MTA DOKTORA PÁLYÁZAT

Stressz-reszponzív adaptációs mechanizmusok működése és kölcsönhatásai

Rövid értekezés

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TARTALOMJEGYZÉK

ÖSSZEFOGLALÁS

A változáshoz (stresszhez) történő alkalmazkodást egy komplex, specializált stresszválaszokra épülő adaptációs mechanizmus segíti. Az öregedés és számos betegség folyamatában az adaptáció elégtelensége figyelhető meg, azonban az egyes stresszválaszok kölcsönhatásai nem tisztázottak. Kutatásaink során különféle stresszadaptációs mechanizmusokat tanulmányoztunk izolált fehérjéktől a humán perifériás limfocitákig.

A Hsp90 hősokkfehérje többszáz jelátviteli (kliens) fehérje szerkezetét stabilizálja. A Hsp90 (Ph.D. munkám során azonosított) C-terminális nukleotidkötőhelyének specificitását jellemeztük; megállapítottuk, hogy a C-terminális inhibitor szelektíven gátolja a szteroidreceptor kliensek funkcióját; rámutattunk, hogy a tumorokban túltermelődő humán Hsp90α a Hsp90β-nál hatékonyabban működik és Hsp90-inhibitorral szemben ellenállóbb.

A kalóriacsökkentés a leghatékonyabb élettartamnövelő hatás, mely számos adaptációs mechanizmust aktivál, többek között a Sir2 szirtuin metabolikus, a HSF1 fehérje homeosztatikus hősokkválasz és az Nrf2 xenobiotikus stresszválasz mesterregulátorokat. Kimutattuk, hogy idős patkány máj citoszol chaperon kapacitása enyhén csökkent és elméleti és számítógépes modellt alkottunk a fehérje homeosztatikus puffer és a hősokkválasz öregedésben játszott szerepéről; kimutattuk, hogy humán időskori cinkszupplementáció és a kalóriacsökkentés mimetikus resveratrol stimulálja a hősokkválaszt, megerősítettük a HSF1 szerepét a kalóriamegvonás-indukálta élettartamnövekedésben és megcáfoltuk a Sir2 túltermelésének élettartamnövelő hatását *Caenorhabditis elegans*-ban; megfigyeltük a denaturált fehérjeszerkezet funkciótól független, emlős sejtek proliferációjára és stressz-indukálta túlélésre gyakorolt negatív hatását; bizonyítottuk, hogy az oxidatív stressz emlős sejteken és *C. elegans*-ban egyaránt az RNS interferencia közvetítésével gátolja a hősokkadaptációt; valamint feltártuk, hogy az Nrf2 ortológ SKN-1 transzkripciós faktor optimális aktivációja szükséges a természetes immunitáshoz, aktivitáscsökkenése pedig részt vesz az immunoszeneszcenciában *C. elegans*-ban.

Eredményeink összekapcsolják a fehérje homeosztázist, az oxidatív stresszt, a kalória csökkentést, a természetes immunitást valamint az öregedést, ezáltal elősegítik a különféle stresszek és stresszválaszok integratív szemléletét. Az azonosított mechanizmusok az emberi öregedés és a korral kapcsolódó betegségek tekintetében is megállhatják helyüket és új terápiás célpontokat kínálhatnak.

KÖSZÖNETNYILVÁNÍTÁS

Köszönetemet szeretném kifejezni mindazoknak, akik hozzájárultak eddigi életutam és tudományos pályám alakulásához, és elnézést kérek mindazoktól, akiket itt név szerint nem említek. Számos általános- és középiskolai tanárom bizalmát éreztem. Selmeczi Lászlóval a Kórélettani Intézet diákköröseként dolgozhattam és részesülhettem a tudomány iránti lelkesedéséből és alázatából. Somogyi János az Orvosi Vegytani Intézetben laboratóriumába befogadott, hálás vagyok megbecsüléséért, beszélgetéseinkért. Mentoromnak, Csermely Péternek köszönöm barátságát, az inspirációt, a gondolat szabadságának tiszteletét, nagylelkűségét, hogy segítette önállósodásomat.

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Szerencsés vagyok, hogy diákköri és doktori éveimet Schnaider Tamással baráti közösségben és szolidarításban, egymástól tanulva tölthettem. Hálás vagyok Tóth Márton posztdoktor kollégámnak barátságáért, az együtt gondolkodásért és a *C. elegans* laboratórium megteremtésében vállalt alapvető szerepéért. Gilányi Bea kollégám érdeme a labor rendezett, olajozott működése, köszönöm megbízható munkáját. Munkatársaim és tanítványaim életük egy részét a közös munkának szentelték, köszönöm, hogy tanulhattam tőlük és általuk (a teljesség igénye nélkül): Putics Ákos posztdoktornak; Alper Arslan, Papp Diána, Nguyen Minh Tu, Gyurkó Dávid és Somogyvári Milán volt és jelenlegi PhD hallgatóknak (közülük többen korábbi diákkörösök és szakdolgozók voltak); Spiró Zoltán és Dancsó Balázs szakdolgozóknak; Vermes Ákos, Rácz Attila, Kovács Levente, Végh Eszter Mária, Maria Chikina, Láng Júlia, Pathó Zoltán, Tihanyi Benedek és Taisz István diákkörös hallgatóknak.

Hálás vagyok sok hazai és külföldi kollégámnak az együttműködések, kihívások és közös felfedezések formáló erejéért. Köszönöm az ismeretlen bíráló kollégák idejét, érdeklődését és jobbító szándékát. Vellai Tibor tudása és folyamatos önzetlen segítsége lehetővé tette a *C. elegans* műhely létrehozását és segíti magas színvonalú működését, melyet köszönök. Örömmel tölt el, hogy a *C. elegans* közösséghez tartozhatom, amelynek baráti és tudásközpontú légköre, valamint a nyílt tudás- és törzsbázis (Wormbase és *Caenorhabditis* Genetics Center) bármilyen merész elképzelés és együttműködés megvalósítását megkönnyíti.

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Sőti Csaba

RÖVIDÍTÉSEK JEGYZÉKE

Δ9CAT: Chloramphenicol-acetiltranszferáz C-terminális 9 aminosavát nélkülöző mutáns

CD3: Cluster of Differentiation 3, T-limfocita marker

CDDP: ciszplatin, cisz-diamin-dikloroplatinum (II)

CTL-2: peroxiszómális *C. elegans* kataláz-2

DCR-1/Dicer: az érett mikroRNS-eket előállító citoszolikus endonukleáz *C. elegans*/humán ortológja

GFP: zöld fluoreszcens fehérje

GHKL: Giráz/Hsp90/His-kináz/MutL fehérjékben előforduló nem-konvencionális ATP-kötő domén

HSF1: hősokk transzkripciós faktor-1

Hsp70: 70 kDa molekulatömegű hősokkfehérje

Hsp90: 90 kDa molekulatömegű hősokkfehérje

Lck: Leukocyte C-terminal Src kinase

NAD^+: nikotinamid-adenin dinukleotid

Nrf2: Nuclear factor (erythroid-derived 2)-Related Factor 2 transzkripciós faktor

PASH-1/Drosha: a hajtű pre-mikroRNS-t előállító sejtmagi endonukleáz *C. elegans*/humán ortológja

PMK-1: a p38 MAP kináz *C. elegans* ortológja

PRDX-2: citoszolikus *C. elegans* peroxiredoxin-2

Raf-1: Rapidly Accelerated Fibrosarcoma Ser/Thr kináz 1

Sir2: Silent Information Regulator 2, NAD^+-függő szirtuin deacetiláz család

SIR-2.1: a Sir2 *C. elegans* ortológja

SIRT1: a Sir2 egyik fő humán ortológja (humán sejtben 7 paralóg van: SIRT1-7)

SKN-1: SKiNhead transzkripciós faktor, az Nrf2 *C. elegans* ortológja

TIR-1: Toll/Interleukin-1 Receptor domén fehérje, a SARM *C. elegans* ortológja

UPR: unfolded protein response, az endoplazmatikus retikulum stresszválasza

BEVEZETÉS

Az élő szervezet alapvető képessége, hogy érzékelje az egyensúlyából kilendítő környezeti hatásokat és azokra megfelelő módon reagáljon, melynek célja egy új, a megváltozott körülményekkel összhangban levő egyensúlyi állapot kialakítása. A hatásokat – Selye János munkája nyomán – stressznek, az ezt érzékelő és az alkalmazkodást segítő mechanizmusokat stresszválasznak, a folyamatot stresszadaptációnak nevezzük. Az utóbbi évtizedek kutatásai feltárták a stresszválasz(ok) moduláris szerveződését: beszélhetünk antioxidatív, xenobiotikus, genotoxikus, fehérje konformációs (hősokk-), metabolikus, stb. stresszválaszokról. Az enyhe stresszek által kiváltott hatékony stresszadaptáció eredménye azonos illetve eltérő stresszekkel szembeni (kereszt-)tolerancia, míg a túlzott stressz gyengíti az alkalmazkodást. Ezek a kereszthatások komplex, hálózatos szerveződést sejtetnek, melyek működésének molekuláris mechanizmusai és kölcsönhatásai nagyrészt feltáratlanok.

