by three overlapping Gaussians (green lines). The sum of the three fitted Gaussian distributions perfectly overlapping the experimentally determined histogram is shown by the black, long dashed line.

unstimulated F1-4 cells might not represent the behavior of the receptor under physiological conditions due to the high overexpression in these cells. We investigated two other model systems with significantly lower levels of ErbB1 expression. F1-10 is another transfected CHO line stably expressing ~50,000 ErbB1-eGFP/cell (25), whereas the human HeLa-ErbB1eGFP line expresses ~200,000 ErbB1-eGFP/cell in a background of 50,000 endogenous, unlabeled ErbB1 receptors. The molecular brightness of ErbB1-eGFP measured in starved F1-10 and HeLa-ErbB1eGFP cells was only 3–5% higher than that of monomeric eGFP, indicating that the majority of ErbB1-eGFP was monomeric in unstimulated cells expressing moderate to low levels of the receptor (Fig. 1*C*, Fig. S1*C*, and Table 1). The brightness of ErbB1-eGFP showed a steeper dependence on its local density in F1-10 than in F1-4 cells (Fig. 2*A* and *C*). Stimulation of both F1-10 and HeLa-ErbB1eGFP cells led to significant increases in the molecular brightness of ErbB1-eGFP (Table 1), and the distribution in EGF-stimulated F1-10 cells deviated significantly from a unimodal Gaussian (Fig. 1*C*), correlating strongly with the local density (mean fluorescence intensity) of ErbB1-eGFP (Fig. 2*C*). Larger clusters of the receptor induced by EGF (Fig. 2*D* and Fig. S1*F* and *G*) were colocalized with clathrin-coated pits after fixation and immunofluorescence labeling (S1*H* and Figs. S2*A*). Such clusters were not observed in either quiescent or stimulated F1-4 cells (Fig. S1*A* and *B*). Three overlapping Gaussians were required to fit the brightness distribution of EGF-stimulated F1-10 cells (Fig. 1*D*), rightmost peak corresponding to ~5 ErbB1-eGFP in clathrin-coated pits.

The Propensity of ErbB2 to Form Homoclusters Is Greater than that of ErbB1. Starved A4erbB2 cells (26) expressing ~1.2·10⁶ and 10⁶ endogenous ErbB1 and ErbB2-mYFP, respectively, showed remarkably heterogeneous clustering of ErbB2 both at the microscopic and molecular levels. Conspicuous “macroclusters” of ErbB2 were observed in microscopic images (Fig. S3). The molecular brightness of ErbB2-mYFP was significantly higher in these macroclusters than outside them (Figs. 3*A* and *C* and 4*A–D*), but the N&B analysis demonstrated that ErbB2 was homo-associated in both locations (Table 1). A similar tendency was

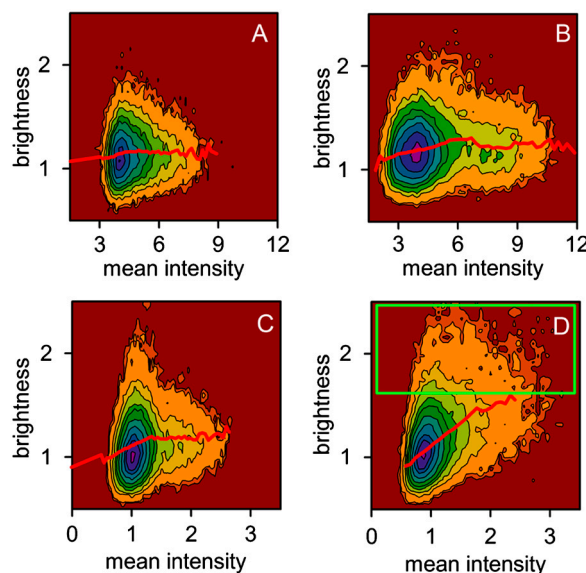


Fig. 2. N&B contour plots of quiescent and EGF-stimulated cells expressing ErbB1. N&B contour plots of starved (A) and EGF-stimulated (B) F1-4 cells, and starved (C) and EGF-stimulated F1-10 cells were calculated. The horizontal scales (mean fluorescence intensity) were divided into 50 bins and the average brightness calculated for each bin is shown by the red trend lines. The gated part in (D) marked by the green box corresponds to the gated pixels marked by red in Fig. S1*G*.

Table 1. Molecular brightness values of ErbB1-eGFP and ErbB2-mYFP

ErbB1	soluble eGFP	F1-4			F1-10		HeLa-ErbB1-eGFP	
		starved	bleached	+EGF	starved	+EGF	starved	+EGF
mol. br. (mol/cluster)	0.118 ± 0.002	0.158 ± 0.002 (1.3)	0.128 ± 0.002 (1.0)	0.217 ± 0.003 (1.8)	0.122 ± 0.001 (1.0)	0.175 ± 0.003 (1.5)	0.126 ± 0.005 (1.0)	0.172 ± 0.005 (1.5)
ErbB2	soluble mYFP	A4erbB2				CHO-ErbB2mYFP		
		starved	+EGF	pertuzumab	pertuzumab+EGF	starved	pertuzumab	
		In mc	0.4 ± 0.02 (6.3)	0.283 ± 0.025 (4.5)	0.35 ± 0.023 (5.6)	0.33 ± 0.017 (5.2)	0.91 ± 0.056 (14.4)	0.75 ± 0.053 (11.9)
mol. br. (mol/cluster)	0.063 ± 0.01	outside mc	0.182 ± 0.008 (2.9)	0.164 ± 0.019 (2.6)	0.16 ± 0.021 (2.5)	0.14 ± 0.023 (2.2)	0.28 ± 0.037 (4.4)	0.28 ± 0.03 (4.4)

Molecular brightness values ($\pm$ SEM) of quiescent and EGF-stimulated cells, and that of soluble, monomeric eGFP and mYFP are shown in the table. The numbers in parentheses represent the average number of ErbB1-eGFP or ErbB2-mYFP in a molecular cluster determined by dividing the molecular brightness values of cells with that of soluble monomeric fluorescent protein. The displayed values were calculated from 5–10 cells. (mc = macrocluster)

observed in CHO cells stably expressing ~300,000 ErbB2/cell but lacking the other ErbB proteins. However, in this cell line the size of molecular homoclusters of ErbB2 was even larger (Table 1), averaging 5–15 inside and 3–4 ErbB2 molecules outside the macroclusters. ErbB2-mYFP in the central and most intense part of the macroclusters had a lower molecular brightness than at the periphery (Fig. S3A and Fig. 4B). We attribute this observation to a lower mobility of ErbB2 in the central and most dense part of the clusters.

Temperature changes are expected to affect the clustering properties of integral membrane proteins that associate with specific lipid environments (15, 27, 28). Macroclusters of ErbB2 gradually increased in size and density after transferring cells from 37°C to room temperature over a period of 2 h (Fig. S3).

Several factors might contribute to increased homoaggregation of ErbB2 compared with ErbB1: (i) the C-terminal PDZ binding motif that interacts with intracellular proteins such as Erbin and Pick1 (29); (ii) linker regions to the mYFP moiety (26); or (iii) the mYFP moiety itself (30). Additional transgenes were constructed: ErbB2-short-mYFP, with a dipeptide linker; ACP-ErbB2, an amino terminal acyl carrier protein (ACP) sequence insertion (31); and C-terminal VPV deletion mutants of both constructs. N&B measurements on transiently transfected HeLa cells expressing ErbB2-short-mYFP yielded data identical to those observed with the original ErbB2-mYFP vector (Fig. S4A and Table S1). Enzymatic covalent labeling of the ACP-ErbB2 in the plasma membrane of transfected HeLa cells with Atto-565-CoA displayed identical clustering properties to mYFP-labeled ErbB2 (Fig. S4B and Table S1). Finally, the cluster sizes of wild-type ErbB2 and Δ VPV-ErbB2 were not significantly different from each other in cells transfected with either mYFP-tagged or ACP-conjugated versions of the vectors (Fig. S4A and B and Table S1). Colocalization experiments with immunolabeling

are described in *SI Text* and confirmed that no fluorescence signal contributions from internal vesicles or binding to extracellular matrix contributed to the molecular brightness determinations while macroclusters colocalize with caveolin (Fig. S5).

ErbB2 Is Removed from Preformed Homoclusters upon EGF Stimulation. A4erbB2 cells were used to measure the effect of EGF stimulation on the molecular clustering of ErbB2. EGF significantly decreased the size of molecular clusters of ErbB2 inside macroclusters without affecting external molecules (Fig. 3B and C and Table 1). Ligand-activated ErbB1 recruited ErbB2 from homoclusters into ErbB1-2 heterodimers. Pertuzumab, an antibody blocking the heterodimerization of ErbB2, decreased the molecular brightness values of ErbB2-mYFP in starved cells and abolished the decrease induced by EGF (Table 1). We conclude that the observed EGF-induced decrease in the size of molecular homoclusters of ErbB2 was the consequence of the recruitment of ErbB2 into heterodimers by ligand-activated ErbB1.

Discussion

According to the widely accepted molecular scheme underlying the biology of ErbB receptor tyrosine kinases, inactive monomeric receptors undergo ligand-induced dimerization and activation (32). Although several lines of evidence (3, 4), support this view, both recent and earlier evidence (16–18, 20–22) have suggested that two aspects of the classical model need to be reconsidered: (i) inactive receptors are not necessarily monomeric, such that (ii) higher-order clusters of inactive and/or activated receptors may exist. We chose the novel technique of N&B analysis (23) to investigate these issues. Clusters detected by N&B analysis are defined in terms of joint mobility, whereas FRET measurements resolve aggregates based on proximity. The underlying principles of N&B and FCS measurements are the same and both measure the distribution and association states of molecules in live cells. N&B data are statistically more reliable due to the larger number of pixels used in the analysis compared with FCS. Fluorescence intensity distribution analysis and photon count histogram analysis have the potential to yield information on the distribution of cluster sizes within single pixels, something that cannot be achieved by N&B analysis. However, their requirement for highly accurate photon statistics necessitates high illumination intensities leading to photodamage (21).

The molecular brightness value of ErbB1-eGFP in unstimulated F1-4 cells expressing the receptor at a high level (>500,000/cell) was higher than that of monomeric eGFP and approached a value characteristic of preformed dimers in pixels with the highest local density. The brightness value indicated the presence of ~30% preformed dimers with no indication of higher oligomers, in agreement with previous FCS results (21). In contrast, unstimulated

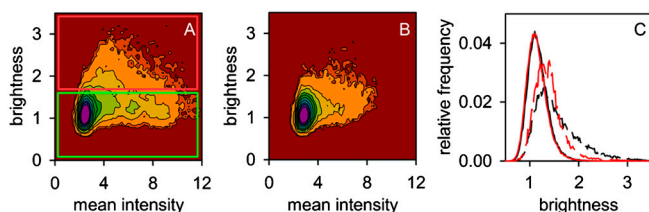


Fig. 3. N&B contour plots of quiescent and EGF-stimulated A4erbB2 cells. Starved A4erbB2 cells were stimulated with 100 nM EGF for 3 min. The N&B contour plots were calculated for the control (A) and EGF-stimulated cells (B). The top and bottom boxes in part A were used to gate pixels with high and low brightness values, respectively, and these pixels are shown in red in Fig. 4C and D, respectively. (C). Brightness values separately analyzed for pixels inside (dashed line) and outside (solid line) ErbB2 macroclusters both in quiescent (black lines) and EGF-stimulated cells (red lines).

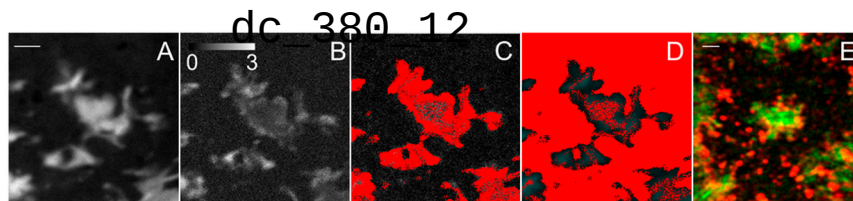


Fig. 4. Molecular brightness is different inside and outside ErbB2 macroclusters. (A–D). A4erbB2 cells were starved in the absence of serum, and 100 images were acquired for N&B analysis. The mean and brightness values are shown in (A) and (B), respectively. Pixels displaying high and low brightness values were gated as shown in Fig. 3, and the masks representing pixels with high (C) and low (D) brightness values were overlaid on the mean intensity image. (E). Starved A4erbB2 cells were fixed, permeabilized, and labeled with a monoclonal antibody against caveolin followed by secondary labeling. The fluorescence intensity of ErbB2-mYFP and the antibody against caveolin are shown in green and red, respectively. [Bars: 2 μm in (A), valid for (A–D); 1 μm in (E)].

ErbB1 was monomeric in two different cell lines (F1-10, HeLa-ErbB1eGFP) expressing the receptor at a lower level (50,000–200,000/cell) found on primary, nononcogenic cells. These observations suggest that the existence of preformed ErbB1 dimers is strongly dependent on the expression level and density of the receptor, and thus, may reflect the establishment of thermodynamic equilibrium. There may also be a contribution of lipid rafts and gangliosides to the tendency of ErbB1 to form preformed dimers (15, 33, 34). The fact that CHO cells do not express GM1 should also be taken into consideration (35).

Stimulation of cells with EGF led to an increase in the cluster size of ErbB1. In the high-expression F1-4 cell line the brightness histogram shifted homogeneously to larger values after EGF stimulation and the mean value of the unimodal distribution was ~1.8-times that of monomeric eGFP, demonstrating an almost complete dimerization of ErbB1 upon EGF treatment. F1-10 cells expressing ErbB1 at a much lower level (~1/10) behaved differently. The EGF-induced increase in the average molecular brightness was lower and the brightness histogram was unsymmetrical. Decomposition of the brightness histogram revealed that the main peak and the brightest subpopulation corresponded to monomeric and pentameric ErbB1, respectively. After fixation pentameric clusters could be colocalized with clathrin-coated pits by antibody labeling. We conclude that EGF-stimulation led to activation and dimerization of ErbB1 followed by immediate recruitment to clathrin-coated pits, leaving behind the remaining inactive and monomeric receptors. In F1-4 cells with high ErbB1-eGFP expression there was rapid recruitment of almost all the ErbB1 molecules into activated dimers after addition of EGF, masking the rather low number of clathrin-coated pits that were presumably saturated (36).

Whereas biochemical data (37) and X-ray crystallography (3, 4) suggest that inactive ErbB1 is monomeric, hetero-FRET (16–18), homo-FRET (19, 22, 38), and FCS (20, 21, 25) measurements have found evidence for dimers, or in a few cases for higher-order clusters. We propose that several cell- and measurement-dependent factors contribute to the discrepancies in the literature: (i) the strong dependence of preformed ErbB1 dimers on expression levels established in this study accounts for the presence of preformed clusters in cells (e.g., A431) with high receptor densities (16, 17); (ii) fixation and labeling by bivalent antibodies may artificially generate receptor aggregates (39); and (iii) the continuous cycle of phosphorylation and dephosphorylation of ErbB1 induced by autocrine stimulation or lack of starvation. ErbB1 is fully inactivated only in serum-deprived cells (40), and some protocols have even used phosphatase inhibitors (41), a situation promoting the formation of activated dimers. Thus, the observation of ErbB1 clusters persisting in nonstarved cells (20) leads to the erroneous conclusion that they preexist in unstimulated cells.

The perception of clusters, extending from dimers to aggregates containing hundreds of proteins (22, 42), may also reflect the size-dependent selectivity of the methods used to investigate them. Dimers observed by X-ray crystallography are direct associations on the molecular level, whereas positive FRET signals

imply molecular proximity but not necessarily specific molecular interactions. Furthermore, hetero-FRET cannot discriminate between small and large aggregates, because cluster size only slightly affects the transfer efficiency (43), whereas homo-FRET is more suitable for the detection of higher-order associations (22, 38, 44). In addition, methods based on the measurement of fluorescence correlations, including N&B analysis, detect joint mobility, which can arise not only from tight molecular associations, but also by mutual confinement by membrane structures (45). The use of image correlation techniques [image correlation spectroscopy or dynamic image correlation spectroscopy (DICS)] (27, 46) to determine molecular associations applied to fixed cells is much less reliable due to fixation artifacts, photon statistics, and the optical resolution of the microscope. The overestimation of clustering or aggregate formation by DICS is apparent even in live cells from the calculated diffusion constant of $2.5 \cdot 10^{-11} \text{ cm}^2/\text{s}$ for ErbB1 (27), compared to values greater by one to two orders of magnitude obtained by fluorescence recovery after photobleaching (47, 48), FCS (21, 25), and single particle tracking (49).

We propose the following model for the ErbB1 receptor, one which is in agreement with most experimental results.

- Inactive ErbB1 is monomeric when expressed at physiological, nononcogenic levels.
- Density-dependent formation of preformed clusters occurs in cells expressing the receptor at high levels. Molecules in these preformed clusters can be held together by two different types of interactions: (i) Mutual confinement by membrane- or cytoskeleton-mediated indirect interactions. Because lipid rafts harbor ErbB1 and influence its association state they are likely candidates for exerting the membrane-mediated confinement of ErbB1 (34, 50). The existence of preformed dimers stabilized by indirect interactions is supported by the observation of such transient protein clusters in which the distance between proteins is larger than the range of direct molecular interactions. Therefore, these clusters do not constitute stable molecular associations such as those arising after ligand binding (45). (ii) Direct protein-protein interactions mediated by the dimerization arm of ErbB1. It has recently been demonstrated that preformed ErbB1 dimers stabilized by their dimerization arms can arise transiently (51) but these are highly dependent upon receptor density.