Doktori értekezésem két részből áll: először a Hsp90 molekuláris chaperon biokémiai és funkcionális jellemzésével, majd a hősokkválasz és más stresszek illetve stresszválaszok kölcsönhatásaival és öregedésben játszott szerepével kapcsolatos munkánkat foglalom össze.

A HSP90 BIOKÉMIAI ÉS FUNKCIONÁLIS JELLEMZÉSE

A 90 kDa molekulatömegű hősokkfehérje (Hsp90) egy konzervált, esszenciális citoszolikus molekuláris chaperon. A sejt összfehérje tartalmának mintegy 2-5%-át alkotja. Két izoformája van, a konstitutívan expresszálódó β és a stresszindukálható α, melyek nagyfokú szekvencia identitást mutatnak, azonban szabályozásuk eltérő. Perifériás sejtekben elsősorban a β izoforma fordul elő, míg intenzíven osztódó, illetve a stresszelt tumorsejtekben az α expressziója erősen indukálódik, így mennyisége a β izoformával összemérhető vagy azt meghaladja. Az eltérő szabályozás és szöveti expresszió funkcionális különbségeket sejtet.

A Hsp90 N-terminális nukleotidkötő, ezt követő flexibilis linker, középső szubsztrátkötő és C-terminális dimerizációs/fehérje interakciós doménekből áll. A Hsp90 specifikus chaperon: többszáz termodinamikailag instabil, jelátviteli kulcsmolekula, ún. ‘kliens’ fehérje (pl. kinázok, transzkripciós faktorok, polimerázok) szerkezetét stabilizálja, melyek szerteágazó biológiai funkcióikat látnak el, és kiemelt szerepet játszanak a sejtproliferációban és túlélésben. Ehhez az N-terminális, GHKL-típusú ATP-kötőhelyének ATPáz aktivitása szükséges. Az N-terminális ATP-antagonista Hsp90-inhibitorok (vezető molekula: geldanamycin) ígéretes specifikus tumor kemoterápiás szerek, azonban a Hsp90 gátlásával a hősokk transzkripciós faktor-1 (HSF1) aktivációja révén rezisztenciához vezetnek. PhD munkám során jellemeztük a Hsp90 chaperon ciklusát moduláló kismolekulákkal illetve ATP-vel való kölcsönhatását. Utóbbi során felfedeztünk egy C-terminális doménen elhelyezkedő nukleotidkötőhelyet, mely az N-terminális kötőhellyel allosztérikus kapcsolatban áll, azonban geldanamycinnel nem, hanem novobiocinnal és ciszplatinnal (CDDP) gátolható.

Célkitűzés

Hsp90-nel kapcsolatos kísérleteink során az alábbi kérdésekre kerestünk választ:

1. Milyen nukleotidspecificitással rendelkezik a Hsp90 C-terminális nukleotidkötőhelye?
2. Milyen hatást fejt ki a C-terminális nukleotidkötőhelyet gátló ciszplatin a Hsp90 kliensfehérje komplexekre?
3. Milyen funkcionális jelentőséggel bír a Hsp90 α és β izoformája?

Eredmények és megbeszélésük

1. A Hsp90 C-terminális nukleotidkötőhelyének jellemzése

A Hsp90 N- és C-terminális nukleotidkötőhelyének jellemzésére a korábban beállított nukleotid-affinitáshasítási módszert és a szilárd fázisú gyantán immobilizált nukleotidokkal történő affinitáskromatográfiás módszert, illetve a kettő kombinációját alkalmaztuk. Timothy Haysteaddel együttműködve megállapítottuk, hogy az N-terminális kötőhely adenin-nukleotidokra (ezek között dinukleotidokra, pl. NAD^+-ra, illetve alarmonokra) specifikus, míg a C-terminális domén széles specificitású, mind purin-, mind pirimidin-nukleotidok kötésére képes (Sőti és mtsai, 2003b).

Munkánk azt sugallja, hogy a C-terminális kötőhelynek *in vivo* egyéb kismolekulájú ligandok kötésében is szerepe lehet, és lehetőséget nyújt a C-terminális kötőhelyre specifikus inhibitorok előállítására, melyek az N-terminális domént nélkülöző izoformákra (pl. Hsp90N) is hatékonyak (Sreedhar és mtsai, 2004, Sőti és mtsai, 2005b).

2. Ciszplatin hatása a Hsp90-kliens kapcsolatra

A C-terminális kötőhelyet gátló ciszplatinnak a Hsp90 kliensekre gyakorolt hatását tanulmányozva Theo Rein csoportjával együttműködésben kimutattuk, hogy a ciszplatin koncentrációfüggően gátolja a Hsp90 kliens glukokortikoid és androgén receptor

stabilizációját és funkcióját. Ezt a hatást a Hsp90 közvetíti. Ciszplatin nem hatott más Hsp90 kliensekre, így a Ser/Thr-kináz Raf-1-re, a Tyr-kináz Lck-ra. Fontos, hogy ciszplatin nem befolyásolja a hősokk választ és a Hsp90α expresszióját reguláló hősokk transzkripciós faktor-1 (HSF1) transzaktivációját (Rosenhagen és mtsai, 2003).

Eredményeink arra utalnak, hogy a C-terminális kötőhely szelektív gátlása a Hsp90 bizonyos klienseivel kialakított kölcsönhatását gátolja. Mindez illusztrálja a C-terminális kötőhely specifikus funkcióját, továbbá első bizonyítékát adja a annak, hogy a Hsp90-nek bizonyos klienseivel kialakított kölcsönhatása – a hősokkválasz aktiválódásának elkerülésével – terápiásan megcélozható.

3. A Hsp90 α és β izoformájának funkcionális eltérései

Az élesztő egyszerűségét, kiváló genetikai manipulálhatóságát és a Hsp90 funkció nagyfokú konzerváltságát kihasználva Peter Piper csoportjával együttműködve kimutattuk, hogy Hsp90-deficiens élesztő modellrendszerben egyedüli Hsp90-ként kifejezett humán Hsp90α és Hsp90β egyaránt képes az életképességet helyreállítani és kliensekkel komplexeket kialakítani. Azonban a két izoforma funkcionálisan eltér: a Hsp90α alacsonyabb affinitású (azaz tranziensebb, hatékonyabb) komplexeket alakít ki, jelenléte jóval hatékonyabban aktiválja a hősokkválaszt, segíti elő a v-src Tyr-kináz kliens aktivációját, és nagyságrendekkel ellenállóbb a Hsp90 N-terminális inhibitor radicicollal szemben (Millson és mtsai, 2007).

Eredményeink a humán Hsp90 izoformák eltérő celluláris funkcióira az elsők között adtak bizonyítékot. Mivel a Hsp90α a tumorokban szelektíven indukálódik és a tumor agresszivitás és rezisztencia számos molekuláris mechanizmusát közvetíti, ezért megfigyeléseink rávilágítanak a tumorok nagyfokú ellenállóképességére, így a Hsp90α specifikus megcélzása, vagy indukciójának megelőzése fontos kemoterápiás stratégia lehet.

STRESSZEK ÉS STRESSZVÁLASZOK KÖLCSÖNHATÁSAINAK VIZSGÁLATA

A fehérje konformációs homeosztázist, így a sejt fehérjehálózatát a fehérje homeosztatikus puffer tartja karban, mely a hősokkválasz köré szerveződik. Hősokkfehérjék (Hsp-k, vagy molekuláris chaperonok) segítik a fehérjék feltekeredését, a komplexek összeszerelődését, transzportját, és ismerik fel a nem-natív szerkezetű fehérjéket, melyeket helyreállítanak vagy a proteaszómális illetve autofág lebontás útjára, illetve az aggregáció felé segítenek. A Hsp90-központú chaperon komplex többszáz instabil szerkezetű jelátviteli kulcsmolekula szerkezetét stabilizálja, a sejt legtöbb kapcsolattal rendelkező fehérjéi közé tartozik, így a celluláris jelátviteli folyamatok zavartalan működését teszi lehetővé (ld. később). A proteotoxikus stressz következtében felszaporodó denaturált fehérjéket megkötik a fő citoszolikus Hsp90 és Hsp70 chaperonok, és gátló komplexükből elengedik a hősokk transzkripciós faktort-1- (HSF1)-et. Az aktiválódó HSF1 indukálja a hősokk-gének, illetve szabályozza a sejt önfenntartását és növekedésében érintett gének (a genom mintegy 3-4%-a) expresszióját, mely erősíti az ellenállóképességet és az immunitást. Ezáltal a hősokkválasz az akut túlélés és betegségekkel szembeni ellenállóképesség egyik fontos általános tényezője.

Az öregedés az élő szervezet funkcióinak progresszív csökkenése. Az öregedéselméletek genetikai programot (celluláris szeneszcencia: Hayflick-szabály, gerontogének), a hibák random, ill. oxidatív stressz-okozta halmozódását (mutáció-akkumuláció és hibakatasztrófa elmélet, szabadgyök-teória) helyezték előtérbe. Öregedés során megfigyelhető az oxidatív stressz és károsodások (pl. sérült fehérjék) mennyiségének növekedése. A sérült fehérjék a posztmitotikus sejtekben öregedés-szerű sorvadást okoznak (pl. mutáns fehérjék okozta neurodegeneratív betegség). Azonban az öregedés a változáshoz történő alkalmazkodás csökkenéseként is értelmezhető, melyre az előző elméleteket magába ölelő kísérleti bizonyítékot a stresszválaszok (hősokkválasz, immunválasz, egyéb önfenntartó

mechanizmusok) válaszkészségének korral járó csökkenése, illetve a stressztolerancia és az élettartam közötti optimumkorreláció szolgáltat. Ez felveti annak lehetőségét, hogy a stresszválaszok, pl. a hősokkválasz farmakológiás aktivációja célpont lehet az egészséges élettartam megnyújtásában és a degeneratív civilizációs betegségek (elhízás, diabétesz, kardiovaszkuláris és neurodegeneratív betegségek, immunproblémák) megelőzésében és kezelésében.

A kalóriacsökkentés, a kalóriabevitel mérsékelt (30-40%-os) csökkentése az eddig ismert leghatékonyabb, emlős modelleken is igazolt élettartamnövelő környezeti hatás. Az inzulin szignálon túl a kalóriacsökkentés sejtszintű közvetítőjeként a 2000-es évek elején a metabolikus szenzor szirtuin (Sir2) NAD^+-függő deacetilázt azonosították. Szirtuin túltermelő élesztő, fonálféreg és ecetmuslica törzsek élettartama megnövekedett, így a szirtuin aktiváció ígéretes humán élethossznövelő farmakológiás célponttá vált. Ezzel egyidőben az eredetileg rákellenes hatásáról ismert polifenol resveratrolban az első élettartamnövelő kismolekulát fedezték fel, mely hatékonynak bizonyult az élesztőtől a túltáplált egerekig. A resveratrolról bizonyították, hogy a Sir2 deacetiláz aktivitását serkenti és a kalóriacsökkentéshez hasonló anyagcsere- és génexpressziós hatásokat hoz létre. Ezen túl a kalóriacsökkentés aktivál egyéb stressz-reszponzív pályákat, pl. indukálja az antioxidáns választ, a hősokkfehérjéket, és a xenobiotikus regulátor Nrf2 transzkripciós faktort. Az erőteljes és koordinált változások azt sugallják, hogy a kalóriacsökkentés egy enyhe metabolikus stresszhatás által egy szervezeti szinten összehangolt önfenntartó program aktivációja az erőforrások szűkössé válásakor, így megértésével, egyéb stresszválaszokkal történő kapcsolatainak feltárásával közelebb juthatunk az egészséges élettartam meghosszabbításához.

A megfelelő immunitás szükséges az akut túléléshez és hozzájárul az egészséges élettartam kialakításához. Az öregedés egyik legmarkánsabb velejárója a gyengülő hatékony immunválasz és erősödő gyulladásos tendencia kombinációjaként jellemezhető

immunszeneszcencia. A kalóriacsökkentés serkenti az immunműködést és késlelteti az immmunszeneszcenciát. Az immunválasz is felfogható egy szervezeti veszélyt érzékelő komplex stresszválaszként (Matzinger-hipotézis), azonban a szervezet önfenntartó stresszválaszaival való kapcsolata nem teljesen tisztázott.

Célkitűzés

Stresszválaszokkal kapcsolatos kutatásaink során az alábbi kérdésekre kerestünk választ:

1. Hogyan változik a chaperon kapacitás előrehaladott korban? Mi a hősokkválasz és fehérje homeosztázis szerepe az öregedés során?
2. Hogyan hat a humán időskori cinkszupplementáció a perifériás limfociták hősokkválaszára?
3. Milyen kapcsolat van a metabolikus szignálutak aktivációja (kalória megvonás, resveratrol, genetikai szirtuin aktiváció) és a hősokkválasz valamint az élettartam között?
4. Milyen hatást fejtenek ki a denaturált (instabil szerkezetű) fehérjék, funkciójuktól függetlenül a fehérje homeosztázisra és a sejtfunkcióra?
5. Hogyan és milyen mechanizmussal hat az oxidatív stressz a hősokkadaptációra?
6. Mi a szerepe az enyhe stresszeknek és a xenobiotikus stresszválasz regulátor Nrf2 ortológ SKN-1-nek a természetes immunitásban és az immunoszeneszcenciában?

Eredmények és megbeszélésük

1. A chaperon kapacitás és a hősokkválasz változása és szerepe az öregedés során

A hősokkválasz indukálhatósága az öregedés során csökken, azonban a chaperonok fehérje denaturációtól védő hatékonysága munkánk kezdetekor ismeretlen volt. Hőérzékeny

luciferáz modellfehérjét felhasználva elsőként vizsgálva a citoszolikus teljes chaperon kapacitást kimutattuk, hogy globális fehérje denaturációt nem okozó körülmények között (39ºC) idős (26 hónapos) patkány máj citoszol preparátumok passzív chaperon (fehérje denaturációt és aggregációt gátló) kapacitása fiatal (10 hetes) állatokhoz képest enyhén csökkent. A két fő citoszolikus chaperon közül a Hsp70 mennyisége nem változott, a Hsp90 mennyisége csökkent (Nardai és mtsai, 2002). A csökkenés hátterében akár a luciferázt stabilizáló Hsp90 szint csökkenése, esetleg oxidált-denaturált fehérjék (köztük a módosult chaperonok) korral járó mérsékelt növekedése állhat.

A sejt és szervezeti öregedés egyik tényezője az aminosavakat módosító, így a fehérje konformációt károsító proteotoxikus stresszek és a fehérje homeosztázis karbantartásában fontos szerepet játszó chaperonok kapacitásának egyensúlytalansága. Ennek modellezésére megalkottuk a „Chaperon túlterhelés" modellt (Sőti és Csermely, 2003), majd ezt kibővítve a az „Öregedés fehérje homeosztázis hipotézisét" (Sőti és mtsai, 2003a; Arslan és mtsai, 2006; Sőti és Csermely 2007b). Ezeknek az információknak a felhasználásával Tom Kirkwood csoportjával együttműködésben egy számítógépes szimulációs modellt fejlesztettünk ki (Proctor és mtsai, 2005). Ezeken túl rámutattunk a chaperonok sejt hálózatában betöltött szervező szerepére, farmakológiai jelentőségére és az öregedés hálózatos modelljében elfoglalt helyére (Sőti és mtsai, 2005a és b; Csermely és Sőti 2006; Sőti és Csermely 2007a).

2. Humán cinkszupplementáció hatása limfociták Hsp70 fehérje expressziójára

A cink, mint nyomelem számos fehérje és enzim integritásához nélkülözhetetlen, így fontos szerepet játszik a sejtproliferációban, a genetikai stabilitásban és az antioxidatív védelemben és az immunválaszban. Idős emberek cinkstátusza és immunfunkciója romlik. Tanulmányunkban, Eugenio Mocchegiani csoportjával együttműködve, idős (64-85 év közötti) emberek középtávú cink (10 mg/nap, 48 napig) táplálékkiegészítésének hatását

vizsgáltuk áramlási citometriával a humán perifériás vérsejtek fő hősokkválasz markerének, a Hsp70 fehérjének mind alap, mind hőindukált expressziójára. Megállapítottuk, hogy a Hsp70 alap és hőindukált szintje – a kiegészítés előtti állapothoz, illetve a nem szupplementált kontroll csoporthoz képest – szignifikánsan növekedett mind az össz-, a CD3+ (T-sejt) és CD3- populációkban. A Hsp70 szintek szignifikánsan magasabbak voltak a magas cink elérhetőséggel rendelkező csoportban (Putics és mtsai, 2008a). Eredményeink felvetik a hősokkválasz szerepét a cinkdeficiencia és cinkpótlás immunrendszerre és immunszeneszcenciára kifejtett hatásaiban.

3. A metabolikus szignálok, szirtuin aktiváció és a hősokkválasz kapcsolata

A növényi stresszhatásra termelődő fitoalexin resveratrol hősokk adaptációra kifejtett hatását vizsgálva emlős sejtkultúrán és humán limfocitákon beláttuk, hogy aktiválja a HSF1-függő transzaktivációt és Hsp70 fehérje expressziót. Ez az aktiváció a citoszolikus hősokkválaszra specifikus, az endoplazmatikus retikulum stresszválaszt (UPR) nem érinti és a hatást nem antioxidáns hatása közvetíti. Resveratrol előkezelés letális hőstressz elleni fokozott védelmet biztosít (Putics és mtsai, 2008b). Mindez felveti, hogy a hősokkválasz a resveratrol, és talán a kalóriacsökkentés élettartamnövelő hatásának egyik közvetítője. Ezeket a hipotéziseinket az ezidőtájt létrehozott *Caenorhabiditis elegans* laboratóriumunkban kezdtük el tanulmányozni.