ErbB2 showed a behavior strikingly different from ErbB1. In the absence of stimulation the receptors were present in preformed clusters. Microclusters of 5–15 ErbB2 receptors were found associated in larger macroclusters of $\sim 1\text{ }\mu\text{m}$ (probably also composed of other proteins as shown in the supplementary data). Outside of these macroclusters 3–5 ErbB2 molecules were hom-associated and little monomeric protein was observed. The size of ErbB2 homoclusters was significantly larger in cells in which ErbB2 was expressed as the only member of the ErbB family (CHO-ErbB2mYFP) compared to A4erbB2 cells that coexpress $\sim 1.2 \cdot 10^6$ ErbB1 with 10^6 ErbB2-mYFP. We assume that the more extensive formation of ErbB2 homoclusters in CHO-ErbB2-

mYFP cells was due to the absence of other competing ErbB proteins. This interpretation is supported by earlier near field optical microscopy data that demonstrated larger ErbB2 clusters in a CHO cell line expressing less than 10^5 receptors/cell compared with the cluster size in MDA453 cells expressing ErbB2, ErbB3, and ErbB4, each at levels of $\sim 10^5$ (42). Macroclusters of ErbB2 colocalized with caveolin and ErbB1, a finding in accordance with the presumed raft-association of ErbB2 (15) and the colocalization of ErbB1 with caveolin in resting cells (50). EGF led to a decrease in the homoaggregation state of ErbB2 within macroclusters by recruitment and heterodimerization without significantly affecting the bulk of the ErbB2, in agreement with previous homo-FRET data (22). In polarized epithelial cells ErbB2 is targeted to the basolateral membrane by PDZ domain-containing proteins such as Erbin and may be clustered at specific sites by interaction with PICK1 (29). We eliminated the C-terminal PDZ binding sequence as well as a valine and proline-rich cloning linker to the mYFP tag with VPV deletion mutants and ACP-ErbB2 constructs. No changes in the cluster size or frequency were detected in these constructs compared with wild-type ErbB2. In addition to the PDZ binding motif several other domains of ErbB2 have been reported to contribute to its peculiar association properties, including the transmembrane domain (13) and the sequence 966–968 in the intracellular domain (52). Although not explicitly shown to be important for the homoassociation of ErbB2 the juxtamembrane domain (53) and the kinase domain (6) of ErbB1 are known to regulate dimerization; therefore the role these regions in inducing ErbB2 homo-cluster formation cannot be excluded. In addition, interactions of the extracellular glycosylation chains and the EGF-like domain of the membrane-bound mucin Muc-4 (54) could also promote ErbB2 homoaggregation.

We observed a time-dependent growth of ErbB2 macroclusters after a temperature shift from 37 to 20°C. Such thermally induced increase in the clustering of ErbB1 (27) and other proteins (28) has been reported previously. The finding of temperature-dependent cluster formation of ErbB2 is in accordance with the observation of lipid phase separation in plasma membrane vesicles in the temperature range of 15–25°C (55) and the raft association of ErbB2 (15). Because incubation at nonphysiological temperatures can drastically alter the natural state of receptor distribution, conclusions regarding clustering of membrane proteins derived from data obtained in experiments involving long-term incubation at room temperature or even short-term incubation on ice should be treated with great caution.

In conclusion, we have shown that although inactive ErbB1 molecules dimerize in a density-dependent manner, these associations are not equivalent to the stable molecular dimers that form after ligand stimulation. ErbB2 has a much stronger propensity for homocluster formation, but is recruited away from these homoaggregates after EGF stimulation and formation of hetero-associations. Our results provide significant insights into the underlying molecular processes taking place during the initial steps of ErbB protein activation. Two-color N&B experiments (56) will reveal the composition of heteroclusters and provide unique insights into the mechanisms of potentiated signaling in cells expressing different sets of ErbB family members.

Materials and Methods

Cell Lines. All cell lines have been described previously (24–26, 38). Characterization of the expression levels are detailed in *SI Materials and Methods*. For microscopy experiments cells were cultured in 2- or 8-well coverglass chambers (Nalgene Nunc International) and stimulated with 100 nM EGF (R&D Systems) at room temperature.

Plasmids. Protocols for generating the ACP-ΔVPV-ErbB2, ErbB2-short-mYFP, and ΔVPV-ErbB2-short-mYFP are given in *SI Materials and Methods*.

Antibodies, Cell Labeling, and Fluorescence Microscopy for Colocalization. Information about the antibodies and experimental conditions for the data shown in Fig. S5 are given in *SI Materials and Methods*.

Number and Brightness Analysis. An IX81 Olympus microscope with the FluoView FV1000 confocal configuration was used to carry out number and brightness analysis according to Digman et al. (23). Live cells were analyzed at room temperature in Tyrode's buffer with 10 mM glucose and 0.1% BSA. Measurements were started immediately after removing cells from 37°C and finished within 30 min to avoid changes in the distribution of receptors at room temperature. Image series of 50–100 single optical slices of the cell membrane adjacent to the coverslip were acquired in the pseudophoton counting mode with a pixel size of 41 nm at laser powers of 70 μW of 488 nm for eGFP, 90 μW of 514 nm for mYFP and 130 μW of 543 nm for Atto565-labeled samples. See *SI Text* for additional details concerning imaging and analysis methods.

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Supporting Information

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SI Text

SI Results. Determination of molecular brightness and cluster size for ErbB2-short-mYFP or ACP-ErbB2 transfected HeLa cells. The PDZ domain binding motif (VPV) was deleted from ErbB2-short-mYFP and ACP-ErbB2, and HeLa cells were transfected with plasmids coding for the Δ VPV-deletion mutants or wild-type ErbB2-short-mYFP or ACP-ErbB2 respectively. Cells expressing ACP-tagged ErbB2 were labeled with Atto565 using Sfp transferase two days after transfection. Atto565-labeled and mYFP-expressing cells were analyzed by the N&B method (Fig. S4). The molecular brightness of ErbB2 was analyzed inside and outside macroclusters and the means $\pm$ standard errors of ~ 10 cells are displayed in Table S1. The molecular brightness of soluble monomeric fluorophores was also determined. To determine the average number of ErbB2 molecules in a molecular cluster (displayed in parentheses, Table S1) cellular molecular brightness values were divided by that of the soluble fluorophore. Because HeLa cells express $\sim 30,000$ endogenous ErbB2, these numbers had to be corrected for the presence of “dark,” unlabeled ErbB2. The total number of ErbB2 in transfected cells was $\sim 5 \cdot 10^5$ and $\sim 3 \cdot 10^5$ in the case of cells transfected with mYFP- and ACP-tagged proteins, respectively (numbers determined by Qifikit). Therefore, the number of molecules/cluster was divided by 0.94 and 0.9 in the two cases. In cells expressing ACP-tagged proteins a further correction was necessary to account for the incomplete labeling of the ACP-tag by Sfp transferase. It was determined in a separate experiment that $\sim 60\%$ saturation of the ACP-tag is achieved with $5 \mu\text{M}$ Atto565-CoA; therefore the number of molecules/cluster was divided by 0.6. The data shown in Table S1 and Fig. S4 show conclusively that the high potential for ErbB2 to form clusters is independent of the C-terminal PDZ binding sequence and not influenced by the mYFP moiety.

Colocalization analysis of ErbB2 with membrane components and/or internal vesicles. We eliminated potential artifacts arising from internal vesicles or receptor binding to the extracellular matrix as causes of increased molecular brightness in ErbB2 cells by colocalization experiments. Upon labeling intact cells with a fluorescent antibody against the extracellular portion of ErbB2, a strong correlation between the antibody and the ErbB2-mYFP signals was observed, confirming that ErbB2 homoclusters reside in the plasma membrane and internal vesicles do not contribute to the observed signal (Fig. S5 A and B). Furthermore, there was no colocalization between ErbB2 macroclusters and focal contacts (Fig. S5 C and D), clathrin-coated pits (Fig. S5 E and F) or endocytic vesicles (Fig. S5 G and H). However, labeling of caveolin revealed a significant degree of colocalization with ErbB2 macroclusters (Fig. S2B and Fig. 4E). Although the correlation coefficient was intermediate in value ($r = 0.5$), visual inspection of the images revealed a tendency of ErbB2 macroclusters to be surrounded by caveolae (Fig. 4E). This observation suggests that the colocalization is driven more by the local membrane environment around caveolae rather than a specific association of ErbB2 with caveolin itself. ErbB1 was also found to colocalize with ErbB2 macroclusters (Fig. S5 I and J).

SI Materials and Methods. ErbB expression levels in stably transfected cell lines. The CHO clones, designated F1-4 and F1-10 (1, 2), express $\sim 6 \cdot 10^5$ and $\sim 5 \cdot 10^4$ ErbB1-eGFP/cell, respectively. Expression levels were characterized by flow cytometry using Qifikit (Dako-Cytomation, DAKO). HeLa-ErbB1eGFP cells express

$\sim 2 \cdot 10^5$ ErbB1-eGFP/cell (3). The cells, designated A4erbB2, express $\sim 1 \cdot 10^6$ ErbB2-mYFP/cell and $\sim 2 \cdot 10^4$ endogenous ErbB2 (4). A4erbB2 cells express $\sim 1.2 \cdot 10^6$ endogenous ErbB1/cell. CHO cells express endogenous ErbB proteins at a level undetectable by flow cytometry or Western blot. The cell line, designated CHO-ErbB2mYFP, expresses $\sim 3 \cdot 10^5$ ErbB2-mYFP/cell (5). For microscopy experiments cells were cultured in 2- or 8-well coverglass chambers (NalgeneNunc International) and stimulated with 100 nM EGF (R&D Systems) at room temperature.

Plasmids, transfection. A 21 amino acid acyl carrier protein (ACP) sequence mt1.3 was derived by Natalie George (6) through mutagenesis and selection from the full length *Escherichia coli* ACP. To generate ACP-ErbB2, which can be labeled by Sfp transferase, this sequence was inserted after residue 23 of the signal peptide of the ErbB2 ORF by In-Fusion cloning into an EcoRV digest of pcDNA3-ErbB2 with the following oligonucleotide sequence: TTTTGGTGGGGCCTGGATTCCCTGGATACCGTG-GAACTGGTGATGGCGCTGGAAGAAGAATT. The Δ VPV deletion mutant (Δ VPV-ACP-ErbB2) was created by PCR of the ACP-ErbB2 ORF and ligated into pcDNA3 with NotI and XbaI at the 5' and 3' ends, respectively. The wild-type and mutant ErbB2 plasmids were confirmed by sequencing.

The original ErbB2mYFP (A206K) in the pEYFP N1 vector has a long, 19 amino acid linker sequence containing several valine and proline residues (4). To eliminate any possible influence of either the linker or the terminal VPV amino acids in ErbB2 we recloned the wild-type ErbB2 and Δ VPV-ErbB2 with NheI/HindIII into mYFP pcDNA3.1zeo+ (mYFP inserted HindIII toXhoI). The names of the resultant vectors are ErbB2-short-mYFP and Δ VPV-ErbB2-short-mYFP. This strategy results in only a lysine-leucine dipeptide linker from the HindIII site between ErbB2 and the mYFP sequence.

For transfection ~ 1 million HeLa cells were electroporated by the Nucleofector device of Lonza using $2 \mu\text{g}$ plasmid DNA, solution R, and program I-013.

Isolation of Sfp transferase, synthesis of Atto565-CoA and labeling of cells expressing ACP-tagged ErbB2 using Sfp transferase. Sfp phosphopantetheinyl transferase-His₆ enzyme was purified from pET29-Sfp (the kind gift of Jun Yin, Harvard Medical School) according to author's protocol (7). Atto565-CoA was synthesized from Atto565-maleimide (Atto-Tec GmbH) and CoA dithium salt (Sigma-Aldrich) according to published protocols (7, 8) and purified on a C18 column by HPLC with an acetonitrile/ammonium acetate gradient. ACP-ErbB2 was labeled in the membrane of expressing cells with $0.2 \mu\text{M}$ Sfp transferase and $5 \mu\text{M}$ Atto565-CoA in the presence of 10 mM MgCl_2 in Tyrode's buffer for 20 min at room temperature followed by washing and subsequent N&B analysis of the cells.

Antibodies, cell labeling, and fluorescence microscopy for colocalization experiments. Mab528, a monoclonal antibody against the extracellular portion of ErbB1, was prepared from the supernatant of the HB-8509 hybridoma cell line obtained from ATCC. The anti-ErbB2 antibody, trastuzumab, was purchased from Roche Ltd. Mab528 and trastuzumab were labeled with AlexaFluor647 (Invitrogen-Molecular Probes) according to the manufacturer's specifications. ErbB1 phosphorylated at Tyr1068 and the light chain of clathrin were detected with the mouse monoclonal antibody 2236L (Cell Signaling) and CON.1 (Dianova), respectively. α -actinin and caveolin were labeled with rabbit polyclonal anti-

bodies (α -actinin: A2543, Sigma–Aldrich; caveolin: ab18199, Abcam). Cells were fixed in 3.7% formaldehyde for 30 min on ice and labeled with the primary antibodies in permeabilization buffer (PBS containing 0.1% (v/v) Triton X-100 and 1 mg/mL BSA) for 30 min followed by secondary labeling with Cy5-conjugated anti-mouse or anti-rabbit IgG.

To investigate the uptake of transferrin cells were starved of iron in iron poor Medium 199 for 24 h and subsequently incubated in the presence of 10 μ g/mL AlexaFluor633-conjugated holotransferrin (Invitrogen–Molecular Probes) for 30 min at 37 °C.

Number and brightness analysis. An IX81 Olympus microscope with the FluoView FV1000 confocal configuration was used to carry out number and brightness analysis according to Digman et al. (9). Live cells were analyzed at room temperature in Tyrode's buffer with 10 mM glucose and 0.1% BSA. Measurements were started immediately after removing cells from 37 °C and finished within 30 min to avoid changes in the distribution of receptors at room temperature. Image acquisition was performed in the pseudophoton counting mode with an UPlanSApo 60 $\times$ (NA = 1.35) objective. The pixel dwell and frame times were 10 μ s and 3.26 s, respectively, for imaging transfected cells, or 2 μ s and 1.1 s, respectively, for imaging soluble eGFP, mYFP, or Atto565. eGFP and mYFP were excited at 488 and 514 nm, respectively, using an AOF-adjusted laser intensity of 0.1% corresponding to $\sim$ 70 and $\sim$ 90 μ W, respectively, delivered to the sample. Atto565

was excited at 543 nm using a laser intensity of 130 μ W measured at the sample. A single frame consisted of 512 $\times$ 512 pixels and the pixel size in the x and y directions was 41 nm. The central part of the images was used for analysis to eliminate artifacts arising from scanner speed nonlinearity at the borders. A single optical slice containing the membrane adjacent to the glass coverslip was imaged 50–100 times. The image stack was analyzed using a custom-written Matlab program (Mathworks Inc.) incorporating functions of the DipImage toolbox (Delft University of Technology). The images were first registered (i.e., corrected for lateral shift) using the correctshift function of DipImage. Then, the mean fluorescence intensity of every slice was determined. Next, the pixel variance taken along the 3rd axis (i.e., the temporal dimension was calculated as a function of the number of slices in the stack). If the mean decreased by more than 10% due to stage shift or photobleaching or if the pixel variance did not converge to zero with increasing stack size, the stack was discarded. The apparent brightness (B) and the molecular brightness (ϵ) of each pixel was calculated according to the following equation:

$$B = \frac{\sigma^2}{\langle k \rangle} = \epsilon + 1$$

where σ^2 and $\langle k \rangle$ are the variance and mean, respectively, of a given pixel signal.

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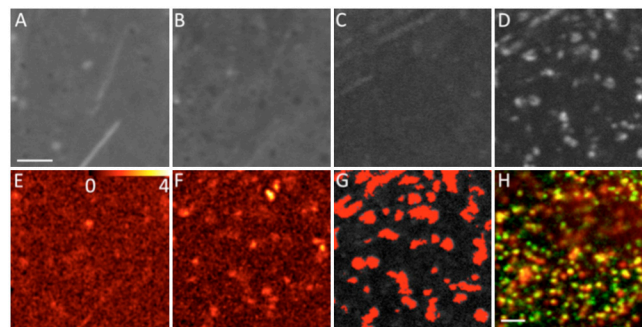


Fig. S1. Mean fluorescence and calculated brightness images of ErbB1 in quiescent and EGF-stimulated cells. (A–D). Mean fluorescence intensity images for F1-4 cells (A, B) and F1-10 cells (C, D). One hundred fluorescence images were recorded for the N&B analysis, and the mean fluorescence images of the starved (A, C) and EGF-stimulated cells (B, D) are shown. (E–F). Brightness values in F1-4 (E) and F1-10 cells (F) stimulated by EGF for 3 min shown on a pixel-by-pixel basis. (G). Pixels displaying high brightness values in EGF-stimulated F1-10 cells marked by red. These pixels are within the gate in Fig. 2D. (H). Colocalization between ErbB1-eGFP (green) and coated pits (red). F1-10 cells stimulated with EGF for 10 min were fixed, permeabilized and labeled by a monoclonal antibody against the light chain of clathrin followed by labeling with a Cy5-tagged secondary antibody. The bar in part A corresponds to 2 μ m, and is valid for A–G. The bar in H is also 2 μ m.

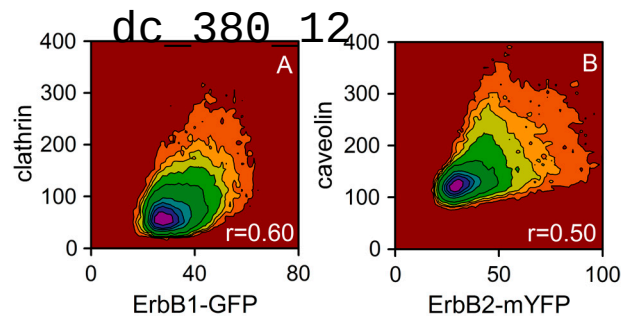


Fig. S2. Colocalization between ErbB1 and clathrin, and ErbB2 and caveolin. Two dimensional histograms showing the correlation between the fluorescence intensities of ErbB1-eGFP vs. clathrin (A) and ErbB2-mYFP vs. caveolin (B) were calculated. F1-10 cells were stimulated with 100 nM EGF for 5 min, fixed in 3.7% formaldehyde, permeabilized, and immune-labeled for coated pits with mAb against the light chain of clathrin (A). Starved A4erbB2 cells were labeled for caveolin by secondary immunofluorescence (B). The correlation coefficients (r) are also displayed in the figure. The corresponding fluorescence images are shown in Fig. S1H and Fig. 4E.

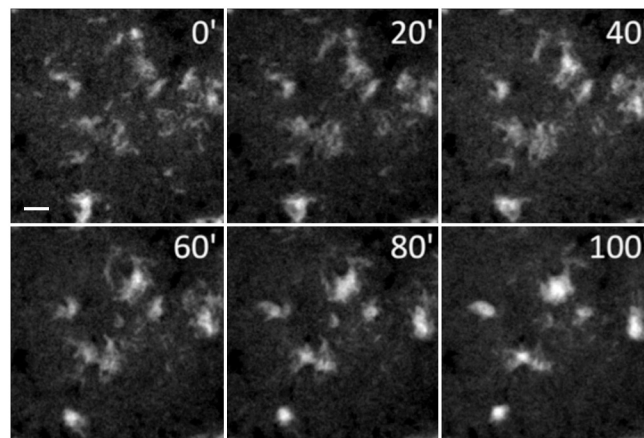


Fig. S3. Large-scale ErbB2 clusters grow at room temperature. A4erbB2 cells were starved in the absence of serum, and the same area of a single cell kept at room temperature was imaged in the confocal microscope at the indicated time points. The bar corresponds to 2 μ m.