A hősokkválasz, metabolikus stressz és kalória csökkentés kapcsolatának áttekintése mellett közöltük azon eredményeinket, melyek egy amerikai kutatócsoporttól függetlenül megerősítik, hogy a teljes kalóriamegvonás a HSF1 jelenlététől függő élettartam növelő hatással rendelkezik *C. elegans*-on. Megállapítottuk, hogy a kalóriamegvonás nem igényli a klasszikus metabolikus szenzor AMP-függő fehérje kináz és Sir2 ortológokat. A HSF1-függő élettartam növekedés a 20 fokos neutrális hőmérséklet alatt és felett egyaránt megfigyelhető

volt (Dancsó és mtsai, 2010). Ez felveti a kalóriamegvonás és a HSF1 klasszikus metabolikus jelátviteltől és fehérje homeosztázistól független szerepét az élettartam növekedésben.

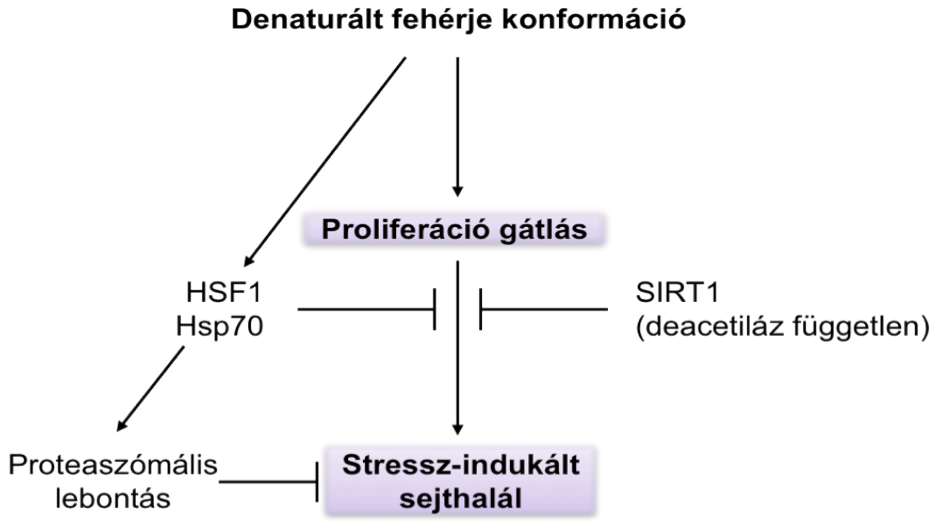
További eredményeink igazolni látszottak, hogy a resveratrol és a szirtuin magas kópiaszámban történő túltermelésének élettartamnövelő hatását *C. elegans*-ban a HSF1 közvetíti (Tóth és mtsai, 2008, 2009 konferencia absztraktok; Dancsó és mtsai, 2010). Ezzel egyidőben anekdotikus hírekre hivatkozva és saját kísérletek kapcsán David Gems kételyeit fejezte ki a szirtuin túltermelés élettartamnövelő hatásával kapcsolatban, ezért a kérdés megnyugtató tisztázására együttműködésbe kezdtünk. Mindkét, az irodalomban szereplő, egy magas és egy alacsony kópiaszámú *sir-2.1* (Sir2 ortológ) transzgén fonálféreg törzset egymástól függetlenül a megfelelő genetikai háttérrel visszakereszteztünk. A téma kiemelt fontosságára tekintettel kölcsönös látogatások során mindkét laboratóriumban mindkét fél által végrehajtott kettősvak kísérleteket végeztünk. A *C. elegans* SIR-2.1 túltermelés élettartamnövelő hatása a visszakeresztezés után eltűnt, melyet nemzetközi együttműködő partnereink is megerősítettek. Egyúttal kimutattuk, hogy az eredeti kísérletekben tapasztalt élettartam növekedés háttér mutációval kapcsolódott, míg Linda Partridge csoportja ecetmuslicában bizonyította, hogy a Sir2 transzgén élettartamnövelő hatása a transzgén konstrukciónak tulajdonítható (Burnett és mtsai, 2011).

Eredményeink a korábbi tanulmányokkal szemben megkérdőjelezik a Sir2 túltermelés élettartamnövelő hatását két gerinctelen modell organizmuson, egyúttal felhívják a figyelmet a genetikai háttér és a potenciális mutációk megfelelő ellenőrzésére.

4. Inert denaturált modellfehérjék celluláris hatásainak viszgálata

A rosszul feltekeredő („misfolded”) fehérje szerkezet alapvető szerepet játszik a konformációs betegségekben és az öregedésben, azonban hogy ennek oka mennyiben a kitekert szerkezetű fehérje funkcióvesztése és a keletkező kóros szerkezetű toxikus molekulák

funkciónyerése, nem tisztázott. Ennek vizsgálatára létrehoztunk két független, az eukarióta sejtben funkcióval nem rendelkező ’inert’ fehérjemolekula destabilizált konformációs mutánsának GFP-fúziós változatát, hogy izoláltan megvizsgáljuk a funkciónyerés hatásait. A GFP-degron és a GFP-Δ9CAT egyaránt kitekert fehérjeként viselkedett: jó részüket a sejt proteaszomális degradációval eliminálta, az erre nem képes sejtekben perinukleáris aggregátumokat képeztek, ahol kolokalizáltak a chaperon Hsp70-nel és indukálták expresszióját. Egyik fehérje sem okozott számottevő sejtpusztulást stresszmentes körülmények között, azonban mindkét fehérje sejtproliferáció gátlást okozott és fokozta a proteotoxikus stressz- (hősokk és proteaszóma inhibitor MG132) indukálta sejthalált. A sejthalált akár a HSF1 és Hsp70, illetve a vad és deacetiláz deficiens H363Y pontmutáns SIRT1 szirtuin túltermelése egyaránt képes volt, míg a SIRT6 paralóg nem volt képes megelőzni. Eredményeink a szirtuinok differenciációját és a SIRT1 nem-katalitikus hatásmechanizmusát sugallják, így az enzimaktivitást (és az anyagcserét) nem befolyásoló terápiás célpontot nyújthat (Arslan és mtsai, 2012; *1. ábra*).

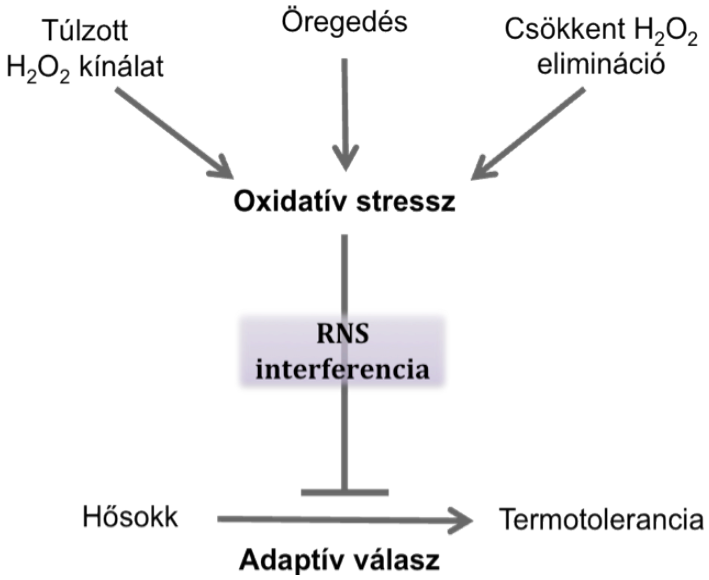


1. ábra ***A denaturált fehérje konformáció celluláris hatásai***

Megfigyeléseink igazolják, hogy egyetlen, az összfehérjék 1-2%-át kitevő, inert denaturált fehérje expressziója a sejtre nem toxikus, azonban az azt lebontani képtelen sejtekben elegendő, hogy gátolja a sejtproliferációt (növekedést) és a stresszadaptációt, azaz az öregedő/szeneszcens sejtekre jellemző viselkedést vált ki (ld. Sőti és mtsai, 2003a). A SIRT1 protektív hatása a metabolikus a fehérje homeosztatikus válaszok keresztkapcsolatára utal. Vizsgálataink létrehoztak egy új emlős modellt és rávilágítanak a denaturált fehérjeszerkezet funkciótól független hatásaira.

5. Az oxidatív stressz hatása a hősokkadaptációra

A fokozott oxidatív stressz kóroki szerepet játszik számos betegségben és az öregedésben, és hatásaiért elsősorban az általa okozott molekuláris károsodást teszik felelőssé. Emlős sejteken és *C. elegans* fonálférgen végzett kísérleteinkben az oxidatív hatású H_2O_2 hősokkadaptációra kifejtett hatását Bart Braeckmannal együttműködve tanulmányoztuk. H_2O_2 előkezelés egyaránt gátolta a Hsp70 expressziót és a hőindukált termotoleranciát emlős sejtekben illetve a hőindukált termotoleranciát *C. elegans* fonálféregben. Ezt a hatást a mikroRNS kötésben szerepet játszó Hsp70 3'UTR riporter konstrukt aktivációjával és a mikroRNS metabolizmusban kulcsszerepet játszó DCR-1/Dicer csendesítésével kapott eredmények szerint az RNS-interferencia közvetíti. Fonálférgekben mind *dcr-1*, mind másik kulcsenzim PASH-1/Drosha csendesítés visszaállítja az endogén peroxid-turnoverben deficiens *ctl-2* kataláz és *prdx-2* peroxiredoxin mutáns fonálférgek termotoleranciáját, illetve késlelteti az öregedő fonálférgek termotolerancia csökkenését (Spiró és mtsai, 2012; *2. ábra*).