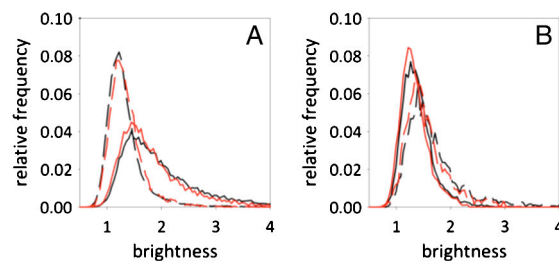


Fig. S4. The high cluster forming potential of ErbB2 is independent of the PDZ domain binding motif and the mYFP tag. A. HeLa cells were transfected with ErbB2-short-mYFP (black lines) or Δ VPV-ErbB2-short-mYFP (red lines) and N&B analysis was carried out two days after transfection. Brightness was determined inside (dashed lines) and outside (continuous lines) macroclusters of ErbB2. (B). HeLa cells were transfected with ACP-ErbB2 (black lines) or ACP- Δ VPV-ErbB2 (red lines), labeled with Atto565 two days after transfection and analyzed using the N&B method. Brightness was determined inside (dashed lines) and outside (continuous lines) macroclusters or ErbB2.

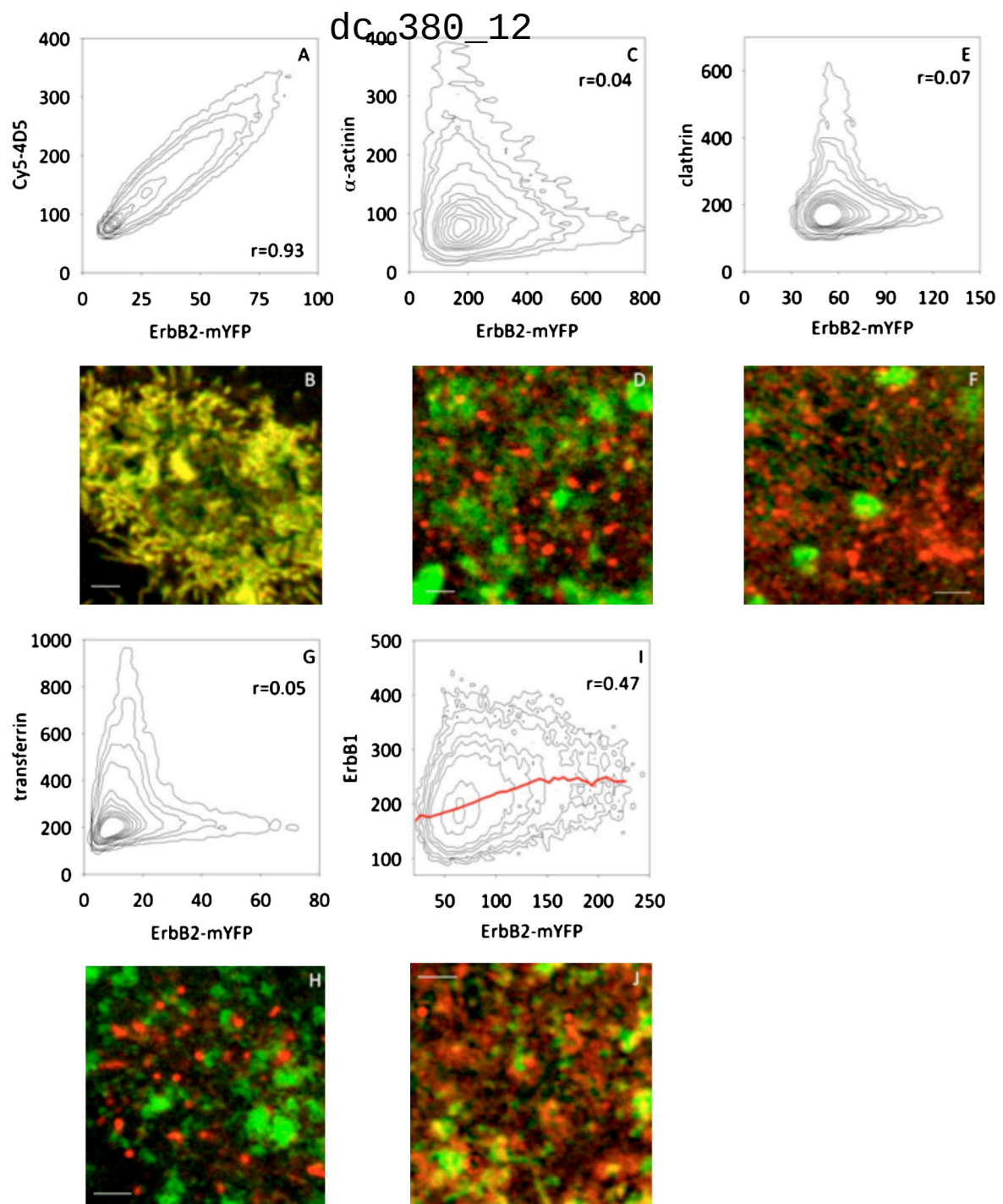


Fig. S5. Colocalization analyses of A4erbB2 cells. (A, B). Starved A4erbB2 cells were labeled with Cy5-tagged trastuzumab against ErbB2 without permeabilization. (C–F). Starved A4erbB2 cells were fixed, permeabilized and labeled with fluorescent antibodies against α -actinin (C, D) or clathrin light chain (E, F). (G–H). Starved A4erbB2 cells were incubated in the presence of fluorescent transferrin and fluorescence images of ErbB2-mYFP and transferrin were recorded. (I, J). Starved A4erbB2 cells were labeled with Cy5-tagged Mab528 (against ErbB1) without permeabilization. The red trend line shows the mean ErbB1 intensity corresponding to a given ErbB2-mYFP fluorescence intensity. The green and red intensities in the fluorescence images correspond to ErbB2-mYFP and the fluorescent antibody, respectively. The correlation between the green and red fluorescence intensities is displayed above the fluorescence images as contour plots. The correlation coefficients (r) are displayed in each contour plot. (Bars: B—3 μ m; D—4 μ m; F—2 μ m; H—2.5 μ m; J—2 μ m)

Table S1. Molecular brightness of Atto565-labeled ACP-ErbB2 and ErbB2-mYFP in wild-type and Δ VPV-ErbB2

	Soluble mYFP		Wild-type ErbB2-short-mYFP	Δ VPV-ErbB2-short-mYFP
Mol. brightness (mol/cluster)	0.063 $\pm$ 0.01	Inside macroclusters	0.89 $\pm$ 0.06 (14.1)	0.96 $\pm$ 0.08 (15.2)
		Outside macroclusters	0.38 $\pm$ 0.05 (6.0)	0.4 $\pm$ 0.04 (6.4)
	Soluble Atto565		Wild-type ACP-ErbB2	Δ VPV-ACP-ErbB2
Mol. brightness (mol/cluster)	0.19 $\pm$ 0.01	Inside macroclusters	0.81 $\pm$ 0.04 (7.8)	0.76 $\pm$ 0.04 (7.3)
		Outside macroclusters	0.44 $\pm$ 0.03 (4.2)	0.39 $\pm$ 0.04 (3.7)

The PDZ domain binding motif (VPV) was deleted from ErbB2-short-mYFP and ACP-ErbB2, and HeLa cells were transfected with plasmids coding for the Δ VPV-deletion mutants or wild-type ErbB2-short-mYFP or ACP-ErbB2 respectively. Cells expressing ACP-tagged ErbB2 were labeled with Atto565 using Sfp transferase two days after transfection and Atto565-labeled and mYFP-expressing cells were analyzed by the N&B method. The molecular brightness of ErbB2 was analyzed inside and outside macroclusters and the means $\pm$ standard errors of $\sim$ 10 cells are displayed. The molecular brightness of soluble monomeric fluorophores was also determined. To determine the average number of ErbB2 molecules in a molecular cluster (displayed in parentheses) cellular molecular brightness values were divided by that of the soluble fluorophore. Because HeLa cells express $\sim$ 30,000 endogenous ErbB2, these numbers had to be corrected for the presence of dark, unlabeled ErbB2. The total number of ErbB2 in transfected cells was $\sim 5 \cdot 10^5$ and $\sim 3 \cdot 10^5$ in the case of cells transfected with mYFP- and ACP-tagged proteins, respectively (numbers determined by Qifikit). Therefore, the number of molecules/cluster was divided by 0.94 and 0.9 in the two cases. In cells expressing ACP-tagged proteins a further correction was necessary to account for the incomplete labeling of the ACP-tag by Sfp transferase. It was determined in a separate experiment that $\sim$ 60% saturation of the ACP-tag is achieved with 5 μ M Atto565-CoA; therefore the number of molecules/cluster was divided by 0.6.

RESEARCH ARTICLE

Comparative analysis of fluorescence resonance energy transfer (FRET) and proximity ligation assay (PLA)

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Both fluorescence resonance energy transfer (FRET) and proximity ligation assay (PLA) are techniques used in the investigation of protein interactions but the latter has not been evaluated in a systematic way, prompting us to compare their performance quantitatively. Proteins were labeled with oligonucleotide- or fluorophore-conjugated antibodies and their proximity was analyzed by flow cytometry in order to obtain statistically robust data. Both intermolecular and intramolecular PLA signals reached saturation at high expression levels. At the same time, the FRET efficiency was independent of, while the FRET signal exhibited a strict linear correlation with the expression levels of proteins. When the density of oligonucleotide- and fluorophore-conjugated antibodies was systematically changed by competition with unlabeled antibodies the FRET signal was linearly proportional to the amount of bound fluorophore-tagged antibodies, whereas the PLA signal was again saturated. The saturation phenomenon in PLA could not be eliminated by decreasing the duration of the rolling circle amplification reaction. Our data imply that PLA is a semiquantitative measure of protein colocalizations due to non-linear effects in the reaction and that caution should be exercised when interpreting PLA data in a quantitative way.

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1 Introduction

The characterization of protein associations and their alterations induced by physiological ligands or drug molecules not only reveals the intricacies of signal transduction networks but also promises to be a profitable tool for the development of efficient therapeutical approaches. A large variety of methods is available for the investigation of the protein interactome including molecular biological techniques (e.g. immunoprecipitation, yeast two-hybrid [1], fluorescence complementation [2]), approaches based on

different manifestations of fluorescence resonance energy transfer (FRET) [3] and variations of fluorescence correlation spectroscopy (FCS) [4, 5]. The latter two techniques based on accurately described physical principles offer quantitative insight into protein clustering but their widespread application for specimens with high fluorescence background (e.g. tissue sections) is hampered by their sensitivity to errors in background subtraction. Therefore, efforts have been made to develop techniques applicable under conditions of high background as well. The VeraTag [6] and proximity ligation assays (PLA) [7, 8] are two novel additions to the proteomics toolbox for the detection of protein complexes under harsh experimental conditions. The VeraTag assay is based on the release of a fluorescent reporter molecule coupled to an antibody via a cleavable linker by singlet oxygen generated by a photosensitizer conjugated to another antibody. The fluorescent reporter, detected by CE is

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Abbreviations: EGF, epidermal growth factor; FRET, fluorescence resonance energy transfer; PLA, proximity ligation assay; RCA, rolling circle amplification

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only generated if the two antibodies bind in close proximity to each other. PLA is unique in that it is based on the amplification of a DNA sequence whose generation is dependent on the molecular proximity of two antibodies labeled by different oligonucleotides (priming and non-priming), which will form a circular DNA molecule with the help of connector oligonucleotides. The amplified DNA sequence is detected by a fluorescently labeled probe. Due to the amplification of the signal by the rolling circle amplification (RCA) reaction and the requirement for the proximity of two antibodies, the PLA technique is both sensitive and specific and provides a high signal to noise ratio. The PLA signal, i.e. the intensity of the fluorochrome-labeled oligonucleotide, is presumed to be proportional to protein associations, but a critical evaluation of this assumption has not been carried out.

One of the best characterized signal transduction networks involving complex molecular interactions is the ErbB family of receptor tyrosine kinases [9]. While ErbB2 is thought to form large-scale protein clusters in quiescent cells, ErbB1 (also known as epidermal growth factor (EGF) receptor) is much less prone to ligand-independent clustering and undergoes growth factor-mediated dimerization and clustering [10–14]. Although the convincing majority of experimental results support the EGF-induced increased homoassociation of ErbB1, a recent publication using PLA reported no elevation of ErbB1 homoclustering upon EGF stimulation [15]. The lack of previous thorough investigation on the quantitative nature of PLA and the aforementioned discrepancy prompted us to compare PLA to FRET, a well-established method for studying protein associations. We show that quantitative interpretation of PLA results is complicated by saturation effects and conclude that PLA is a semiquantitative measure of protein colocalizations.

2 Materials and methods

2.1 Cells, antibodies and growth factors

SKBR-3 and A431 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and grown under subconfluence according to their specifications. The cells were plated in μ -Slide 8-well ibiTreat chambers (Ibidi, Martinsried, Germany) for microscopic analysis or harvested by trypsinization for flow cytometric measurements.

The following anti-ErbB1 antibodies were used in the study: cetuximab purchased from Merck (Darmstadt, Germany); mAb 528 (Mab528) produced by the HB-8509 hybridoma cell line obtained from the ATCC; monoclonal antibody EGFR455 produced by hybridoma 455 obtained from the European Collection of Cell Cultures (Wiltshire, UK). Several anti-ErbB2 antibodies were applied for PLA and FRET experiments: trastuzumab was

purchased from Roche (Budapest, Hungary); pertuzumab and 7C2 were kind gifts from Genentech (South San Francisco, CA, USA). Antibodies produced by hybridoma cell lines were purified by protein A affinity chromatography. EGF was purchased from R&D Systems (Minneapolis, MN, USA). A protocol for activation of cells with EGF is given in the Supporting Information.

2.2 Preparation of fluorophore-conjugated antibodies and proximity probes

Conjugation of primary antibodies with AlexaFluor488, AlexaFluor546 and AlexaFluor647 (Invitrogen, Carlsbad, CA, USA) was carried out according to the manufacturer's specifications. The proximity probes (PLA probes) were prepared according to a method described previously [15]. Detailed description of the protocol is given in the Supporting Information.

2.3 Labeling of cells for in situ PLA

One million freshly harvested cells were washed in cold PBS and fixed in 1% formaldehyde for 10 min on ice. After washing twice in cold PBS, they were incubated at 37°C in blocking buffer (PBS containing 1% BSA and 5 μ g/mL sonicated salmon sperm DNA; Invitrogen) for 30 min. After centrifugation and removal of the blocking buffer, the cells were labeled with proximity probes and fluorescent antibodies at a concentration of 10–20 μ g/mL dissolved in antibody diluent (250 μ g/mL BSA and 2.5 μ g/mL salmon sperm DNA in PBS) for 1–2 h at 37°C. To remove unbound proximity probes, the cells were washed twice in TBST (20 mM Tris, 150 mM NaCl, pH 7.4, 0.05% Tween-20) followed by incubation with connector oligonucleotides (hybridization stock from Duolink Detection kit 563, Olink Bioscience, Uppsala, Sweden, Cat. no. 90104) for 15 min at 37°C. After washing in TBST, the samples were incubated with Duolink Ligation solution at 37°C for 15 min followed by washing in TBST and incubation with Duolink Amplification solution for 60 min at 37°C. Afterward, the cells were washed once in TBST and the product of the RCA reaction was visualized by adding 25 nM Cy5-labeled detector oligonucleotide (5'-Cy5- CAG TGA ATG CGA GTC CGT CT-3', TriLink Biotechnologies, San Diego, CA, USA) dissolved in detection buffer (0.1 μ g/mL salmon sperm DNA, 250 ng/mL BSA, 0.05% Tween-20, 2 $\times$ SSC). Finally, the cells were washed in TBST followed by fixation in 1% formaldehyde.

2.4 Measurement and analysis of PLA

A FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA), equipped with two lasers emitting at 488

and 635 nm, was used to analyze cells labeled with proximity probes and fluorophore-conjugated antibodies. The Cy5 signal of the oligonucleotide used to detect the PLA signal was excited at 635 nm and measured in the FL4 channel through a 661/16 nm band-pass filter. The fluorescence of AlexaFluor488-conjugated antibodies was excited at 488 nm and recorded in the FL1 channel through a 530/30 band-pass filter. Data of 20 000 cells recorded in list-mode format were analyzed by the ReFlex software [16]. Two-dimensional histograms (contour plots) were calculated in Matlab (Mathworks, Natick, MA, USA). The correlation of the PLA signal with the intensity of fluorophore-conjugated antibodies was analyzed by calculation of trend lines in Matlab. Briefly, the range of the x variable (intensity of AlexaFluor488-tagged antibody) was divided into 30 bins, and the average intensity of Cy5-labeled oligonucleotides (plotted on the y axis) in each bin was calculated and plotted as a function of the AlexaFluor488 intensity.

2.5 FRET

FRET was measured with a FACSArray flow cytometer (Becton Dickinson). Antibodies labeled with AlexaFluor546 and AlexaFluor647 were used as donor and acceptor, respectively. The donor, FRET and acceptor fluorescence intensities were measured in the *yellow*, *far red* and *red* channels, respectively. The *yellow* and *far red* intensities were excited with a 532-nm solid-state laser and detected using a 585/42 nm band-pass and a 685-nm long-pass filter, respectively. The *red* intensity was excited at 635 nm using a diode laser and measured using a 661/16 nm band-pass filter. The necessary controls, calibration samples and evaluation principles have been described elsewhere [17]. List-mode data of 2000 cells were analyzed using the ReFlex software [16]. All parameters were calculated on a cell-by-cell basis. FRET efficiency (E) is defined as the fraction of donor molecules relaxing by FRET. It is inversely related to the negative 6th power of the average separation distance between the donor and acceptor and is interpreted as a measure of the extent of association of the molecules [3, 18]. The unquenched donor intensity (I_d) is the fluorescence intensity of the donor in the absence of FRET and is proportional to the expression level of the protein labeled by the donor-tagged antibody. FRET intensity (I_{FRET}) is calculated according to the following equation:

$$I_{\text{FRET}} = I_d E \alpha \quad (1)$$

where α is a constant characteristic of instrument settings and the donor–acceptor pair [17]. I_{FRET} is the fluorescence signal measured in the FRET channel of the flow cytometer after correction for spectral spillover. Contour plots and trend lines were calculated in Matlab (Mathworks).

3 Results

3.1 Initial observations on the lack of correlation between FRET and PLA

As an initial approach to the comparison of PLA and FRET results, we compared the two methods in detecting EGF-induced increased homoassociation of ErbB1. While the homocustering of ErbB1 was significantly increased by EGF according to FRET results on cells labeled by two different antibodies (Mab528 and EGFR455), PLA did not reveal any significant change in the homoassociation of the receptor upon growth factor stimulation (Supporting Information Fig. S1A). Next, we analyzed the correlation between FRET and PLA results obtained from intermolecular and intramolecular measurements using another member of the ErbB family, ErbB2. Cells were labeled with donor- and acceptor-conjugated antibodies against different epitopes of ErbB2 to measure intramolecular FRET. Next, cells were labeled with donor-conjugated and acceptor-conjugated anti-ErbB2 antibodies against the same epitope of the protein to analyze homoassociation. The FRET efficiency was significantly higher when measuring intramolecular distances, while a different tendency was observed in the case of PLA measurements (Supporting Information Fig. S1B). Although the measurements were carried out by flow cytometry, the cells were also checked with fluorescence microscopy. As shown by the representative image in Supporting Information Fig. S2, the distributions of immunofluorescence labeling and the PLA signal were in accordance with published data.