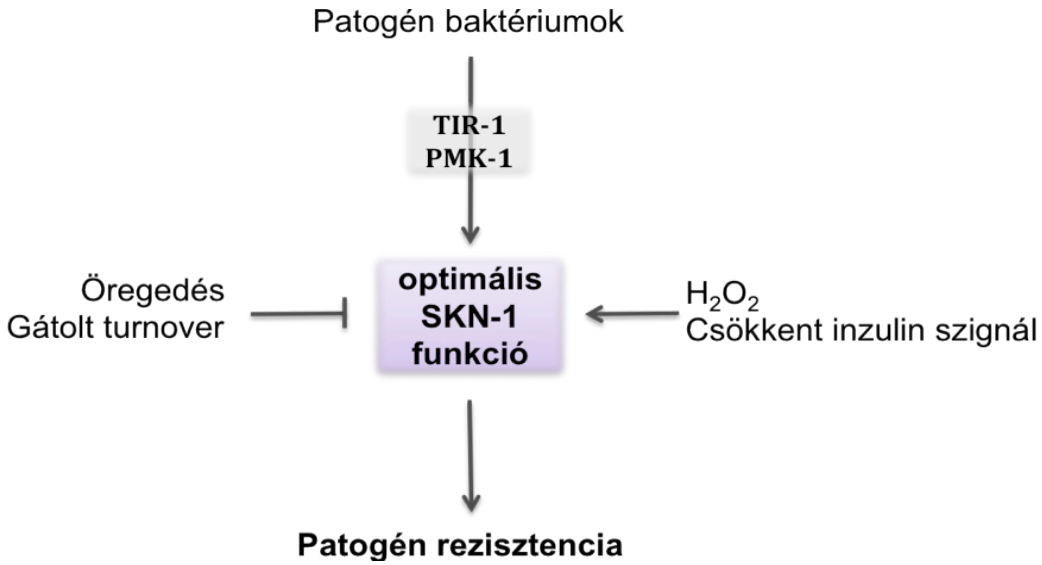
2. ábra ***Az oxidatív stressz hatása a hősokkadaptációra***

Eredményeink feltárnak egy szabályozott, az RNS interferencia révén megvalósuló keresztkapcsolatot, mely hozzájárulhat az oxidatív stressz potenciális hatásaihoz betegségekben és az öregedés során. A keresztgátlás jelentősége egyelőre ismeretlen, de egy adaptációt segítő stresszválasz független stressz általi gyengítése példát ad a komplex rendszerek működése kapcsán fellépő túlzott kooperáció öregítő hatására (Kiss és mtsai, 2009). Az RNS interferencia, illetve a résztvevő, azonosítandó mikroRNS-ek új terápiás célpontokat kínálhatnak.

6. Az SKN-1/Nrf2 szerepe a természetes immunitásban és az immunszeneszcenciában

A természetes immunitás kiváló modellje a *C. elegans* fonálféreg, egyszerű immunrendszerén túl azért is, mert számos humán fakultatív patogénnel való kölcsönhatását és az ellenük kialakított rezisztenciát és immunválaszt tudjuk rajta tanulmányozni. Az utóbbi időben derült fény arra, hogy a megfelelő természetes immunitás az antimikrobiális effektorokon túl a gazdaszervezet épségét megőrző-helyreállító mechanizmusokra is

támaszkodik. Ilyen mechanizmus a xenobiotikus stresszválasz, mely a szervezetre potenciálisan káros anyagokat, pl. oxidatív vagy elektrofil ágenseket közömbösít. Ennek mesterregulátora az SKN-1/Nrf2 transzkripciós faktor. Kísérleteink során kimutattuk, hogy enyhe oxidatív (H_2O_2) és metabolikus (csökkent inzulin jelátvitel) stresszek fokozzák a humán opportunista patogén *Pseudomonas aeruginosa* elleni patogén rezisztenciát, melyekhez az SKN-1 jelenléte szükséges. SKN-1 hiányos fonálférgek csökkent túlélést mutatnak mind a Gram-negatív *P. aeruginosa*, mind Gram-pozitív *Enterococcus faecalis* baktériumokon. *P. aeruginosa* hatására az intesztinális SKN-1 nukleáris transzlokációja és transzaktivációja következik be, melyhez szükség van a TIR-1 adapter és PMK-1/p38 MAP kináz fehérjékre és egy eleddig ismeretlen tényezőre. Az SKN-1 transzkripciós aktivitása az életkor előrehaladtával már a reproduktív időszak alatt drasztikusan csökken, és a korral csökkenő expressziót mutató SKN-1-függő gének között túlreprezentáltak a *P. aeruginosa* fertőzés által regulált gének. Az SKN-1 turnoverének gátlásával létrehozott excesszív aktivációja azonban – ugyan fokozza az oxidatív rezisztenciát – de csökkenti a patogén rezisztenciát (Papp és mtsai, 2012; *3. ábra*).



3. ábra ***Az SKN-1 szerepe a patogén rezisztenciában***

Eredményeink feltárják a xenobiotikus stresszválasz regulátor SKN-1 fehérje természetes immunitásban és immunoszeneszcenciában játszott szerepét, melynek a humán immunitásban és immun-öregedésben is szerepe lehet. Az SKN-1 túlaktivációjának patogén rezisztenciára kifejtett antagonisztikus hatása illusztrálja az erőforrások optimális allokációjával fellépő konfliktust, ami az oxidatív stressz öregedés-okozó hatásának kivédése révén gyengítheti a patogén rezisztenciát (Kiss és mtsai, 2009). Az SKN-1/Nrf2 új terápiás stratégia illetve screen célpontja lehet.

AZ ÚJ TUDOMÁNYOS EREDMÉNYEK ÖSSZEFOGLALÁSA

Tudományos munkám legfontosabb kutatási eredményeit az alábbi tézisekben foglalom össze:

Hsp90-nel kapcsolatos eredmények

1. Jellemeztük a Hsp90 C-terminális nukleotidkötőhelyét és megállapítottuk, hogy széles nukleotid-specificitással rendelkezik.

2. Kimutattuk, hogy a Hsp90 C-terminális kötőhelyét gátló ciszplatin szelektíven gátolja a szteroid receptor kliensek stabilizációját, és nem érinti a Hsp90 kinázokkal és HSF1-gyel alkotott komplexeit.

3. A humán Hsp90α és β funkcionális jellemzése során rávilágítottunk a Hsp90α hatékonyabb chaperon működésére és fokozott Hsp90-antagonista radicicollal szembeni rezisztenciájára.

Stresszválaszokkal kapcsolatos eredmények

4. Kimutattuk, hogy idős patkány májból izolált citoszol chaperon kapacitása enyhén csökkent.

5. A fehérje homeosztatikus puffer, a hősokkválasz és az öregedés kapcsolatáról elméleti és számítógépes szimulációs modelleket alkottunk.

6. Megállapítottuk, hogy az időskori humán cinkszupplementáció stimulálja a perifériás limfociták alap és hőindukált Hsp70 expresszióját.

7. Kimutattuk, hogy a kalória csökkentés-mimetikum resveratrol emlős sejtekben aktiválja a hősokkválaszt.

8. Rámutattunk, hogy a kalória megvonás *C. elegans*-ban hőmérsékletfüggetlenül és HSF1-függő módon hosszabbítja meg az élettartamot.

9. Két szirtuin túltermelő transzgén *C. elegans* törzsben igazoltuk, hogy nem a szirtuin túltermelés növeli meg az élettartamot, hanem a genetikai háttérben található mutáció.

10. Létrehoztunk két emlős modellt az inert denaturált fehérjék funkciótól független hatásainak vizsgálatára és kimutattuk, hogy a denaturált szerkezet gátolja a sejtproliferációt és a stresszadaptációt, melyet a hősokkválasz és a SIRT1 szirtuin túltermelése kivéd.

11. Felderítettük, hogy az oxidatív stressz emlős sejtekben és *C. elegans*-ban az RNS interferencia közvetítésével gátolja a hősokkadaptációt.

12. Feltártuk, hogy a xenobiotikus regulátor SKN-1/Nrf2 transzkripciós faktor optimális aktivációja szükséges a természetes immunitáshoz, aktivitáscsökkenése pedig részt vesz az immunoszeneszcenciában *C. elegans*-ban

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Chaperone function and chaperone overload in the aged. A preliminary analysis

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Abstract

Chaperones have an important role in the repair of proteotoxic damage, which is greatly increased in aged subjects. Chaperone levels and expression were subject of numerous studies in aged organisms. However, there were only very few attempts to measure chaperone activity in aged animals. Here, we report our initial studies showing a decreased chaperone capacity of liver cytosol from aged rats compared to those of young counterparts. The amount of Hsc70/Hsp70 was not significantly different in livers of young and aged rats. On the contrary, old animals showed a significant decrease in their hepatic Hsp90 content, which may explain their decreased chaperone activity. The observed decrease in chaperone capacity may also reflect a direct proteotoxic damage of chaperones, or an increase in chaperone occupancy, i.e. a 'chaperone overload' due to the increased amount of damaged hepatic proteins in aged rats. Experiments are in progress to elucidate the mechanism of the observed age-induced changes in chaperone function.

Keywords: Molecular chaperones; Heat shock proteins; Stress proteins; Hsc70; Hsp70; Hsp90; Luciferase; Protein aggregation; Protein denaturation; Chaperone overload

1. Introduction

Chaperones are ubiquitous, highly conserved proteins, which either assist in folding of newly synthesized or damaged proteins in an ATP-dependent, active process or work in an ATP-independent, passive mode sequestering damaged proteins for future refolding or digestion (Bukau and Horwich, 1998; Hartl, 1996). Chaperones are especially needed and their synthesis is induced after an environmental stress leading to proteotoxic damage.

Aging is characterized by an increased rate of protein modification such as oxidation, glycation, deamidation of asparaginyl and glutaminyl residues and the subsequent formation of isopeptide bonds, etc. (Stadtman and Berlett, 1998; Wright, 1991). Susceptibility to various proteotoxic damages is further increased due to transcriptional and translational errors and the resulting folding defects (Dukan et al., 2000).

Due to the decrease in proteasome function during aging (Conconi et al., 1996; Bulteau et al., 2001) as well as the impaired lysosomal protein degradation in aged rats (Cuervo and Dice, 2000), damaged proteins accumulate in cells of aged and may cause a chaperone overload. Here, the competition of

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damaged proteins may disrupt the folding assistance of other chaperone targets, such as: (1) newly synthesized proteins; (2) 'constantly damaged' (mutant) proteins; and (3) constituents of the cytoarchitecture (Csermely, 2001a,b).

Despite of the large number of studies on chaperone levels and induction in aged organisms (reviewed by Söti and Csermely (2002) and Verbeke et al. (2001)) direct studies on chaperone function in aged organisms are largely restricted to the eye lens chaperone, α-crystallin. Chaperone activity of α-crystallin is decreased in senile human lenses (Cherian and Abraham, 1995). As another of the sporadic examples of chaperone function in aged animals, Hsp90 fails to protect the proteasome in aged animals (Conconi et al., 1996).

In our present studies we have pursued an initial test of passive chaperone function by an indirect method, by measuring the attenuation of heat-induced luciferase denaturation by liver cytosolic preparations from young and aged rats. Our data show a decreased chaperone capacity of liver cytosol from aged rats compared to those of young counterparts, which is the first data on total chaperone function of cytosolic chaperones in aging.

2. Materials and methods

2.1. Materials

Anti-Hsc/Hsp70[1] antibody (rabbit, polyclonal) was a kind gift of Zoltán Pénzes (Biorex R&D Co., Veszprém, Hungary) (Kurucz et al., 1999). Anti-Hsp90α/β antibody (goat, polyclonal, sc-1055) was a Santa Cruz (Santa Cruz, CA, USA) product. Anti-luciferase antibody (goat, polyclonal) was purchased from Promega (Madison, WI, USA). Secondary anti-rabbit, and anti-goat antibodies were DAKO A/S products (Glostrup, Denmark). Chemicals used for polyacrylamide gel electrophoresis and protein determination were from Bio-Rad (Richmond, CA, USA). All other chemicals (including luciferase) used were from Sigma Chemicals Co. (St Louis, MO, USA).

[1] Abbreviations used: Hsc70, the constitutive form of the 70 kDa heat shock protein; Hsp70, inducible form of the 70 kDa heat shock protein; Hsp90, 90 kDa heat shock protein.

2.2. Animals

Young (10 weeks ± 3 days old) and aged (26 months ± 2 weeks old) Wistar rats were from Charles River Inc. (Hungary). Animals were kept and sacrificed according to the Guidelines of the Hungarian Council of Health Sciences (permission no. 39/1999).

2.3. Isolation of rat liver cytosol

Livers were removed and homogenized by a Potter–Elvehjem homogenizer in two volumes of an ice-cold buffer consisting of 20 mM Hepes, pH 7.4 and a complete protease inhibitor cocktail (Roche, Mannheim, Germany). Liver homogenates were filtered through a cheesecloth and centrifuged at 4 °C for 10 min at 700 × *g*. Supernatants were centrifuged in a Beckman J2-HS centrifuge at 4 °C for 10 min at 12,000 × *g*. Postmitochondrial supernatants were cleared from microsomes in a Beckman Optima TL ultracentrifuge using a TLA 100.4 rotor at 4 °C for 60 min at 100,000 × *g*. The supernatant cytosol was immediately aliquoted, frozen in liquid nitrogen and stored at −80 °C. Extreme care was exercised to use the thawed aliquots immediately and never re-freeze them. Protein content of the obtained cytosolic samples was measured using the Bradford (1976) method with bovine serum albumin as standard.

2.4. Protection of luciferase from heat denaturation by cytosolic chaperones

Cytosolic proteins at a final concentration of 40 mg/ml were mixed with 400 nM of firefly luciferase in a final volume of 200 μl of a buffer consisting of 20 mM Hepes, pH 7.5 and 50 mM potassium acetate and incubated at 39 °C. At time points indicated 2 μl aliquots were removed from the incubation mixture, and their luciferase activity was measured by adding them to 36 μl of the reaction mixture containing 25 mM Tricin (pH 7.8), 10 mM $MgSO_4$, 0.2 mM EDTA, 20 mM DTT, 0.26 mM Coenzyme A, 1 mM ATP and 30 μg/ml luciferin. Luciferase activity was measured in a BioOrbit Galaxy 1258 luminometer (Turku, Finland) with an integration time of 10 s, at a normal gain setting.

2.5. Protection of luciferase from aggregation by cytosolic chaperones

Cytosolic proteins at a final concentration of 40 mg/ml were mixed with 400 nM of firefly luciferase in a final volume of 200 μl of a buffer consisting of 20 mM Hepes, pH 7.5 and 50 mM potassium acetate and incubated at temperatures indicated. At time points indicated all the 200 μl samples were removed from the thermostatized tubes, and centrifuged in a Beckman Optima TL ultracentrifuge using a TLA 100.1 rotor at 4 °C for 10 min at 350,000 × *g*. Supernatants were carefully removed. Luciferase content of the supernatants and ice-cold control samples without incubation/centrifugation procedures were assessed by subjecting the samples of SDS PAGE and consecutive Western blotting. Blots were visualized by anti-luciferase antibody using the ECL chemiluminescence kit.

2.6. Assessment of chaperone levels

80 μg of cytosolic proteins were subjected to SDS PAGE on a 10.5% gel using a BioRad MiniProtean B system. Gels were blotted with a home-made semidry blotting apparatus to Protran nitrocellulose membrane (Schleicher & Schuell Co., Keene, NH, USA). Blots were blocked by 2% bovine serum albumin and visualized by anti-Hsc/Hsp70 and anti-Hsp90α/β antibodies using the ECL chemiluminescence kit. Loading efficiency was controlled by assessing the identical amount of the externally added luciferase to the samples. Photographic images were quantitated by densitometry using an LKB Ultroscan XL laser densitometer (Bramma, Sweden).

2.7. Statistical evaluation

Statistical evaluation of luciferase heat denaturation data was performed by the Student's *t*-test.

3. Results

Parallel with our ongoing experiments to measure the active (ATP-dependent) chaperone activity of cytosolic preparations, we also assessed the passive (ATP-independent) chaperone activity of liver cytosols using an indirect method. Here, we report our initial results of these, latter experiments. For this we had to find a test protein, which is much more susceptible to denaturation than most of the cytosolic proteins, including molecular chaperones. Luciferase, a commonly used firefly enzyme was suitable for this purpose, since it rapidly loses its activity when incubated at 39 °C, where most of cellular proteins (potential other targets and chaperones) still remain intact (Freeman et al., 2000).

The effect of cytosol from young and old rats on the heat-induced luciferase denaturation is shown in Fig. 1. Luciferase denaturation is complete in 30 min irrespective of the presence of protecting cytosolic chaperones. At all other time points measured cytosol from livers of young (10 weeks old) rats shows a better protection than that of old (26 months old) animals. The difference between the protected luciferase activity is significant after 5 min of incubation reaching a level of significance of $p < 0.03$.