3.2 Different dependence of FRET and PLA on expression levels when measuring ErbB2 homoassociations

Given the lack of correlation between FRET and PLA results, we compared the dependence of FRET and PLA on the density of the fluorophore-tagged or oligonucleotide-conjugated antibody. SKBR-3 cells were labeled with a mixture of trastuzumab-priming and trastuzumab-non-priming antibodies to analyze the homoassociation of ErbB2. Cells were also labeled with a fluorescent antibody against ErbB2 to measure the expression level of the protein. An obvious saturation phenomenon was observed at high, albeit not extreme, expression levels of ErbB2 in contour plots and trend lines showing the dependence of the PLA signal on ErbB2 expression level (Fig. 1). The fact that the splay (curved part) in the trend line was present in the middle range of the fluorescence distribution corresponding to the mean fluorescence intensity implies that the PLA signal may be saturated at expression levels often observed in cancer cells. The tendency of the PLA signal to saturate at high expression levels was observed at three different ratios (1:3, 1:1, 3:1) of the antibodies labeled with priming and

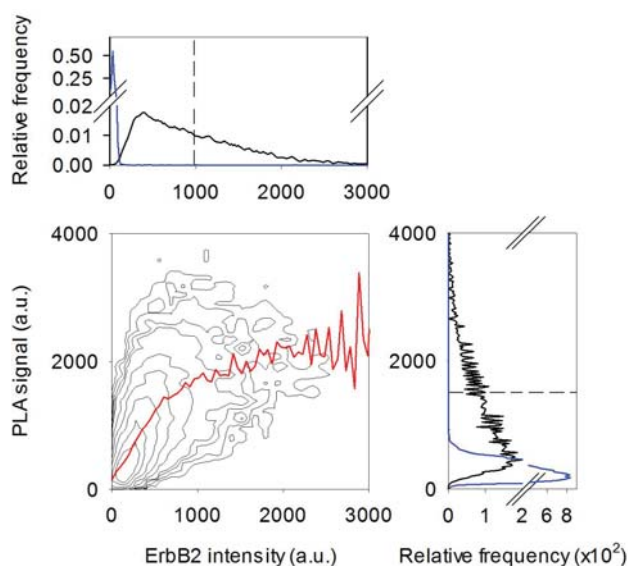


Figure 1. Saturation of PLA signal at high expression levels of ErbB2 measured by flow cytometry. SKBR-3 cells were labeled with a mixture of trastuzumab-priming and trastuzumab-non-priming antibodies to detect PLA for the homoassociation of ErbB2. The PLA signal was measured by Cy5-tagged detection oligonucleotides. Concomitant with the proximity probes, cells were also labeled with AlexaFluor488-7C2 to measure the expression level of ErbB2. A red trend line displaying the mean PLA signal as a function of ErbB2 expression is overlaid on the contour plot. Contour levels were defined logarithmically but the frequency distribution histograms, displayed at the corresponding axes of the contour plots, show the real distributions of fluorescence intensities. The black histograms in the marginal plots along the horizontal and vertical axes display the distributions of AlexaFluor488-7C2 intensity and the PLA signal, respectively. The dashed reference lines in the marginal plots display the mean of the black histograms. The blue histogram in the upper marginal graph shows the fluorescence intensity of unlabeled cells measured in the AlexaFluor488-channel. The blue histogram in the right marginal plot displays the PLA signal of negative control cells labeled with trastuzumab-priming antibody only.

non-priming oligonucleotides (Supporting Information Fig. S3).

In striking contrast, the FRET intensity, calculated according to Eq. (1), for ErbB2 homoassociation was not saturated and the trend line displayed a linear relationship between the FRET intensity and the expression level of ErbB2 even at high fluorescence intensities (Fig. 2A). Since the FRET intensity is proportional to both the amount of the fluorophore-labeled antibodies (I_d) and their proximity (E), it is analogous to the PLA signal. The fact that the dependence of the FRET intensity and the PLA signal on the expression level of the investigated protein is different suggests that the two phenomena reveal protein associations in distinctly different ways. In contrast to FRET intensity, FRET efficiency was independent of protein expression level (Fig. 2B). Although such a relationship is theoretically possible [19], it is in contrast to our previous results obtained by micro-

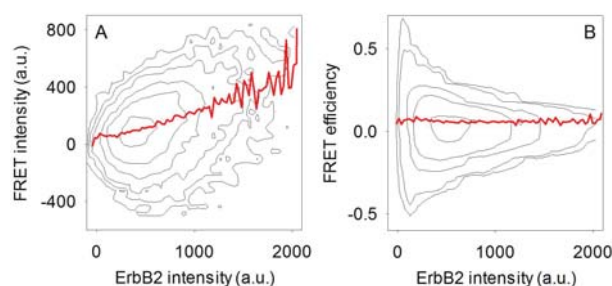


Figure 2. FRET exhibits linear behavior even at high expression levels of ErbB2. SKBR-3 cells were labeled with a mixture of donor-tagged and acceptor-tagged trastuzumab to measure the homoassociation of ErbB2 using flow cytometric FRET. The unquenched donor fluorescence intensity (ErbB2 intensity on the horizontal axes), representing the cellular expression level of the protein, was calculated. The FRET intensity and the FRET signal are measures of the extent of ErbB2 homoassociation. Contour levels in the two-dimensional histograms were defined logarithmically. The red trend lines display the average FRET intensity or FRET efficiency as a function of ErbB2 expression level.

scopic FRET measurements in which the homoassociation of ErbB2 in a pixel was proportional to the local density of the protein in accordance with the law of mass action [20]. Therefore, we analyzed the correlation between ErbB2 expression level and cell size estimated by forward angle light scattering. The unquenched donor intensity (I_d , a measure of ErbB2 expression) and the FRET intensity were proportional to cell size (Supporting Information Fig. S4). This finding is in accordance with the assumption that high expression level does not lead to high surface density since cells exhibiting high expression levels have high surface area. We concluded that the lack of dependence of FRET efficiency for ErbB2 homoassociation on the expression level is due to the constant surface density of the protein in cells with different expression levels.

3.3 FRET and PLA exhibit different dependence on expression levels in the case of intramolecular measurements

The proximity between two epitopes of the same molecule is expected to provide a consistent point of reference for comparing the reliability of PLA and FRET measurements since intramolecular distances do not depend on expression levels. Therefore, the proximity between the epitopes of two anti-ErbB2 antibodies, pertuzumab and trastuzumab, was analyzed by PLA and FRET. Similar to the aforementioned findings, intramolecular PLA exhibited saturation which was apparent in the brightest quarter of the fluorescence distribution corresponding to intensities ~ 2 times higher than the mean (Fig. 3). In contrast, FRET intensity showed a strong linear correlation with the expression level and the

FRET efficiency was constant at all expression levels when measuring the proximity of the same epitopes using FRET (Fig. 4). We concluded that the saturation phenomenon in PLA manifests itself in intra- and intermolecular measurements as well.

3.4 Antibody density distinctly influences FRET and PLA measurements

To adjust the antibody density on the cell surface in a controlled way and measure the dependence of PLA on antibody density, cells were labeled with pertuzumab-non-priming, trastuzumab-priming and AlexaFluor488-trastuzumab antibodies. The total concentration of trastuzumab-priming and AlexaFluor488-trastuzumab antibodies was kept constant, but the ratio of trastuzumab-priming was changed from 100 to 0%. The amount of cell-bound trastuzumab-priming antibody was calculated from the decrease in the fluorescence intensity of AlexaFluor488-

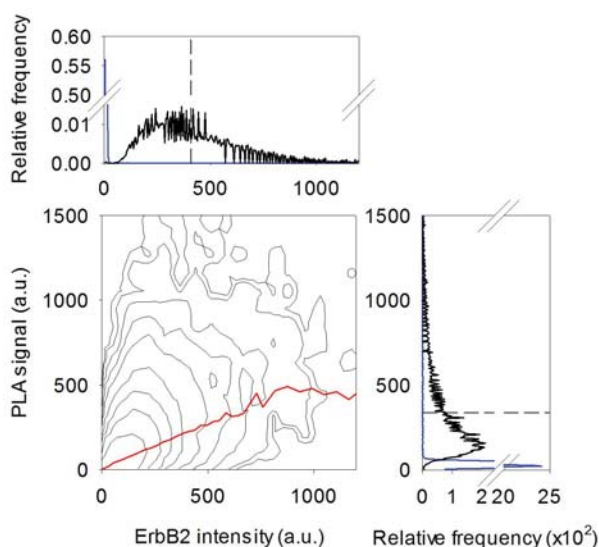


Figure 3. The PLA signal is saturated at high densities of proximity probes against epitopes of the same molecule. Two epitopes on the ErbB2 molecule were labeled with pertuzumab-priming and trastuzumab-non-priming antibodies to detect an intramolecular PLA signal measured by Cy5-tagged detection oligonucleotides. At the same time, cells were also labeled with AlexaFluor488-7C2 to measure the expression level of ErbB2. The red trend line superimposed on the two-dimensional histograms shows the mean PLA signal as a function of the ErbB2 expression level. Contour levels were defined logarithmically. The black histograms in the marginal plots on the top and the right display the distributions of AlexaFluor488-7C2 intensity and the PLA signal, respectively. The dashed reference lines in the marginal plots display the mean of the black histograms. The blue histogram in the upper marginal graph shows the fluorescence intensity of unlabeled cells measured in the AlexaFluor488-channel. The blue histogram in the right marginal plot displays the PLA signal of negative control cells labeled with trastuzumab-non-priming antibody only.

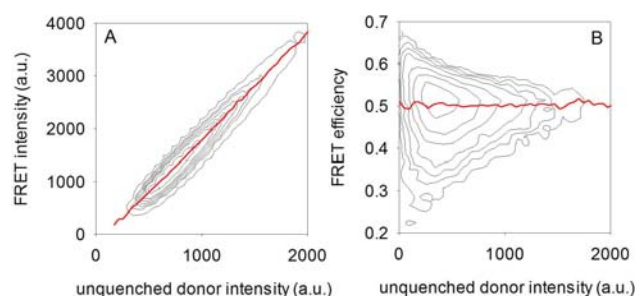


Figure 4. Intramolecular FRET efficiency is constant while FRET intensity is a linear function of antibody density. SKBR-3 cells were labeled with donor-tagged trastuzumab and acceptor-tagged pertuzumab to measure intramolecular FRET between two epitopes of ErbB2 by flow cytometry. The unquenched donor fluorescence intensity, the FRET intensity and the FRET efficiency were calculated on a cell-by-cell basis. Contour levels in the two-dimensional histograms were defined logarithmically. The red trend lines display the average FRET intensity or FRET efficiency as a function of the unquenched donor fluorescence intensity.

trastuzumab. The PLA signal saturated when plotted as a function of the density of the trastuzumab-priming antibody (Fig. 5A). A principally similar experiment was performed using FRET as well. The pertuzumab-binding epitope of ErbB2 was saturated with donor-conjugated antibodies while acceptor-tagged and unlabeled antibodies competed for the trastuzumab-binding epitope. The FRET efficiency showed a strict linear dependence on the density of the acceptor-conjugated antibody (Fig. 5B). We concluded that the saturation phenomenon is reproducibly observed in PLA experiments.

3.5 Saturation effects in PLA are not significantly influenced by the duration of RCA

We tested whether the saturation phenomenon in PLA is related to depletion of nucleoside-triphosphate substrates during RCA. Therefore, we analyzed the dependence of the PLA signal on the expression level at three different incubation times for RCA (30, 90 and 150 min). The PLA signal increased with the incubation time, albeit not linearly (Fig. 5C). Although the degree of saturation tended to increase with the incubation time, it was obviously present in the case of the shortest incubation time as well (Fig. 5C). We concluded that substrate depletion during the amplification process in PLA cannot account for the saturation effects described above.

4 Discussion

The recent development of in situ PLA provided researchers with the possibility of detecting minuscule amounts of proteins and protein-protein interactions in cell lines and

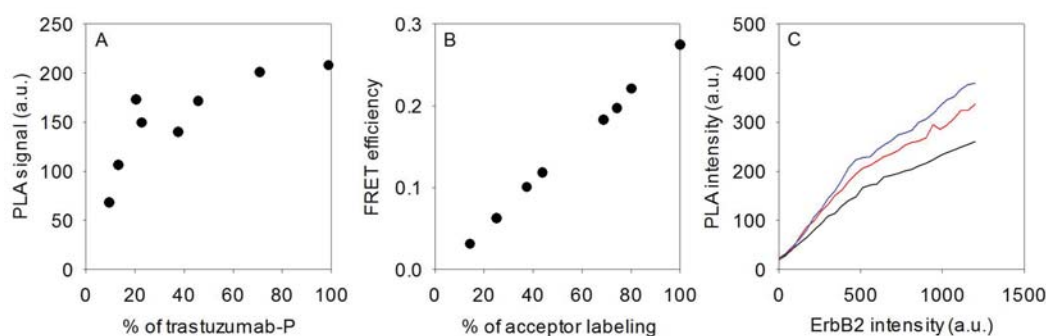


Figure 5. Saturation effects in PLA as a function of antibody density and duration of rolling circle amplification. (A) SKBR-3 cells were labeled with pertuzumab-non-priming antibody and a mixture containing varying amount of trastuzumab-priming and AlexaFluor488-trastuzumab antibodies. The intramolecular PLA signal between trastuzumab-priming and pertuzumab-non-priming antibodies was measured by a Cy5-tagged detection oligonucleotide. The percentage of the trastuzumab epitope occupied by trastuzumab-priming antibodies was calculated by subtracting the fluorescence intensity measured in the AlexaFluor488-channel from the fluorescence intensity of the sample labeled only by AlexaFluor488-trastuzumab and normalizing the difference to the latter intensity. (B) Intramolecular FRET efficiency was measured between donor-tagged pertuzumab and acceptor-tagged trastuzumab in SKBR-3 cells. The pertuzumab epitope was saturated with donor-conjugated antibody, while the saturation of the trastuzumab-binding epitope with acceptor-conjugated antibody was varied by adding an increasing concentration of unlabeled trastuzumab into the antibody mixture. The mean FRET efficiency of 20 000 cells measured by flow cytometry is displayed as a function of the relative density of the acceptor-tagged antibody (100% = acceptor signal in the absence of competing unlabeled antibody). (C) Intramolecular PLA signal was measured between trastuzumab-priming and pertuzumab-non-priming antibodies using a Cy5-tagged detection oligonucleotide. The duration of the rolling circle amplification reaction was 30 min (black curve), 90 min (red curve) or 150 min (blue curve). Cells were also labeled with AlexaFluor488-conjugated 7C2 to measure the expression level of ErbB2. Two-dimensional histograms were calculated as previously described and trend lines displaying the mean PLA signal as a function of ErbB2 expression level were determined.

tissues due to the high signal-to-noise ratio of fluorescently labeled samples [7, 21]. PLA has been applied to a variety of proteins under different experimental conditions. Among others, the homo- and heterodimeric interactions between members of the ErbB receptor tyrosine kinase family have been shown by flow cytometric in situ PLA [15]. However, the authors failed to detect any significant EGF-induced change in the homoclustering of ErbB1, a finding in stark contrast to previously published data obtained by FRET, fluorescence correlation spectroscopy, number and brightness analysis and molecular biology [11, 12, 22–26]. We also found that EGF treatment did not increase ErbB1 homo-association detected by flow cytometric in situ PLA, whereas our FRET measurements revealed significant growth factor-mediated enhancement in the homoclustering of ErbB1. This discrepancy convinced us that a systematic comparison between FRET and PLA is needed. We repeatedly observed that the PLA signal for the homoassociation of ErbB2 was saturated at medium to high expression levels while the FRET signal remained linear even at this expression density (Figs. 1 and 2). Since intramolecular measurements between different epitopes of the same molecule are not expected to display cell-to-cell variation, the investigations were also carried out using such an experimental system. While the FRET signal displayed an even stronger linear correlation with the expression level, PLA again displayed the saturation phenomenon at high fluorescence intensities (Figs. 3 and 4). By experimentally modifying the surface density of PLA probes and fluorescent antibodies the saturation effect was only observed in PLA experiments

(Fig. 5). These findings provide strong evidence for the non-linear dependence of the PLA signal on the density and association of proteins while supporting the predicted correlation between FRET and protein clustering. The threshold for the saturation behavior was at fluorescence intensity values $1\text{--}2 \times$ higher than the mean corresponding to $1\text{--}2$ million molecules/cell considering the expression level of ErbB2 in these cells [27]. Although this expression level is high, such an overexpression is not unusual in cancer cells.

FRET is based on solid physical principles. It involves the non-radiative transfer of energy from an excited donor to an appropriate acceptor within a range of 2–10 nm. In a homogenous system of a single donor–acceptor pair, the rate of FRET is inversely proportional to the sixth power of the distance between the donor and the acceptor for which FRET has been termed a “spectroscopic ruler” [3, 17, 18, 28]. In a cellular system containing a mixture of donor–acceptor complexes, FRET efficiency is influenced by the average donor–acceptor distance in protein clusters, the stoichiometry of molecular complexes and the fraction of clustered molecules. Despite this complexity, several successful models have been established in which quantitative predictions have been made for the dependence of FRET on protein clustering [12, 29–31]. In some cases, intermolecular FRET has been shown to display saturation as a function of cluster size [30] or fluorophore density [19]. In addition, even intramolecular FRET can exhibit anomalous behavior at high illumination intensities [32] or high fluorophore densities [33]. However, to the best of our

knowledge, such effects have not been reported for intramolecular FRET between two epitopes of the same molecule under conditions similar to our experimental system. According to our results, FRET efficiency for intramolecular measurements was independent of the amount of the fluorophore, while FRET intensity (defined according to Eq. 1) was linearly proportional to it. Since the quantitative relationship between the PLA signal and experimental conditions (density of proximity probes, incubation times, protein clustering) is unknown, we can only speculate about the reason for the saturation effect observed in PLA experiments. Steric hindrance between densely packed proximity probes might prevent enzymes from taking part in the amplification process. The fact that steric hindrance and non-linear effects indeed take place in PLA, most likely in the amplification phase, is implied by the “quantal” appearance of spots of PLA signals (“blobs”) of uniform size and brightness. Although the proximity of two epitopes of the same molecule is expected to be homogenous on the cell surface, the PLA signal is not evenly distributed. If the amplification process “kicks in,” the PLA signal develops to a uniform blob. If the amplification process is prevented from starting due to steric hindrance or statistical reasons the PLA signal will be completely absent. Consequently, in situ PLA allows the detection of only a fraction of the interacting molecules since it depends not only on molecular proximities but also on the equilibrium of association/dissociation reactions and enzymatic processes [7]. In addition to these presumed non-linear effects, depletion of the dNTP substrate might also contribute to the saturation phenomenon to some extent. However, we have shown that substrate depletion is unlikely to play a significant role in the saturation effects. As a consequence of the aforementioned saturation effects, the lack of an increase in the PLA signal upon treatment has to be interpreted with caution. It may reflect that the treatment was indeed ineffective in increasing protein associations, but may also be caused by saturation of the PLA signal.

Although in situ PLA offers great advantages in determining protein interactions, post-translational modifications and protein associations in patient samples [7], one should be aware of its limitations. While the RCA reaction provides a strong signal-to-noise ratio by amplifying a circular DNA sequence ~1000-fold [8, 21], it also seems to be the reason for the saturation phenomenon. Although it has been suggested that PLA, similar to FRET, could be used as a “molecular ruler” by varying the type of protein binder (IgG or Fab) and the length of the oligonucleotide [8], the method seems to fall short of the requirements for such quantitative measurements. We conclude that due to the saturation effects, PLA is recommended as a semi-quantitative tool for measuring protein associations.

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The authors have declared no conflict of interest.

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PROTEOMICS

Supporting Information for Proteomics

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and Peter Nagy

**Comparative analysis of fluorescence resonance energy transfer (FRET)
and proximity ligation assay (PLA)**

dc_380_12

Supplementary material to

**Comparative analysis of fluorescence resonance energy transfer (FRET) and proximity ligation
assay (PLA)**

Maria-Magdalena Mocanu, Tímea Váradi, János Szöllősi, Peter Nagy

SUPPLEMENTARY MATERIALS AND METHODS**Preparation of oligonucleotide-labeled proximity probes**

Primary antibodies at a concentration of 4 mg/ml in PBS were conjugated for two hours at room temperature with a 30-fold molar excess of succinimidyl 4-hydrazinonicotinate acetone hydrazone (SANH; ThermoScientific). Unbound SANH was removed by gel filtration using Bio-Gel P-6 columns (BioRad, Hercules, CA) equilibrated with conjugation buffer (100 mM MES, 150 mM NaCl, pH 4.8). A 3.5-fold molar excess of aldehyde-modified priming and non-priming oligonucleotides (TriLink Biotechnologies, San Diego, CA) were conjugated for three hours at room temperature with SANH-conjugated antibodies (priming oligonucleotide: 5'-aldehyde-AAA AAA AAA ATA TGA CAG AAC TAG ACA CTC TT-3', non-priming oligonucleotide: 5'-aldehyde-AAA AAA AAA AGA CGC TAA TAG TTA AGA CGC TTU UU-3', U=2'-O-Methyl RNA). The excess of oligonucleotides was removed by HPLC using a Superdex-75 column or by gel filtration using Sephadex superfine G-100 columns (Pharmacia, Uppsala, Sweden). The concentration of selected pooled fractions was estimated by gel electrophoresis and binding of oligonucleotide-conjugated primary antibodies to cells was checked by immunofluorescence.

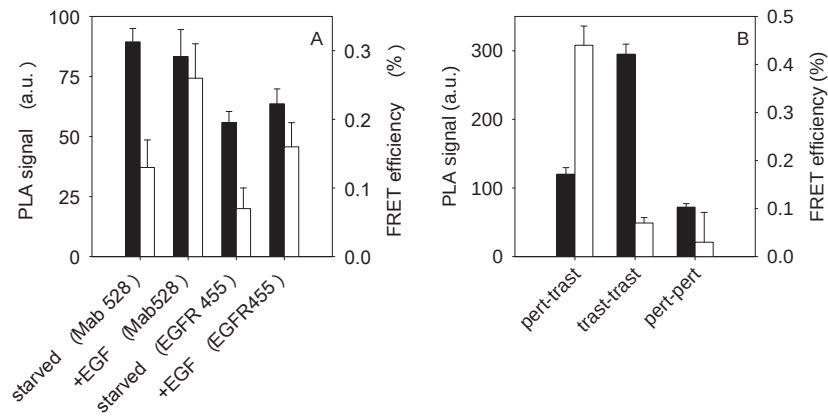
Measurement of PLA by confocal microscopy

A Zeiss LSM 510 confocal laser scanning microscope (Carl Zeiss AG, Jena, Germany) was used to image cells. Hoechst 33342 nuclear staining was excited with a UV laser at 351 nm and its emission was detected through a 385-470 nm band-pass filter. Alexa488 was excited with an argon ion laser at 488 nm and its emission was detected through a 505-550 nm band-pass filter. Cy5 was excited at 633 nm and its emission was detected using a 650 nm long-pass filter. Confocal images were acquired with the pinhole size adjusted to 1 Airy unit using a 63× (NA=1.4) oil immersion objective.

Activation of cells with EGF

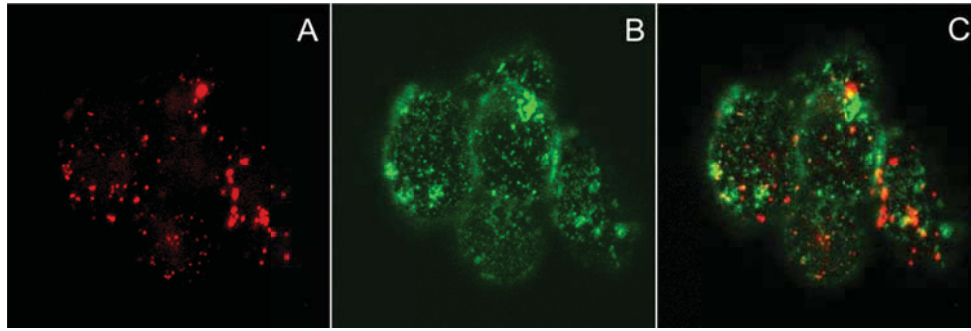
A431 cells were starved for 24h in medium containing 0.1% fetal calf serum before the experiment, harvested by trypsinization followed by washing and treatment with 100 nM EGF for 3 minutes at 37°C. After the stimulation the cells were labeled for FRET or PLA measurements.

SUPPLEMENTARY FIGURES

Figure S1 Discrepancies between PLA and FRET measurements.

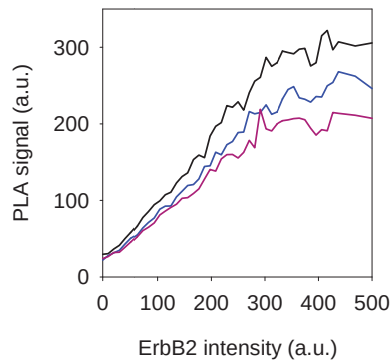
A. Starved A431 cells were labeled with a mixture of donor-tagged and acceptor-tagged Mab528 or with a mixture of donor-conjugated and acceptor-conjugated EGFR455. Both antibodies recognize an extracellular epitope on ErbB1, but Mab528 competes with EGF binding, whereas EGFR455 does not. FRET efficiency was measured using flow cytometry in quiescent and EGF-stimulated cells (white bars). In parallel, control and stimulated A431 cells were labeled with a mixture of Mab528-priming and Mab528-non-priming antibodies or with EGFR455-priming and EGFR455-non-priming antibodies and the PLA signal was measured by flow cytometry (black bars). **B.** SKBR-3 cells were labeled with the indicated mixture of primary fluorophore- or oligo-tagged antibodies. “trast” and “pert” stand for trastuzumab and pertuzumab, respectively. The first antibody was labeled with donor or priming oligonucleotide, while the second was acceptor-tagged or conjugated to the non-priming oligonucleotide. The PLA signal (black bars) and the FRET signal was (white bars) were measured in different samples. Error bars indicate the standard error of the mean.

Figure S2 Representative microscopic image of proximity probe-labeled cells.



SKBR-3 breast tumor cells were labeled with trastuzumab-priming, pertuzumab-non-priming and AlexaFluor488-7C2 antibodies against ErbB2. The PLA signal was visualized by a Cy5-labeled detection oligonucleotide (A). The immunofluorescence image of AlexaFluor488-7C2 and the overlay image are shown in B and C, respectively.

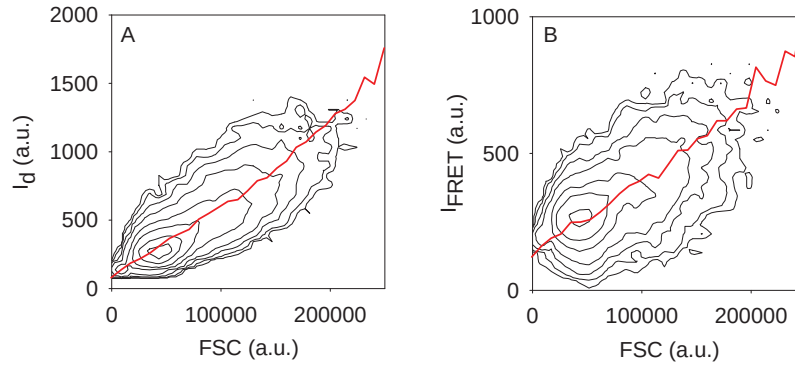
Figure S3 The saturation phenomenon in PLA is independent of the ratio of priming and non-priming oligonucleotides.



SKBR-3 breast tumor cells were labeled with pertuzumab-priming, pertuzumab-non-priming and AlexaFluor488-7C2 antibodies against ErbB2. The ratio of the priming and non-priming antibodies was 3:1 (black line), 1:1 (blue line) and 1:3 (red line) in three separate experiments. The PLA signal was measured with a Cy5-tagged detection oligonucleotide using flow cytometry. The fluorescence intensity of the AlexaFluor488-7C2 antibody was used as a measure of ErbB2 expression level. A two-dimensional histogram of the PLA vs. ErbB2 intensities was calculated and trend lines showing the mean PLA signal as a function of ErbB2 intensity are shown in the graph.

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Figure S4 Donor fluorescence intensity is proportional to forward scatter.



SKBR-3 cells were labeled with a mixture of donor-tagged and acceptor-tagged pertuzumab against ErbB2 and FRET was measured by flow cytometry. The unquenched donor fluorescence intensity (I_d) and the FRET intensity (I_{FRET} , see equation 1 in the paper) were calculated and plotted as a function of forward scatter (FSC) in two-dimensional histograms. The red trend lines display the mean unquenched donor intensity (A) or FRET intensity (B) as a function of FSC.



Molecular and Cellular Pharmacology

ErbB protein modifications are secondary to severe cell membrane alterations induced by elisidepsin treatment

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ABSTRACT

Elisidepsin is a marine-derived anti-tumor agent with unique mechanism of action. It has been suggested to induce necrosis associated with severe membrane damage. Since indirect evidence points to the involvement of ErbB receptor tyrosine kinases and lipid rafts in the mechanism of action of elisidepsin, we investigated the effect of the drug on the distribution of ErbB proteins and systematically compared the elisidepsin sensitivity of cell lines overexpressing ErbB receptors. Stable expression of a single member of the ErbB family (ErbB1–3) or co-transfection of ErbB2 and ErbB3 did not modify the elisidepsin sensitivity of CHO and A431 cells. However, elisidepsin induced the redistribution of ErbB3 and two GPI-anchored proteins (transfected GPI-anchored eGFP and placental alkaline phosphatase) from the plasma membrane to intracellular vesicles without comparable effects on ErbB1 and ErbB2. Elisidepsin increased the binding of a conformational sensitive anti-ErbB3 antibody without modifying the binding of other ErbB2 or ErbB3 antibodies, and it decreased the homoassociation of both ErbB2 and ErbB3. We also found that elisidepsin decreased the fluorescence anisotropy of a membrane specific fluorescent probe and induced a blue shift in the emission spectrum of Laurdan pointing to significant changes in the order of the plasma membrane possibly associated with the formation of liquid ordered domains. Although the distribution of ErbB proteins is preferentially altered by elisidepsin, our data question their role in determining sensitivity to the drug. We assume that induction of liquid ordered domains is the primary action of elisidepsin leading to all the other observed changes.

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1. Introduction

Despite ground-breaking discoveries about the molecular background of malignancy and promising new therapeutic approaches cancer remains the leading cause of death in developed countries (Albrecht et al., 2008). In addition to rational drug design screening the rich resources of natural habitats also provides a valuable source for drug discovery and development. Kahalalide F has been isolated from *Elysia rufescens*, an indopacific mollusc acquiring and accumulating it from algae (*Bryopsis pennata*) on which the *Elysia* mollusc feeds (Faircloth and Cuevas, 2006). Due to the scarcity of the natural source elisidepsin (Irvalec; PM02734) with a closely related structure has been synthesized (Provencio et al., 2009) which is currently undergoing phase II clinical investigations (Martin-Algarra et al., 2009).

It has been observed that Kahalalide F induces the disruption of lysosomal membranes (Garcia-Rocha et al., 1996), nuclear fragmen-

tation (Suarez et al., 2003) and necrotic cell death (Janmaat et al., 2005; Molina-Guijarro et al., 2009; Suarez et al., 2003). It was also suggested that Kahalalide F and elisidepsin act by inhibiting Akt activity (Janmaat et al., 2005; Ling et al., 2009). A recent paper about elisidepsin reported that *S. cerevisiae* lines mutated in genes involved in the regulation of vesicular trafficking were the most sensitive to the compound (Herrero et al., 2008). RNA interference-mediated knock-down of fatty acid 2-hydroxylase (FA2H) expression increased resistance to elisidepsin suggesting that the enzyme plays a role in the mechanism of action of the drug (Herrero et al., 2008). Fatty acid 2-hydroxylation has been implicated in hydrogen bond formation and stabilization of lipid rafts (Brown and London, 1998).

The ErbB family of receptor tyrosine kinases comprises four members (ErbB1–4) whose ligand-induced or overexpression-driven activation involves the formation of an extensive network of dimers and larger clusters (Citri and Yarden, 2006; Nagy et al., 1999; Szabó et al., 2008). They play a key role in the initiation and progression of several human cancers (Holbro et al., 2003) and are the targets of receptor-oriented therapies (Di Cosimo and Baselga, 2008). It has previously been hypothesized that ErbB protein expression determines Kahalalide F and elisidepsin sensitivity. Some authors pointed

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to ErbB3 as an exclusive factor in determining Kahalalide F sensitivity (Janmaat et al., 2005), while others claimed to have found a correlation between elisidepsin sensitivity and the expression levels of ErbB1, ErbB2 and ErbB3 (Ling et al., 2009). According to these data ErbB3 was efficiently dephosphorylated and degraded by elisidepsin (Ling et al., 2009).

To investigate whether ErbB proteins play a role in determining elisidepsin sensitivity, we analyzed the importance of ErbB1–3 in elisidepsin-induced responses. Here we show that although elisidepsin indirectly affects the association and distribution of ErbB2 and ErbB3, their expression is insufficient to increase the sensitivity of CHO and A431 cell lines to the drug. On the other hand, elisidepsin induced significant alterations in the order of the plasma membrane. We assume that these alterations are the primary actions of elisidepsin which can potentially lead to all the other observed changes including the redistribution of ErbB and GPI-anchored proteins.

2. Materials and methods

2.1. Cells and plasmids

SKBR-3, MCF-7, CHO and A431 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and grown according to their specifications. The CHO-ErbB2-3 cell line stably expressing both ErbB2 and ErbB3 was generated by successive transfections of ErbB2 and ErbB3 into CHO cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The ErbB2–pcDNA3 plasmid obtained from Yosef Yarden (The Weizmann Institute of Science, Rehovot, Israel) was transfected into CHO cells and transfected cells were selected with 1 mg/ml G418. Cells stably expressing ErbB2 in their membrane isolated by flow cytometric sorting of cells showing positive staining with trastuzumab were transfected with ErbB3–pcDNA6. The ErbB3–pcDNA6 plasmid was generated from the YFPN1-ErbB3 plasmid obtained from József Tözsér (Department of Biochemistry and Molecular Biology, University of Debrecen, Hungary). Briefly, the ErbB3 sequence was amplified by PCR using YFPN1-ErbB3 as the template. The two PCR primers contained restriction sites for KpnI and NotI. The amplified ErbB3 sequence was ligated into a pcDNA6 plasmid (Invitrogen) linearized by KpnI and NotI restriction enzymes. Cells stably transfected with ErbB3–pcDNA6 were selected with 30 µg/ml blasticidin, and were further enriched by flow cytometric sorting of cells showing positive staining with H3.90.6. CHO-ErbB2 and CHO-ErbB2-3 cells were found to express $\sim 2 \times 10^5$ ErbB2 and the double-transfected cell line expressed $\sim 10^5$ ErbB3 proteins according to flow cytometric characterization by Qifikit (Dako, Glostrup, Denmark). Untransfected CHO cells do not express ErbB1 or ErbB3, but display a very low endogenous expression of ErbB2 undetectable by flow cytometry and Western blotting (Tzahar et al., 1996; Zurita et al., 2004). A431-erbB1-eGFP, A431-erbB2-mYFP and A431-erbB3-citrine cell lines, stably expressing ErbB1-eGFP (ErbB1 fused to enhanced GFP), ErbB2-mYFP (ErbB2 fused to monomeric YFP) and ErbB3-citrine, respectively, were kindly provided by Donna Arndt-Jovin (Max Planck Institute for Biophysical Chemistry, Göttingen, Germany) and have been described elsewhere (Lidke et al., 2004). The ErbB expression profile of the cell lines used in the experiments is summarized in supplementary Table 1. For microscopic experiments cells were cultured in chambered 8-well cover slips (Nalge Nunc International, Rochester, NY). For flow cytometry cells were harvested by trypsinization.

2.2. RNA interference

Cells were transfected with validated “MISSION shRNA” plasmid coding a short hairpin RNA (shRNA) against ErbB3 (TRCN0000009835, NM_001982.x-4705s1c1; sequence: CCGGAGGTTAGGAGTAGATATT-GACTCGAGTCAATATCTACTCTAACCTCTTTTTC; Sigma-Aldrich, St. Louis, MO). Transfection was carried out with the Nucleofector device

of Lonza (Cologne, Germany) using solution T and program X-001 for A431 cells and solution C and program E-009 for SKBR-3 cells. Cells were seeded in 96-well plates after transfection and allowed to express shRNA for 60 h followed by a 3-day treatment with elisidepsin.