Another important consequence of ATP-independent chaperone function is the prevention of protein aggregation. Therefore, a different test of passive

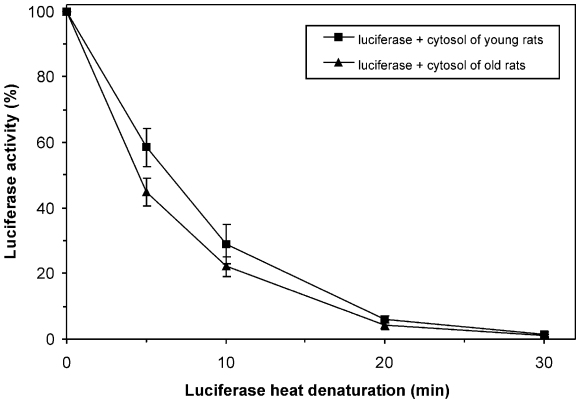

Fig. 1. Protection of luciferase activity against heat denaturation by cytosol of young (10 weeks) and old (26 months) Wistar rats. Isolation of rat liver cytosol, incubation of luciferase and measurement of luciferase activity were performed as described in Section 2. Squares: luciferase activity in the presence of cytosol from young (10 weeks) rats. Triangles: luciferase activity in the presence of cytosol from old (26 months) rats. Data were obtained from duplicate measurements from three rats per group. There is a significant difference between luciferase activity in the presence of cytosol from young and old rats at 5 min of incubation with a level of significance $p < 0.03$.

chaperone function giving complementary results to the experiments shown in Fig. 1, is the assessment of the heat-induced aggregation of luciferase. In initial experiments, we obtained a measurable aggregation of the enzyme (i.e. partition to the pellet after ultracentrifugation at 350,000 × *g* for 10 min) both at 39 and 42 °C, but not at 0 °C, where no aggregation was detected neither in the presence of young nor in the presence of aged cytosols (data not shown). Measurement of luciferase aggregation in the presence of cytosolic proteins from livers of young and old rats at 42 °C showed a larger amount of non-aggregated luciferase in the presence of cytosols from young rats (Fig. 2; 0.21 ± 0.04 and 0.15 ± 0.04 for young and old animals, respectively). However, the difference was not significant ($p < 0.104$). Examination of luciferase aggregation at less stringent conditions (39 °C) did not give conclusive results (data not shown).

As an obvious reason for the weaker luciferase protection of liver cytosol from aged rats, a decreased chaperone content comes to mind. To analyze this possibility we have measured the levels of the two most abundant cytosolic chaperones, Hsc/Hsp70 and Hsp90α/β (Bukau and Horwich, 1998; Hartl, 1996). As it is shown in Fig. 3A, there were no significant differences in the level of Hsc/Hsp70 in cytosol from young and old rats (the antibody we used recognizes both the constitutive and inducible forms of the rat 70 kDa heat shock protein). On the contrary, old animals contained a significantly lower amount of Hsp90α/β than their young counterparts (the antibody we used recognizes both the alpha and beta isoforms of the rat 90 kDa heat shock protein; Fig. 3B; 0.60 ± 0.09 and 0.26 ± 0.10; for young and old animals, respectively, $p < 0.011$).

4. Discussion

The most important finding of the present paper is a decreased chaperone capacity of liver cytosol from aged rats compared to those of young animals, which is the first data on total chaperone function of cytosolic chaperones in aging.

The attenuated chaperone function in aged rats might have at least three independent reasons: (1) the amount of chaperones was diminished; (2) chaperones were damaged; (3) chaperones became occupied by an increased amount of damaged proteins or peptides. Analyzing the first possibility, we measured the levels of the two most important cytosolic chaperones, Hsc/Hsp70 and Hsp90. The significant decrease in Hsp90 content may explain the diminished hepatic chaperone capacity of aged rats. Existing data on chaperone levels in livers of aged rats are rather contradictory: Wu et al. (1993) measured an unchanged Hsc70 level, while Cuervo and Dice

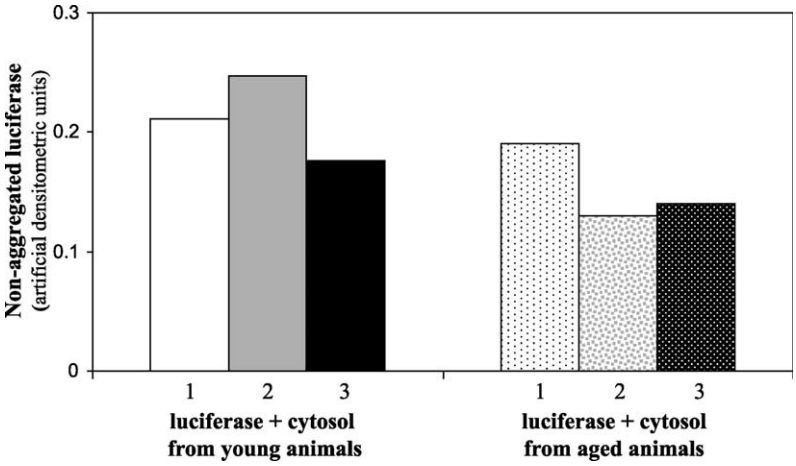

Fig. 2. Prevention of luciferase aggregation by cytosol samples from livers of young (10 weeks) and old (26 months) Wistar rats. Isolation of rat liver cytosol, incubation of luciferase at 42 °C, and measurement of luciferase aggregation were performed as described in Section 2. Bars show the amount of non-aggregated luciferase in the presence of cytosolic proteins of each examined animal in artificial densitometric units.

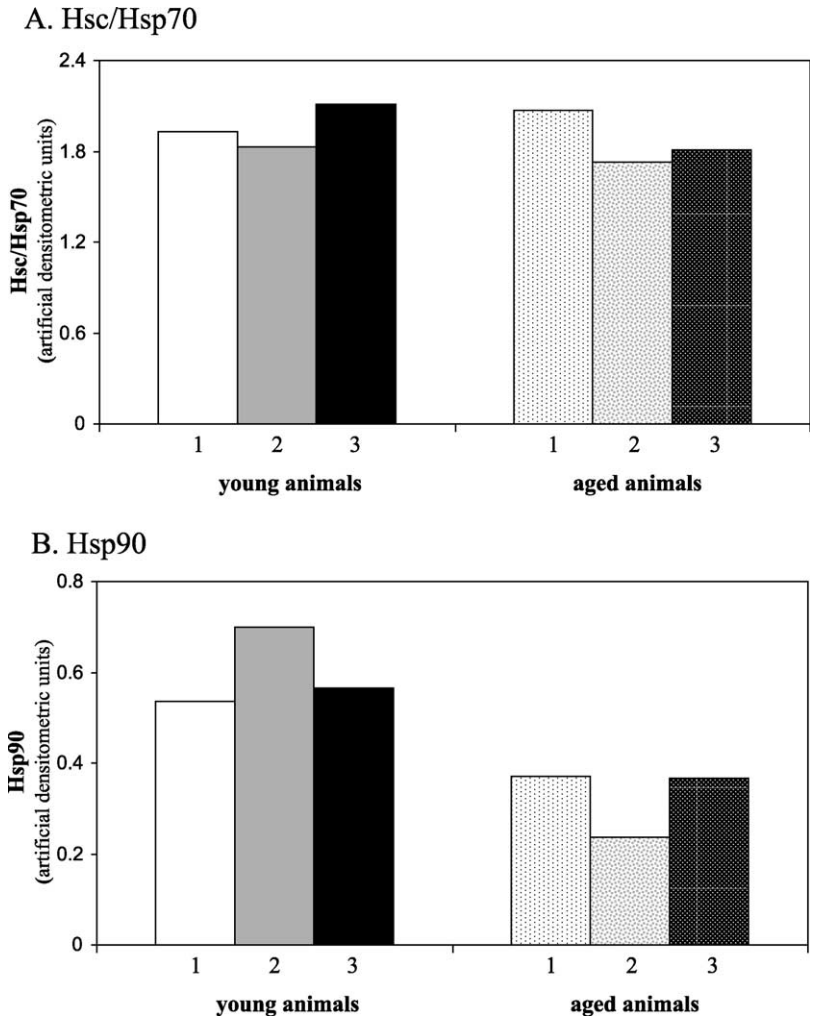

Fig. 3. Level of major cytosolic chaperones from livers of young (10 weeks) and old (26 months) Wistar rats. Isolation of rat liver cytosol, and measurement of Hsc/Hsp70 and Hsp90α/β levels were performed as described in Section 2. Bars show the amount of the respective cytosolic heat shock protein of each rat in artificial densitometric units. Panel A: Hsc/Hsp70 levels. Panel B: Hsp90α/β levels.

(2000) and Hall et al. (2001) showed an increased Hsc70 (and Hsp70) content of old rats, while to our best knowledge no report assessed hepatic Hsp90 levels of old rats. Higher chaperone levels may reflect an adaptation mechanism to the growing number of unfolded polypeptide chains, which titrate out the chaperones from the heat shock factor complex, and induce a constitutive stress response. This rather permanent stress probably 'wears out' the mechanism to mobilize the stress response, which may explain why the *induction* of various chaperones is impaired in aging (Söti and Csermely, 2002). The intrinsic variability of the experienced stress and the various ages and strains used in different studies may explain the observed variability of chaperone levels in aged rats.