2.3. Antibodies and chemicals

The anti-ErbB2 antibody, trastuzumab (Herceptin®) was purchased from Roche (Budapest, Hungary). Anti-ErbB3 antibodies H3.90.6, H3.90.12 and H3.105.5 were obtained from LabVision/Thermo Fisher Scientific (Fremont, CA). Monoclonal anti-PLAP (placental alkaline phosphatase, A2951) and anti-actin (A4700) antibodies were purchased from Sigma-Aldrich. The conjugation of primary antibodies with AlexaFluor488, AlexaFluor546 and AlexaFluor647 (Molecular Probes/Invitrogen, Eugene, OR) dyes was carried out according to the manufacturer's specifications. Alexa647-conjugated F(ab')₂ fragment of goat anti-mouse IgG was purchased from Molecular Probes/Invitrogen (Eugene, OR). Heregulin-β1 was obtained from R&D Systems (Minneapolis, MN). 4'-(trimethylammonio)-diphenylhexatriene (TMA-DPH) and Laurdan (6-dodecanoyl-N,N-dimethyl-2-naphthylamine) were purchased from Sigma-Aldrich. TMA-DPH and Laurdan were dissolved in tetrahydrofuran and dimethyl sulfoxide, respectively. Elisidepsin was manufactured by PharmaMar (Madrid, Spain) and dissolved in dimethyl sulfoxide at a concentration of 1 mg/ml.

2.4. Western blotting

Cells were lysed in Ripa buffer supplemented with protease and phosphatase inhibitor cocktails (Roche). Protein extracts were resolved in denaturing polyacrylamide gels and electroblotted to PVDF membranes. Expressions of ErbB2 and ErbB3 receptors were detected using mAb#2248 and mAb#4754, respectively (Cell Signaling Technology, Danvers, MA).

2.5. Measurement of cell viability

The short-term cytotoxic effect of elisidepsin was tested by microfluorometric propidium iodide uptake assay. Cells were seeded at high density in black, clear bottom 96-well microtiter plates and allowed to grow to confluency. Fresh culture medium (supplemented with 25 mM HEPES, pH 7.4, and 50 µg/ml propidium iodide) in the absence or presence of different concentrations of elisidepsin was added in quadruplicates and the uptake of propidium iodide was quantified by plate fluorimetry at excitation and emission wavelengths of 531 and 632 nm, respectively, at 37 °C using a Victor3 Multilabel Counter (Perkin Elmer, Waltham, MA). The long-term effect of elisidepsin on cell viability was assayed by measuring the oxidation of a water-soluble tetrazolium salt by mitochondrial dehydrogenases using the cell proliferation reagent WST-1 (Roche Diagnostics GmbH, Mannheim, Germany). Cells (7×10^3) were plated into single wells of 96-well plates 24 h before the experiment. Cells were treated with a dilution series of elisidepsin for 2 h in triplicate followed by incubation for 72 h in cell culture medium in a CO₂ incubator at 37 °C. The absorbance of the WST-1 reagent was measured by an ELISA reader at 450 nm and 620 nm. The IC₅₀ value, the concentration leading to the death of 50% of the cells, was determined by fitting the following equation to the normalized absorbance data using the ‘fit’ command of Matlab (Mathworks Inc., Natick, MA):

$$A_{min} + \frac{A_{max} - A_{min}}{1 + 10^{n(\log(c) - \log(IC_{50}))}} \quad (1)$$

where A_{min} and A_{max} are the lowest and highest absorbance values, respectively, n is the Hill coefficient, c is the concentration of elisidepsin and log is logarithm with base 10.

2.6. Statistical analysis of cell viability

One-way analysis of variance (ANOVA) was used to compare the IC50 values and the propidium iodide fluorescence intensities (at 30 and 60 min) of the different cell lines using SigmaStat (Systat Inc., San Jose, CA).

2.7. Fluorescence resonance energy transfer (FRET)

FRET was measured with a FACSArray flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Antibodies labeled with AlexaFluor546 and AlexaFluor647 were used as donor and acceptor, respectively. The donor, FRET and acceptor fluorescence intensities were measured in the Yellow, Far Red and Red channels, respectively. The Yellow and Far Red intensities were excited with a 532 nm solid state laser and detected using a 585/42 nm bandpass and a 685 nm longpass filter, respectively. The Red intensity was excited at 635 nm using a diode laser and measured using a 661/16 nm bandpass filter. The necessary controls, calibration samples and evaluation principles have been described elsewhere (Nagy et al., 2006). The FRET efficiency was calculated on a cell-by-cell basis using the ReFlex software (www.freewebs.com/cytoflex) (Szentesi et al., 2004).

2.8. Confocal microscopy

A Zeiss LSM510 confocal laser scanning microscope (Carl Zeiss AG, Jena, Germany) was used to image fluorescently stained cells. Alexa-Fluor488 was excited with the 488 nm line of an argon ion laser and its fluorescence was detected above 505 nm. The emission of eGFP excited at 488 nm was recorded with a 505 long-pass filter. mYFP and citrine were excited at 514 nm and detected above 530 nm. Confocal stacks were acquired with the pinhole size adjusted to 1 Airy unit and image distances of 0.5 μ m along the Z axis using a 63 $\times$ (NA = 1.4) oil immersion objective. Image analysis and the preparation of orthogonal projections were carried out with a custom-written Matlab (Mathworks Inc., Natick, MA) program incorporating DipImage commands (Delft University of Technology, Delft, The Netherlands).

2.9. Measurement of fluorescence anisotropy and generalized polarization

Trypsinized cells were resuspended in Hank's buffer at a concentration of 10^7 /ml and labeled with 2 μ M TMA-DPH or 2.5 μ M Laurdan at room temperature for 20 min. After TMA-DPH labeling cells were diluted in Hank's buffer without washing to a concentration of 10^6 /ml for fluorescence anisotropy measurements, whereas Laurdan-labeled cells were washed once and resuspended at a concentration of 10^6 /ml in Hank's buffer. Fluorescence measurements were carried out with a Fluorolog-3 spectrofluorimeter (Horiba Jobin Yvon, Edison, NJ). The temperature of the cuvette holder was adjusted to 37 $^{\circ}$ C by a circulating water bath. TMA-DPH was excited at 352 nm and its emission was measured at 430 nm. The fluorescence anisotropy (r) of TMA-DPH was measured in the L-format according to the following formula (Kuhry et al., 1983; Lakowicz, 2006):

$$r = \frac{I_{vv} - G I_{vh}}{I_{vv} + 2G I_{vh}} \quad (2)$$

where I_{vv} and I_{vh} are the vertical and horizontal components, respectively, of the fluorescence excited by vertically polarized light, and G is a correction factor characterizing the different sensitivity of the detection system for vertically and horizontally polarized light:

$$G = \frac{I_{hv}}{I_{hh}} \quad (3)$$

where I_{hv} and I_{hh} are the vertical and horizontal components, respectively, of the fluorescence excited by horizontally polarized

light. Laurdan was excited at 350 nm and its emission was detected in the blue range of its emission spectrum at 435 nm (I_{blue}) and at the red edge at 500 nm (I_{red}). Generalized polarization (GP) of Laurdan fluorescence was calculated according to the following formula (Harris et al., 2002; Parasassi et al., 1991; Sanchez et al., 2007):

$$GP = \frac{I_{blue} - I_{red}}{I_{blue} + I_{red}} \quad (4)$$

3. Results

3.1. Expression of ErbB1–3 does not alter the sensitivity of CHO and A431 cells to elisidepsin

In order to investigate the role of ErbB2 and ErbB3 in elisidepsin sensitivity we have established two CHO-derived cell lines stably expressing ErbB2 (CHO-ErbB2) or ErbB2 and ErbB3 (CHO-ErbB2–3). Western blotting confirmed that the cell lines expressed the transfected proteins (Fig. 1A). Further flow cytometric analysis demonstrated that CHO cells do not express any of the ErbB proteins at levels detectable by this technique (data not shown) in agreement with previous reports (Tzahar et al., 1996; Zurita et al., 2004). CHO-ErbB2–3 cells were found to express $\sim 2 \times 10^5$ ErbB2 and $\sim 10^5$ ErbB3. The expression levels of ErbB2 in CHO-ErbB2 and CHO-ErbB2–3 were comparable (Supplementary Table 1).

In order to investigate the long-term effect of elisidepsin on viability the parental CHO cells and the two derivative cell lines were treated with different concentrations of elisidepsin for 2 h and the cells were allowed to grow for 3 days in the absence of the drug. The three cell lines were found to be equally sensitive to elisidepsin (IC50 in CHO cells (mean $\pm$ standard error of the mean): $10 \pm 0.7 \mu$ M; IC50 in CHO-ErbB2 cells: $10.5 \pm 0.9 \mu$ M; IC50 in CHO-ErbB2–3 cells: $10.3 \pm 0.8 \mu$ M; $p > 0.1$ using analysis of variance; Fig. 1B). The effect of elisidepsin on cell survival/proliferation was not significantly affected by increasing the incubation time from 2 h to 3 days (data not shown) implying that the drug exerts its cytotoxic effects very early during drug exposure. In order to analyze the short-term effect of elisidepsin on viability and to investigate the time course of elisidepsin-induced killing we followed the uptake of propidium iodide in the presence of different concentrations of the drug for one hour. Both the extent and kinetics of elisidepsin-induced membrane permeabilization were comparable in the three cell lines since comparison of fluorescence intensities using analysis of variance did not provide evidence for significant differences among them ($p > 0.1$, Fig. 1C).

In order to confirm the lack of effect of ErbB protein expression on elisidepsin sensitivity in another cell line we checked the short- and long-term effects of the drug on A431 cells and its subclones stably transfected with ErbB1-eGFP, ErbB2-mYFP or ErbB3-citrine (A4erbB1, A4erbB2 and A4erbB3 cells, respectively). The ErbB expression profile of these cell lines is summarized in supplementary Table 1. Similar to CHO cells introduction of ErbB proteins (ErbB1–3) into A431 did not change the long-term sensitivity of cells to elisidepsin (IC50 in A431: $8.2 \pm 0.8 \mu$ M; IC50 in A4erbB1: $9.4 \pm 0.5 \mu$ M; IC50 in A4erbB2: $9.9 \pm 1.0 \mu$ M; IC50 in A4erbB3: $9.9 \pm 0.9 \mu$ M; $p > 0.1$ using analysis of variance; Fig. 2A). The short-term effectiveness of elisidepsin was not altered by overexpression of any of the ErbB proteins either ($p > 0.1$ using analysis of variance; Fig. 2B, Fig. S1). We concluded that the elisidepsin-induced cytotoxic effect is a rapid event and the expressions of ErbB1, ErbB2 and ErbB3 do not influence the sensitivity of cells to the drug.

The lack of any effect of ErbB3 overexpression in cells showing no or weak expression of the protein on elisidepsin sensitivity strongly implies that ErbB3 does not influence elisidepsin sensitivity. However, several previous reports presented evidence for the role of ErbB3 in the mechanism of action of the drug. Therefore, we

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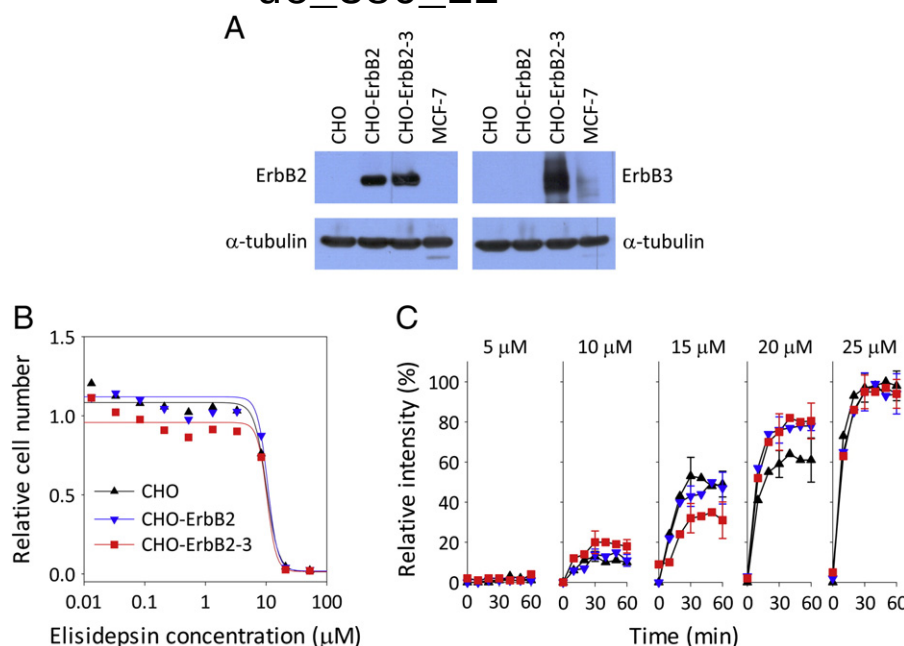


Fig. 1. Expression of ErbB2 and ErbB3 proteins in CHO cells does not increase their sensitivity for elisidepsin. A. Expression of ErbB2 and ErbB3 verified by Western blotting. Whole cell lysates of MCF-7 breast cancer cells, untransfected and transfected CHO cells were separated by SDS-PAGE followed by blotting with an anti-ErbB2 or anti-ErbB3 antibody. Membranes were stripped and reprobed with an anti- α -tubulin antibody. B. Long-term effect of elisidepsin on the viability of wild-type and transfected CHO cells. Untransfected CHO, CHO-ErbB2 and CHO-ErbB2-3 cells were plated in 96-well plates and treated in triplicate with elisidepsin for 2 h. Cells were cultured for another 3 days in the absence of elisidepsin and cell numbers were determined at the end of the experiment using the WST-1 reagent. Counts were normalized to untreated cells and are shown by the symbols as a function of elisidepsin concentration. The lines show the result of fitting according to Eq. 1. C. Short-term effect of elisidepsin on the viability of untransfected and transfected CHO cells. Confluent cultures of CHO, CHO-ErbB2 and CHO-ErbB2-3 cells were treated with the indicated concentrations of elisidepsin for 60 min and the uptake of propidium iodide was followed by microfluorimetry during the treatment. Fluorescence intensities were normalized to the maximum measured intensity in the presence of the highest concentration of the drug. The cell lines are marked by the same symbols as in part B. Error bars, shown only at 30 and 60 min for discernibility, indicate the standard error of the mean of four independent measurements. Two ANOVA tests were performed using fluorescence intensities at 30 and 60 min and they did not provide evidence for a significant difference between the cell lines ($p > 0.1$).

wanted to confirm our conclusion using RNA interference-mediated knock-down of ErbB3 production. In addition to A431 we have chosen SKBR-3 cells displaying a lower IC₅₀ value for elisidepsin ($1.5 \pm 0.2 \mu$ M, data not shown). Two days after transfection with an ErbB3 shRNA plasmid the expression level of ErbB3 was reduced by ~85% corresponding to an expression level of ~500 and ~2500 in A431 and SKBR-3 cells, respectively (Fig. S2). The IC₅₀ of the transfected cells did not significantly differ from control cells (IC₅₀ in mock-transfected SKBR-3: $2.64 \pm 0.8 \mu$ M; IC₅₀ in ErbB3 shRNA-transfected SKBR-3: $2.82 \pm 1.1 \mu$ M; IC₅₀ in mock-transfected A431: $9.48 \pm 1.2 \mu$ M; IC₅₀ in ErbB3 shRNA-transfected A431: 8.55

$\pm 1.4 \mu$ M) corroborating that elisidepsin responsiveness does not depend on ErbB3 expression (Fig. 3).

3.2. Elisidepsin specifically increases the binding of a conformation-sensitive anti-ErbB3 antibody

Our results presented in the previous section refute evidence for the role of ErbB proteins in elisidepsin sensitivity. However, it is still possible that the reported effects of elisidepsin on ErbB proteins are not artifacts, but rather indirect effects of the drug. Therefore, we performed several experiments to reveal if elisidepsin exerts any

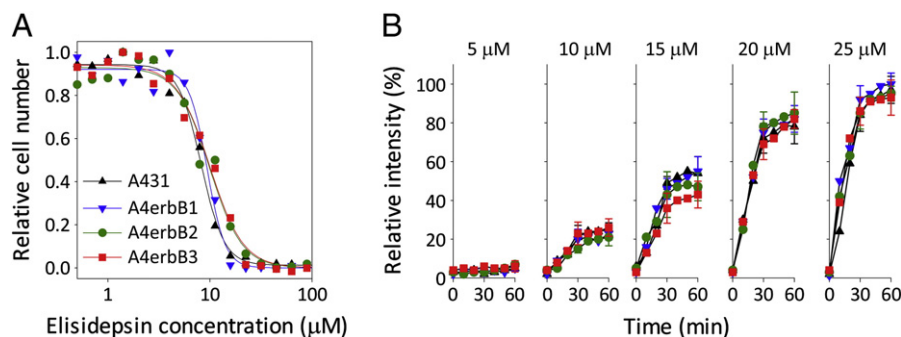


Fig. 2. Transfection of A431 cells with ErbB proteins does not influence their sensitivity for elisidepsin. A. Long-term effect of elisidepsin on the viability of wild-type and transfected A431 cells. Untransfected, ErbB1-eGFP- (A4erbB1), ErbB2-mYFP- (A4erbB2) and ErbB3-citrine-transfected (A4erbB3) A431 cells were plated in 96-well plates, treated with different concentrations of elisidepsin in triplicate for 2 h followed by culturing in the absence of the drug for 3 days. Cell numbers normalized to untreated cells are plotted as a function of elisidepsin concentration. The symbols and the lines show the measured data points and the result of fitting according to Eq. 1, respectively. B. Short-term effect of elisidepsin on the viability of untransfected and transfected A431 cells. Confluent cultures of A431, A4erbB1, A4erbB2 and A4erbB3 cells were treated with the indicated concentrations of elisidepsin for 60 min and the uptake of propidium iodide was followed by microfluorimetry during the treatment. Fluorescence intensities were normalized to the maximum measured intensity in the presence of the highest concentration of the drug. The cell lines are marked by the same symbols as in part A. Error bars, shown only at 30 and 60 min to avoid clutter, indicate the standard error of the mean of four independent measurements. Statistical analysis was carried out as described in the legend to Fig. 1.

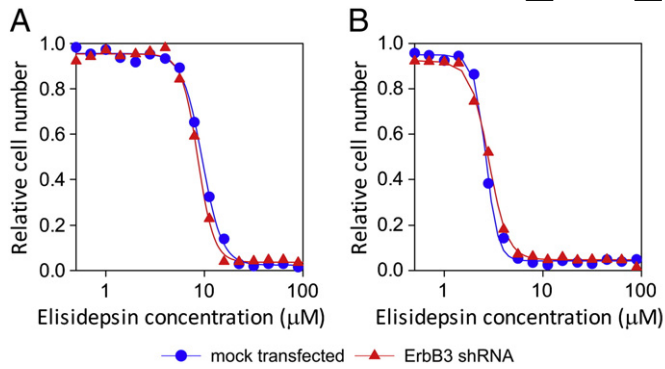


Fig. 3. RNA interference-mediated suppression of ErbB3 expression does not modify elisidepsin sensitivity. A431 (A) and SKBR-3 (B) cells were transfected with a plasmid expressing a shRNA against ErbB3. Control, mock transfected cells were treated identically in the absence of the plasmid. Sixty hours after transfection cells were treated with the indicated concentrations of elisidepsin for 2 h in triplicate followed by culturing in the absence of the drug for another 3 days.

influence on ErbB proteins. First, we tested whether the binding of antibodies against ErbB2 or ErbB3 is altered by treatment of cells with 10 μ M elisidepsin. Elisidepsin-treated and control CHO-ErbB2-3 cells were fixed, permeabilized and stained with fluorescent antibodies followed by flow cytometric analysis. Since the staining was carried out with permeabilized cells, the measured fluorescence intensities reflected the total amount of the antigen in single cells. Although elisidepsin did not alter the binding of two anti-ErbB2 (trastuzumab, pertuzumab) and two anti-ErbB3 antibodies (H3.90.6, H3.90.12), it significantly enhanced the amount or accessibility of the epitope for another antibody against ErbB3, H3.105.5 (Fig. 4). In the absence of elisidepsin the binding of the H3.105.5 antibody to CHO-ErbB2-3 cells was negligible which was significantly increased by elisidepsin. The increased binding of H3.105.5 was abolished by heregulin, the ligand of ErbB3 which is known to compete with the H3.105.5 antibody. Our results indicate that elisidepsin may induce a conformational change of ErbB3 leading to an altered accessibility of the epitope for the H3.105.5 antibody.

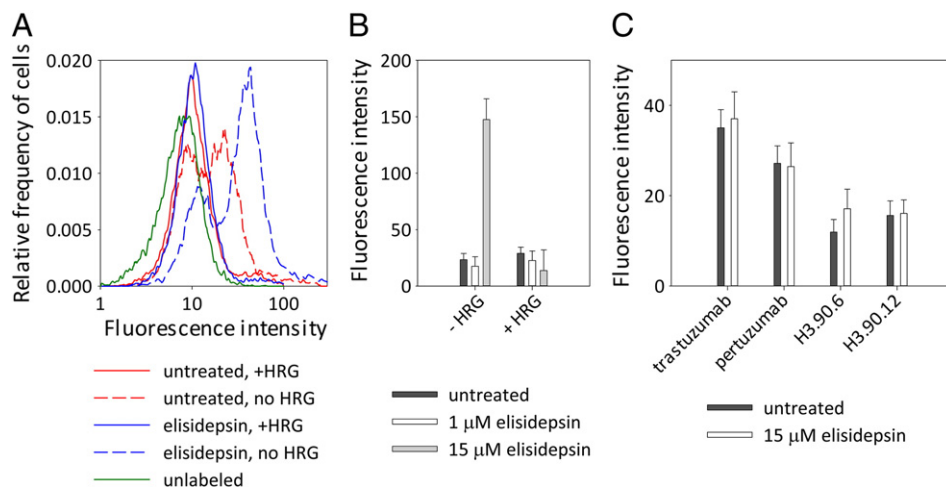


Fig. 4. Elisidepsin-induced changes in the binding of anti-ErbB2 and anti-ErbB3 antibodies. A. Control CHO-ErbB2-3 cells were fixed, permeabilized and labeled by fluorescent anti-ErbB3 antibody H3.105.5 in the absence or presence of heregulin (HRG). Cells treated with 15 μ M elisidepsin for 30 min were also labeled by H3.105.5 in the absence or presence of heregulin. The fluorescence histograms of 10,000 labeled cells and that of unlabeled cells recorded by flow cytometry in a typical experiment are plotted in the figure. The means of these measurements are shown in B. B. Fixed and permeabilized control CHO-ErbB2-3 cells and those treated with 1 μ M and 15 μ M elisidepsin were stained with fluorescent H3.105.5 in the presence (+ HRG) and absence (– HRG) of heregulin. Background-corrected mean fluorescence intensities of three independent measurements ($\pm$ standard error of the mean) are shown in the figure. C. Fixed and permeabilized control (CHO-ErbB2-3) cells and those treated with 15 μ M elisidepsin were stained with fluorescent trastuzumab, pertuzumab (both against ErbB2), H3.90.6 or H3.90.12 (both against ErbB3). Background-corrected mean fluorescence intensities of three independent flow cytometric measurements ($\pm$ standard error of the mean) are plotted in the figure.

3.3. Elisidepsin decreases the homoassociation of ErbB2 and ErbB3

The direct or indirect involvement of a protein in the action of a drug is often reflected in the altered association state of the protein. Since the previous results imply that ErbB3 is affected by elisidepsin, we tested whether the homo- and heteroassociations of ErbB2 and ErbB3 are altered by elisidepsin treatment. Flow cytometric FRET measurements revealed that while the homoassociations of both ErbB2 and ErbB3 were significantly decreased by elisidepsin, their heteroassociation did not change (Fig. 5). The FRET efficiencies for the heteroassociation of ErbB2 and ErbB3 were markedly different when the donor and acceptor antibodies were swapped. This finding is in accordance with the dependence of FRET efficiency on the quantity of the acceptor (Kenworthy and Eddin, 1998). The elisidepsin-induced effect on the homoassociations of ErbB2 and ErbB3 were dose-dependent and the drug concentrations required for the cytotoxic effect and the impact on the homoassociations were comparable. These findings imply that the observed changes in the homoassociations of ErbB2 and ErbB3 are indirectly linked to the mechanism of action of elisidepsin although neither ErbB2 nor ErbB3 is necessary for elisidepsin sensitivity.

3.4. Elisidepsin induces the redistribution of ErbB3 and GPI-anchored proteins into the intracellular space

FRET measurements are sensitive for the interactions of proteins on the molecular scale, but the distribution of molecules and their clustering on the micrometer scale are not revealed. In order to show the effect of elisidepsin on this latter dimension of associations control and elisidepsin-treated CHO-ErbB2-3 cells were fixed, permeabilized and stained with fluorescent antibodies against ErbB2 and ErbB3. Confocal microscopy convincingly showed that elisidepsin did not change the distribution of ErbB2, while that of ErbB3 was significantly altered in that the drug induced the redistribution of the protein from the plasma membrane to the intracellular space. Sometimes a distribution pattern typical of nuclear localization was also observed (Fig. 6). In order to corroborate the selective effect of elisidepsin on the distribution of ErbB3 we investigated A431 cells stably transfected with one of ErbB1–3 fused to spectral variants of GFP. While

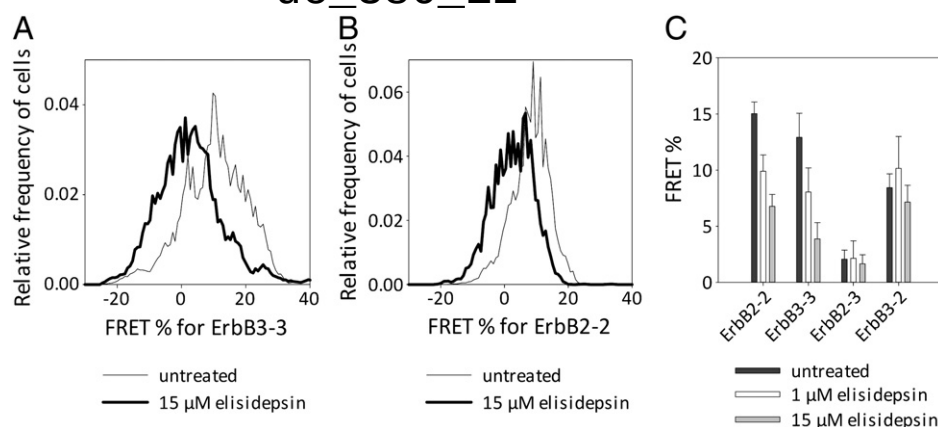


Fig. 5. Elisidepsin-induced changes in the homo- and heteroassociation of ErbB2 and ErbB3. A. CHO-ErbB2-3 cells were treated with 15 μ M elisidepsin for 30 min followed by labeling control and the elisidepsin-treated cells with a mixture of donor- and acceptor-conjugated anti-ErbB3 antibodies (H3.90.6). The efficiency of FRET in 10,000 cells was determined by flow cytometry. B. Control CHO-ErbB2-3 cells and those treated with 15 μ M elisidepsin for 30 min were labeled with a mixture of donor- and acceptor-tagged anti-ErbB2 antibodies (trastuzumab) and the FRET efficiency of 10,000 cells was determined by flow cytometry. C. Control cells and those treated with 1 μ M or 15 μ M elisidepsin were analyzed by flow cytometric energy transfer measurements. ErbB2-2 and ErbB3-3 designate the homoassociation of ErbB2 and ErbB3, respectively. ErbB2-3 stands for the heteroassociation of ErbB2 and ErbB3 in the sample in which ErbB2 and ErbB3 were labeled by donor-conjugated and acceptor-conjugated antibodies, respectively. The donor and acceptor fluorophores were reversed in the sample designated by ErbB3-2, i.e. ErbB2 and ErbB3 were labeled by acceptor-conjugated and donor-conjugated antibodies, respectively. The columns and the error bars represent the means and their standard errors, respectively, determined from three independent measurements.

elisidepsin (10 μ M) did not affect the typical membrane localization of ErbB1 and ErbB2, ErbB3 accumulated intracellularly upon treatment with the drug displaying a distribution pattern typical of intracellular vesicles (Fig. 7). Since lipid rafts were also implicated in the mechanism of action of elisidepsin (Herrero et al., 2008) and the GPI-anchor is known to localize proteins to lipid rafts (Brown and London, 1998; Sharma et al., 2004), we investigated the effect of the drug (10 μ M) on the distribution of GPI-eGFP transiently transfected to A431 cells. GPI-eGFP was present both in the plasma membrane and in intracellular vesicles in untreated cells, but an almost exclusive localization in vesicles was observed after elisidepsin treatment

(Fig. 7). In order to exclude the possibility that GPI-anchored eGFP does not represent the native distribution of GPI-anchored proteins we repeated the experiments by immunofluorescent labeling of an endogenous GPI-anchored protein, placental alkaline phosphatase (PLAP), in A431 cells. In untreated cells PLAP exhibited preferential membrane localization which was changed to a pattern characteristic of vesicular or diffuse intracellular distribution (Fig. 8). These results imply that elisidepsin selectively induces the redistribution of GPI-anchored proteins and ErbB3 from the plasma membrane.

3.5. Elisidepsin induces abrupt changes in the order of the plasma membrane

The finding that elisidepsin exerts many effects on the interactions and distribution of ErbB proteins is in contrast with the lack of effect of ErbB protein overexpression on elisidepsin sensitivity. We attempted to relieve this apparent contradiction by assuming that all the changes induced by elisidepsin observed by us and others, including the redistribution of membrane proteins and membrane permeabilization, are the consequences of primary membrane effects caused by the drug. Since drugs whose primary target is the plasma membrane have been shown to induce changes in the fluidity or order of the lipid bilayer (Balogh et al., 2005; Engelke et al., 1997; Sear, 2009), we used two fluorescent probes to investigate this aspect of the mechanism of action of elisidepsin. The fluorescence anisotropy of TMA-DPH specifically reports on the microviscosity (fluidity) of the plasma membrane since it cannot cross the cell membrane and enter the membrane of intracellular organelles due to its positive charge (Harris et al., 2002; Kuhry et al., 1983; Matkó and Nagy, 1997). Treatment of A431 cells with 10 μ M elisidepsin induced an almost instantaneous decrease in the fluorescence anisotropy of TMA-DPH indicating an increased membrane fluidity followed by a gradual and incomplete return of anisotropy to the initial value in ~20 min (Fig. 9A). The generalized polarization of Laurdan is a sensitive measure of the order of the plasma membrane and of the extent of penetration of water molecules into the plasma membrane (Harris et al., 2002; Parasassi et al., 1991; Sanchez et al., 2007). The generalized polarization of Laurdan was already increased by elisidepsin in one minute, it peaked at ~2 min and gradually and partially declined toward the initial value in ~20 min (Fig. 9B). The increased generalized polarization of Laurdan indicates a higher order of the plasma membrane and a restricted access of water to Laurdan in the plasma membrane.

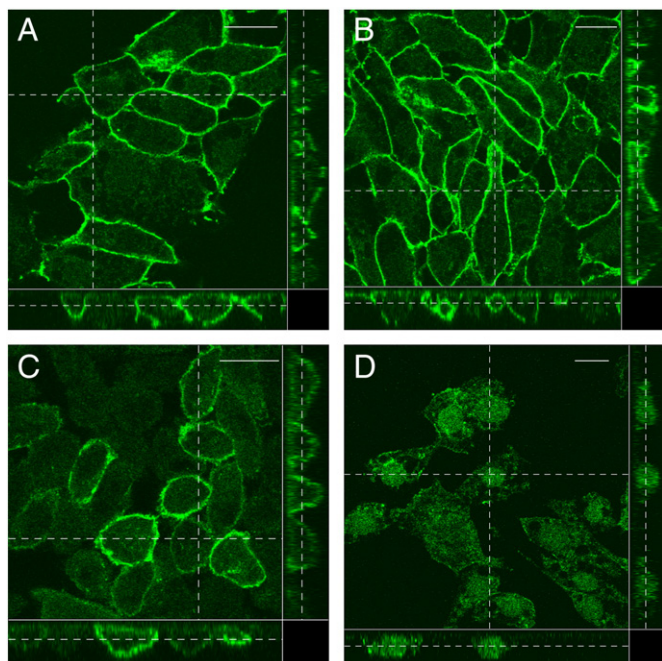


Fig. 6. Elisidepsin induces redistribution of ErbB3 without affecting ErbB2 in CHO cells. A,C: Control (A) CHO-ErbB2-3 cells and those treated with 10 μ M elisidepsin for 30 min (C) were fixed, permeabilized and stained with AlexaFluor647-labeled trastuzumab against ErbB2. B,D: Control (B) and elisidepsin-treated (D) CHO-ErbB2-3 cells were fixed, permeabilized and stained with AlexaFluor647-labeled H3.90.6 against ErbB3. Orthogonal sections of confocal microscopy image stacks are shown. Bar = 10 μ m.

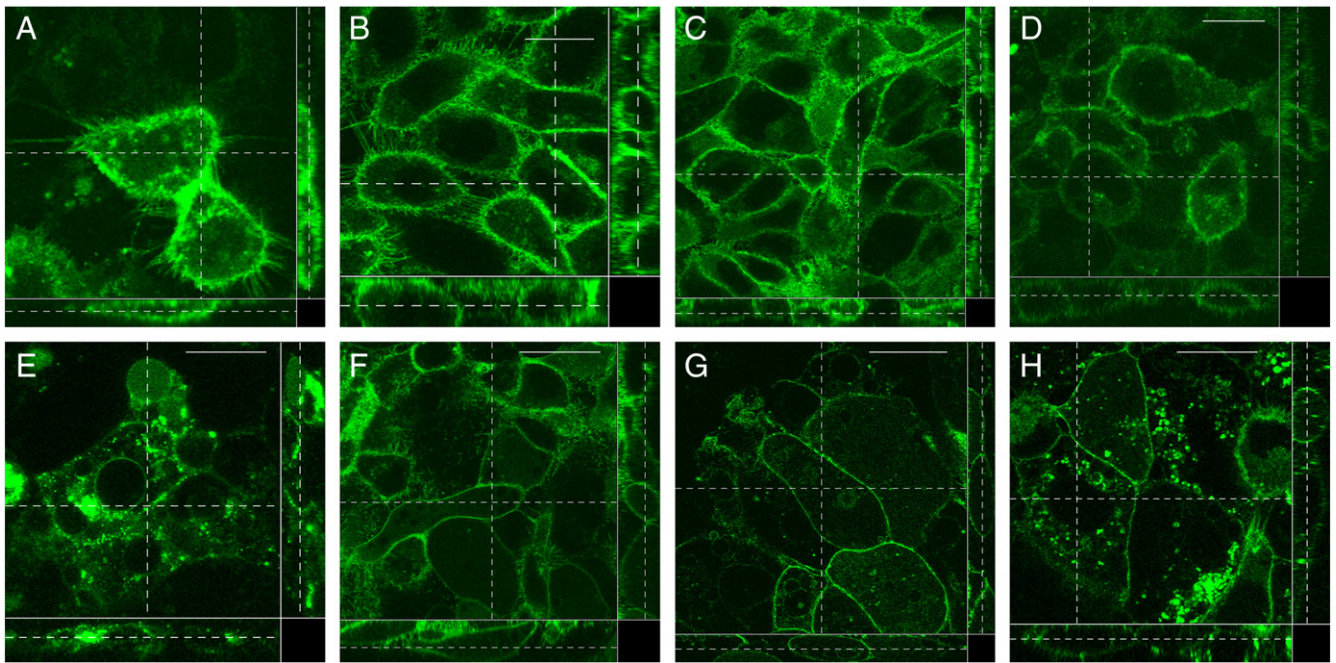


Fig. 7. Elisidepsin preferentially induces internalization of GPI-anchored eGFP and ErbB3-citrine in A431 cells. A431 cells expressing GPI-eGFP, ErbB1-GFP, ErbB2-mYFP or ErbB3-citrine were treated with 10 μ M elisidepsin for 30 min and confocal microscopy images of control (top row) and treated cells (bottom row) were acquired. Bar = 10 μ m. A,E: Control (A) and elisidepsin-treated (E) A431 cells transiently transfected with GPI-eGFP. B,F: Control (B) and elisidepsin-treated (F) A431 cells stably expressing ErbB1-eGFP (A4erbB1 cells). C,G: Control (C) and elisidepsin-treated (G) A431 cells stably expressing ErbB2-mYFP (A4erbB2 cells). D,H: Control (D) and elisidepsin-treated (H) A431 cells stably expressing ErbB3-citrine (A4erbB3 cells).

Dimethyl sulfoxide, the vehicle of elisidepsin, did not induce any significant change in the anisotropy of TMA-DPH or the generalized polarization of Laurdan.

In order to establish that the observed membrane effects of elisidepsin are not unique to the A431 cell line we repeated the experiments with SKBR-3 cells displaying higher sensitivity to elisidepsin. The overall tendency of the elisidepsin-induced changes in the anisotropy of TMA-DPH and in the generalized polarization of Laurdan in SKBR-3 cells was similar to that observed in A431 cells (Fig. 9C,D). The effect size was slightly bigger in SKBR-3 than in A431 cells and the changes were less transient in the former case. The abrupt and specific changes in the order and fluidity of the lipid bilayer support our assumption that the primary target of elisidepsin is the plasma membrane.

4. Discussion

Here we demonstrate that although elisidepsin induced changes in the homoassociation and cellular distribution of ErbB and GPI-

anchored proteins, its cytotoxic effect was independent of the expression of ErbB1–3. On the other hand, elisidepsin was found to induce significant alterations in the order and fluidity of the plasma membrane. All of the observed effects were detected at comparable doses of the drug implying that they are either parts or consequences of its mechanism of action. Previous reports pinpointed ErbB3 (Janmaat et al., 2005) or ErbB1–3 proteins (Ling et al., 2009) as key factors determining Kahalalide F and elisidepsin sensitivity. The fact that the elisidepsin sensitivity of CHO- and A431-derived cell lines did not correlate with their ErbB protein expression levels argues against a decisive role of ErbB receptors in determining elisidepsin responsiveness. In particular, the finding that overexpression of ErbB2 or ErbB2 and ErbB3 in CHO cells (displaying no expression of ErbB1, -3 and -4 and very weak expression of ErbB2) did not alter elisidepsin sensitivity is a strong evidence for the lack of involvement of ErbB2 and ErbB3 in conferring elisidepsin sensitivity. The lack of influence of ErbB3 on elisidepsin responsiveness was also confirmed by showing that RNA interference-mediated knock-down of ErbB3 expression failed to alter the IC₅₀ value for elisidepsin. We excluded the

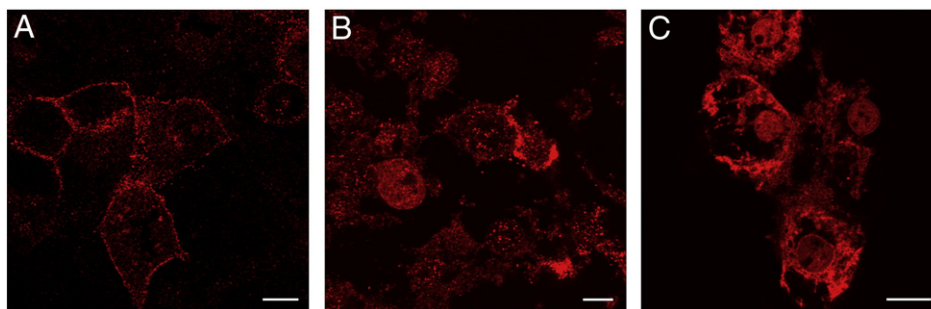


Fig. 8. Distribution of PLAP in control and elisidepsin-treated A431 cells. Control (A) and elisidepsin-treated (10 μ M, 30 min; B–C) A431 cells were fixed, permeabilized and labeled with a monoclonal antibody against PLAP. Visualization of the distribution of the protein was achieved by secondary staining with AlexaFluor647-conjugated goat anti-mouse IgG. Bar = 10 μ m.

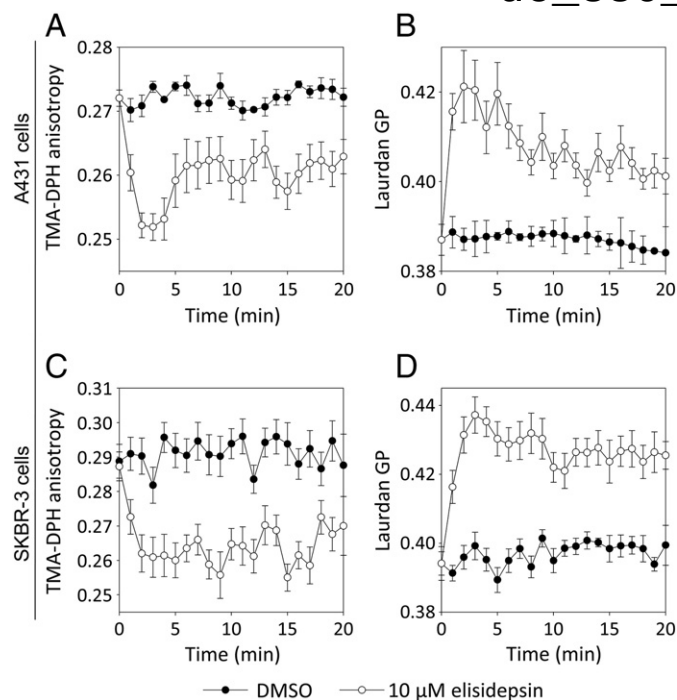


Fig. 9. Elisidepsin-induced changes in the order of the plasma membrane. The fluorescence anisotropy of TMA-DPH (A,C) and the generalized polarization of Laurdan (B,D) were measured in cells treated with 10 μ M elisidepsin or with an identical volume of DMSO. The mean $\pm$ standard error of three independent measurements is plotted in the top (A,B) and bottom (C,D) figures displaying the results obtained with A431 and SKBR-3 cells, respectively.

possibility that the transfected ErbB proteins were non-functional by showing that EGF and heregulin induced tyrosine phosphorylation of the transfected proteins (data not shown). Consequently, we think that the reported correlations between elisidepsin responsiveness and ErbB receptor expression levels were indirect effects related to drug-induced alterations in the cell membrane.

The IC₅₀ values in the cell lines we tested were somewhat higher than those published earlier ranging between 0.1 and 9 μ M (Ling et al., 2009). This difference could be due to different methodologies used in the previous studies compared to ours. In fact elisidepsin was applied for several days (usually 72 h) in previous experiments obtaining lower IC₅₀ values than those determined by us, while we treated cells for a shorter period of time. It is worth noting that the half-life of Kahalalide F is ~30 min in patients, so the brief *in vitro* treatment with elisidepsin is likely to mimic the *in vivo* situation (Martin-Algarra et al., 2009).

Elisidepsin induces characteristic changes in the order of the plasma membrane almost immediately after administration while changes in the association and distribution of membrane proteins (GPI-anchored and ErbB proteins) take place after a longer period of time. Therefore, we assume that the alterations in the distribution and association of ErbB and GPI-anchored proteins are the consequences of the elisidepsin-induced change in the structure of the membrane. Flow cytometric FRET investigations revealed that elisidepsin induces a decrease in the homoassociations of ErbB2 and ErbB3 without measurable changes in their heteroassociations. Both ErbB2 and ErbB3 have been found to form large-scale associations involving tens of proteins in quiescent cells (Landgraf and Eisenberg, 2000; Szabó et al., 2008). We assume that large protein clusters are preferentially disrupted as a result of the membrane alterations induced by elisidepsin since membrane-mediated interactions are thought to play a fundamental role in driving the formation of large protein aggregates (Kusumi et al., 2010). On the other hand, direct protein–protein interactions, which are not expected to be subverted by the membrane damage caused by elisidepsin, are responsible for the

creation of small protein clusters. Although conventional flow cytometric hetero-FRET measurements are not exquisitely suited for the determination of cluster size (Anikovskiy et al., 2008), the fact that the FRET value for the heterodimerization of ErbB2 and ErbB3 is smaller than those for their homoassociations is in agreement with the assumption that ErbB2–3 heteroclusters are smaller than their homoassociations.

We have also observed an increase in the binding of H3.105.5, a conformation-sensitive antibody against ErbB3, upon elisidepsin treatment. The specificity of this increase is underlined by the lack of elisidepsin-induced effect on the binding of other antibodies and the fact that heregulin reverses the increased binding of H3.105.5. We speculate that the membrane effects of elisidepsin might induce a change in the conformation of ErbB3 or in the accessibility of the epitope for H3.105.5. Elisidepsin-induced disruption of large-scale ErbB3 clusters (Landgraf and Eisenberg, 2000), supported by our FRET measurements, may lead to these conformational changes and consequent unmasking of the H3.105.5-binding epitope.

On the other hand, GPI-anchored proteins (GPI-eGFP, PLAP) and ErbB3 were preferentially redistributed upon elisidepsin treatment from the plasma membrane to intracellular vesicles or sometimes even to the nucleus. Although elisidepsin-induced formation of large intracellular vesicles and their progression from the plasma membrane toward the nucleus have been previously reported (Faircloth and Cuevas, 2006), we did not observe the internalization of every kind of membrane protein since ErbB1 and ErbB2 persisted in the plasma membrane after elisidepsin treatment. Although we cannot explain the selectivity profile of elisidepsin in inducing membrane protein internalization, we suspect that elisidepsin-induced membrane effects lead to the specific redistribution of raft-associated membrane proteins including GPI-anchored and ErbB3 proteins (Nagy et al., 2002).

The changes in the fluorescence anisotropy of TMA-DPH and in the generalized polarization of Laurdan immediately after application of the drug preceded cell death and membrane permeabilization reported by propidium iodide uptake by several minutes. All the effects of elisidepsin, including its cytotoxic effect, were very fast. The rapid induction of cell death preceded by changes in the order and fluidity of the plasma membrane are in agreement with the assumption that the primary target of elisidepsin is the plasma membrane and all other effects are the consequences of the primary membrane effects. Fluorescence anisotropy reports the mobility of the fluorescent probe, whereas the generalized polarization of Laurdan is sensitive to the hydration of the plasma membrane (Harris et al., 2002; Parasassi et al., 1991; Sanchez et al., 2007). The combination of decreased fluorescence anisotropy (higher mobility) and increased generalized polarization of Laurdan (lower hydration) is assumed to be characteristic of liquid ordered domains (Harris et al., 2002; Vest et al., 2006) which is followed by detachment of the membrane from the cytoskeleton and membrane permeabilization (“membrane damage”). Elisidepsin-induced increased lipid order has also been reported in a recent publication (Molina-Guijarro et al., 2011).

The elisidepsin-induced changes in the fluorescence anisotropy of TMA-DPH and the generalized polarization of Laurdan in two cell lines (A431 and SKBR-3) having significantly different IC₅₀ values for the drug were identical in direction and similar in magnitude. Elisidepsin was applied at a concentration slightly higher than the IC₅₀ of A431 and significantly larger than that of SKBR-3. In this concentration range effects associated with the mechanism of action of the drug are expected to be manifest. The slightly larger magnitude of changes in anisotropy and generalized polarization in SKBR-3 cells may have been caused by the lower IC₅₀ value of this cell line compared to A431.

Cancer cells display higher sensitivity to Kahalalide F, and presumably to elisidepsin, than their non-tumoral counterparts (Janmaat et al., 2005; Suarez et al., 2003), a finding in apparent disagreement with the

nonspecific membrane effects of elisidepsin. However, cancer cells display characteristic changes in their fatty acid and ganglioside composition. Among others increase in the ratio of saturated/non-saturated fatty acids and accumulation of less-complex gangliosides have been observed (Lopez and Schnaar, 2009; Yin et al., 2006; Yin et al., 2010). In addition, upregulation of fatty acid 2-hydroxylase (FA2H) in malignant tumors has been reported which may lead to the cancer specific cytotoxic effects of elisidepsin (Hama, 2010; Yin et al., 2010). However, tumor hypoxia is known to decrease the rate of hydroxylation due to shortage of oxygen which acts against the tumor-specificity of elisidepsin by reducing the activity of FA2H (Yin et al., 2010). Whether tumor hypoxia could influence the sensitivity of tumor cells to elisidepsin is currently unknown.

In summary, the lack of any correlation between elisidepsin sensitivity and the expression levels of ErbB1–3 questions the role of ErbB proteins in determining elisidepsin responsiveness. Moreover, we also present evidence showing that the preferential changes in the distributions and association states of ErbB and GPI-anchored proteins are secondary to alterations in the cell membrane induced by elisidepsin. Further investigations are required to elucidate how elisidepsin responsiveness is related to structural and dynamic changes of the cell membrane.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at [doi:10.1016/j.ejphar.2011.05.064](https://doi.org/10.1016/j.ejphar.2011.05.064).

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Supplementary material

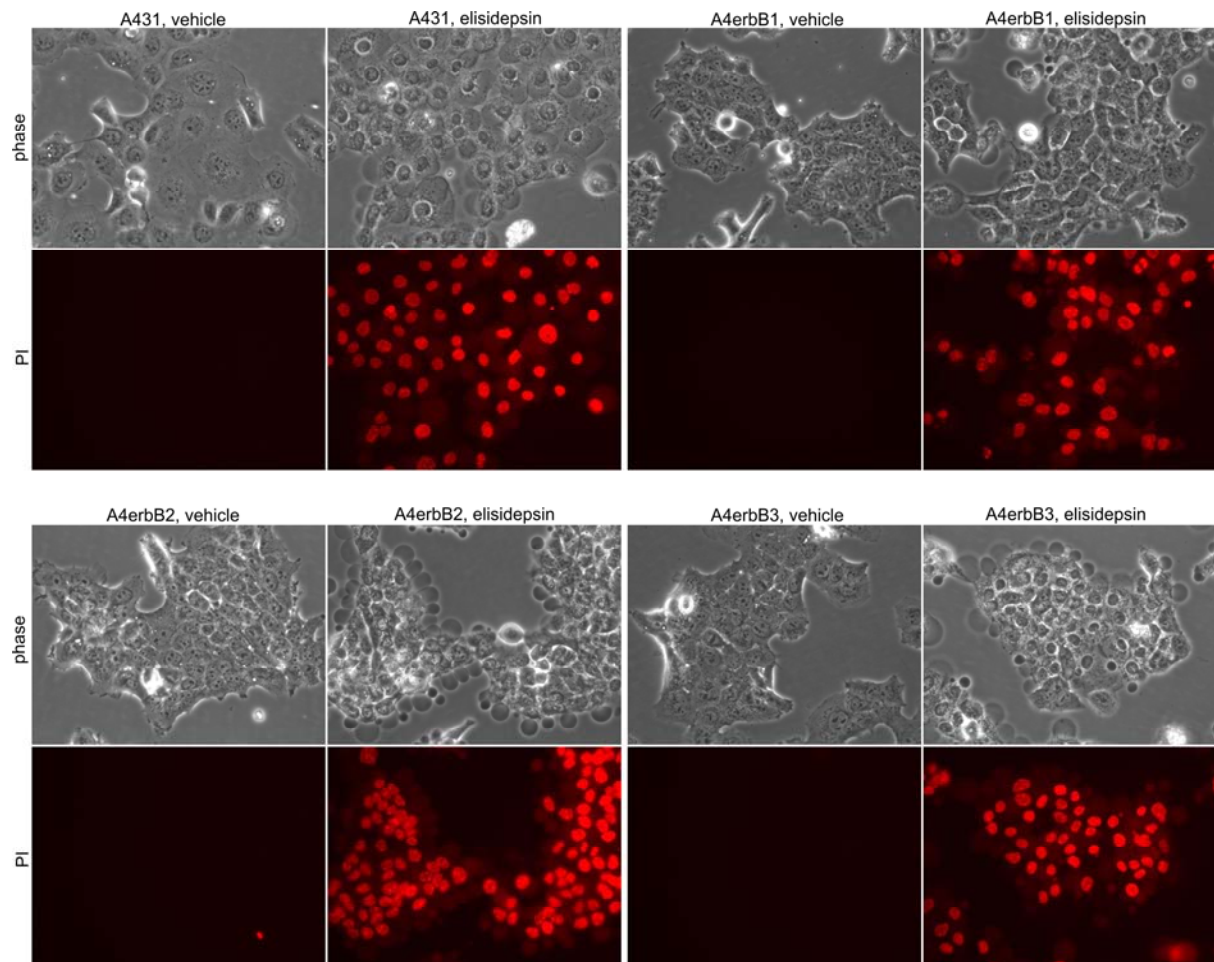
Table S1 ErbB expression profile of the cell lines used

Cell line	Number of proteins/cell ($\times 10^3$)		
	ErbB1	ErbB2	ErbB3
CHO	0	~0	0
CHO-ErbB2	0	200	0
CHO-ErbB2-3	0	200	100
A431	2000	20	3
A4erbB1	3100	20	1.5
A4erbB2	1200	950	1.5
A4erbB3	2000	20	800
SKBR-3	50	800	15

The expression level of ErbB proteins was determined by flow cytometry using Qifikit (Dako, Glostrup, Denmark). The numbers represent the mean of three independent experiments.

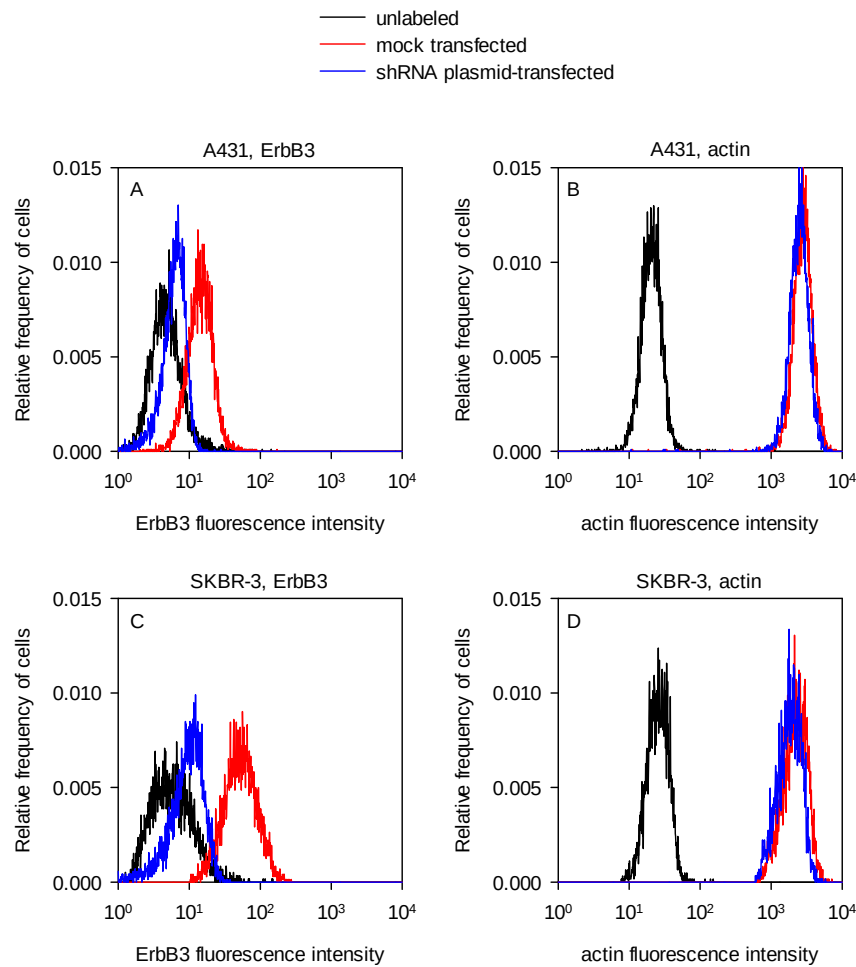
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Fig. S1 Fluorescence microscopic investigation of the short-term effect of elisidepsin on parental and transfected A431 cells



Untransfected, ErbB1-eGFP- (A4erbB1), ErbB2-mYFP- (A4erbB2) and ErbB3-citrine-transfected (A4erbB3) A431 cells were treated with elisidepsin or vehicle in the presence of propidium iodide for 30 min followed by acquiring fluorescence and phase contrast images.

Fig. S2 RNA interference-mediated knock-down of ErbB3 expression



A431 and SKBR-3 cells were transfected by electroporation with a plasmid producing a shRNA against ErbB3. 60 hours after transfection cells were harvested, fixed, permeabilized and stained for ErbB3 (panels A,C) using a primary fluorescently labeled antibody against ErbB3 (H3.90.6). In order to show the lack of any significant effect of transfection and shRNA production on the expression of an irrelevant gene fixed and permeabilized cells were labeled with a monoclonal antibody against actin (clone AC40, Sigma-Aldrich) followed by secondary staining with an AlexaFluor647-labeled anti-mouse antibody (panels B,D).