The observed decrease in chaperone capacity may also reflect a direct proteotoxic damage of chaperones, or an increase in chaperone occupancy, i.e. a 'chaperone overload' due to the increased amount of

damaged hepatic proteins in aged rats. Macario and Conway de Macario (2002) raised the idea of 'sick chaperones' in aged organisms in a recent review. Indeed: chaperones are interacting with a plethora of other proteins (Csermely, 2001a), which requires rather extensive binding surfaces. These exposed areas may make chaperones a preferential target for proteotoxic damage: chaperones may behave as 'suicide proteins' during aging. On the other hand, the dramatic increase of potential targets, the 'chaperone overload' (Csermely, 2001b), saturates the remaining chaperone capacity and worsens the situation probably further. Experiments are in progress to elucidate the mechanism of the observed age-induced changes in chaperone function by analyzing the amount of damaged proteins, and the structure/function relationships of chaperones.

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The Heat Shock Protein 90-Targeting Drug Cisplatin Selectively Inhibits Steroid Receptor Activation

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Cisplatin is an antineoplastic drug that binds to DNA, thereby inhibiting cell division and tumor growth. Cisplatin may also disrupt the function of some proteins, including heat shock protein 90 (Hsp90). We report that cisplatin dose-dependently inhibited transcriptional activity of the androgen receptor and the glucocorticoid receptor (GR) in transient reporter assays. A truncated, hormone-independent GR was only partially inhibited at significantly higher doses of cisplatin. Cisplatin treatment of neuroblastoma cells led to an immediate inhibition of hormone binding by GR, followed by proteasome-dependent degradation of the receptor. Other Hsp90-regulated proteins, *i.e.* the phosphokinases raf-1, lck, and c-src, were not affected, indicating a specific functional interference of cisplatin with the steroid receptors GR and androgen receptor. Cisplatin did not elicit a stress response, in contrast to geldanamycin. Immunoprecipitation revealed that cisplatin disrupts binding of GR to Hsp90. Moreover, cisplatin-treated Hsp90 was unable to associate with untreated ligand binding domain of GR. Reticulocyte lysate was able to restore hormone binding of GR *in vitro*, but not when the lysate was pretreated with geldanamycin. Our data reveal that cisplatin influences steroid receptors also independently of its DNA-mediated effects and, thus, suggest a novel modes of action for this cytostatic drug. (*Molecular Endocrinology* 17: 1991–2001, 2003)

TOGETHER WITH ITS various derivatives, cisplatin [cis-diamminedichloroplatinum (II), CDDP] is one of the most commonly applied antineoplastic drugs today. It is a long-standing paradigm that the cytotoxic effect of cisplatin is primarily due to its well-described formation of adducts with DNA (1–3) that cause G2 arrest in the cell cycle, usually leading to apoptosis and inhibition of tumor growth (4, 5). It has been speculated that protein targets (6) may also be important for the observed effects of CDDP (5), an idea strongly supported by a recent report (7). CDDP has also been reported recently to bind to heat shock protein 90 (Hsp90) (8), thereby reducing its chaperoning activity. The CDDP-binding region has been localized near the C terminus of Hsp90, distant from the N-terminal binding site of geldanamycin (GA) (9), another antineoplastic agent that also reduces Hsp90 chaperoning activity (10). These results suggest that CDDP and GA may inhibit the chaperoning activity of Hsp90 by different mechanisms.

Hsp90 is one of the most abundant molecular chaperones in the eukaryotic cytosol and is known to regulate the stability and function of a wide spectrum of intracellular proteins. Hsp90 client proteins are involved in a variety of biological processes including development, cell cycle control and steroid hormone signaling (11–13). The two best-characterized classes in the steadily growing list of Hsp90-regulated proteins are transcription factors, *e.g*. steroid receptors such as the glucocorticoid (GR) and androgen receptor (AR), and protein kinases including raf1, src, and lck (11, 12). GR is an essential factor in many developmental and physiological processes (14) and has been used in numerous studies to elucidate the contribution of chaperones to proper folding and function of signal transduction molecules (15). In the absence of its ligand, the glucocorticoids, GR is part of a multiprotein complex comprising the receptor, Hsp90, and several other chaperones and cochaperones (16). This complex keeps the receptor protein in an inactive, yet ligand-activable state (17). Upon binding to hormone, GR is translocated to the nucleus and both positively and negatively regulates transcription of a variety of genes (18, 19). Similar Hsp90 heterocomplexes have been found to be important for other factors, *e.g*. heat shock factor 1 (HSF1) and protein kinases like raf1, lck, and c-src (20–23).

There is evidence that some physiological effects of CDDP may result from its targeting of Hsp90. For example, CDDP is commonly used as antineoplastic agent for a variety of prostate cancer types which in turn are often associated with hyperactivity of the Hsp90-dependent androgen receptor (AR). Moreover,

Abbreviations: AR, Androgen receptor; CDDP, cisplatin cis-diamminedichloroplatinum (II); FCS, fetal calf serum; GA, geldanamycin; GR, glucocorticoid receptor; HA, herbimycin A; HSF1, heat shock factor 1; Hsp90, heat shock protein; PMSF, phenylmethylsulfonyl fluoride; RAD, radicicol; RL, reticulocyte lysate.

CDDP-resistance in cells transfected with the Hsp90-dependent protein kinase v-src can be overcome by the Hsp90-targeting drugs radicicol (RAD) or herbimycin A (HA) (24). Interestingly, RAD, HA, and its homolog GA originally were identified as naturally occurring antitumor antibiotics (25–27) and characterized later as tyrosine kinase inhibitors (28–30). Subsequently, it has become clear that they actually act through binding to Hsp90 (9, 31–35). Moreover, it is a long-standing, although unexplained, observation that glucocorticoids like dexamethasone decrease CDDP-induced emesis if added to an antiemetic pharmacological regimen (36). Thus, one can hypothesize that dexamethasone, as a potent ligand of GR, fights CDDP-induced emesis by counteracting CDDP-induced defects in GR function.

To evaluate whether important aspects of CDDP function involve Hsp90-dependent proteins, we investigated the effects of CDDP on the steroid receptors GR and AR. We demonstrate that CDDP inhibits GR- as well as AR-mediated transcriptional activation in neuroblastoma cells. Hormone binding of GR is reduced by CDDP due to perturbation of the chaperone heterocomplex and followed by proteasomal degradation of GR. However, HSF1 is not activated by CDDP and the protein levels and activation of the kinases raf1, c-src, and lck are not changed by CDDP, in contrast to GA. Impairment of GR binding to hormone by CDDP can be reversed *in vitro* with untreated reticulocyte lysate, but only if its Hsp90 is intact. Our results provide new insights into the molecular mechanisms of Hsp90 inhibition by CDDP, which may lead to an improvement of CDDP pharmacotherapy in the future.

RESULTS

Dose-Dependent Inhibition of the Glucocorticoid and Androgen Receptors by Cisplatin

To test the hypothesis that cisplatin (CDDP) inhibits the activity of Hsp90-dependent steroid hormone receptors such as the glucocorticoid receptor (GR) or the androgen receptor (AR), we used a reporter gene assay in transient transfections of cultivated cells (37). Neuroblastoma SK-N-MC cells were transfected with a plasmid expressing the receptor (either GR or AR), a reporter plasmid with the mouse mammary tumor virus promoter controlling the firefly luciferase structural gene, and a β-galactosidase encoding plasmid to monitor transfection efficiency and general transcriptional activity. Cells were incubated in medium with 10 nM cortisol for stimulation of GR or 10 nM dihydrotestosterone for stimulation of AR together with increasing concentrations of CDDP. The stimulation of the reporter gene activity in the presence of CDDP was related to the stimulation in the absence of CDDP, which was set to 100% for each titration. Both GR- and AR-dependent transcription were severely impaired (Fig. 1, A and B, respectively). About half-maximal activity of the receptors was observed at a concentration of CDDP of 0.1–1 μM (GR) or 1 μM (AR), and virtually complete inhibition was reached at concentrations of 100 μM CDDP. CDDP did not affect the enzymatic activity of the luciferase reporter (control not shown).

To evaluate the specificity of the inhibitory effect on GR and AR, we tested two other reagents, the CDDP structural isomer transplatin and cyclophosphamide, which is another DNA-targeting antineoplastic drug. Transplatin and cyclophosphamide failed to affect GR-dependent activity up to a concentration of 100 μM (Fig. 1, C and D). We also tested whether CDDP reduced the viability of the SK-N-MC cells under our assay conditions and found no effect on the MTT assay up to 100 μM CDDP (data not shown).

The inability of other DNA targeting drugs, such as cyclophosphamide, to inhibit GR-dependent transcription from the mouse mammary tumor virus promoter does not completely rule out the possibility that the effect of CDDP is due to its interaction with DNA (38). To begin to characterize the inhibitory effect of CDDP on GR we used a truncated GR that is devoid of the ligand binding domain. This receptor is not regulated by Hsp90, does not bind to hormone, and displays constitutive transcriptional activity to a moderate extent (39). CDDP also dose-dependently inhibited this receptor (Fig. 1E). However, in contrast to the full-length GR (Fig. 1A), higher doses were needed and the effect was not as strong. We conclude that, whatever the mechanism for the inhibitory effect of CDDP on the truncated GR is, it cannot fully account for the effect on full-length GR.

A likely target for the additional effect of CDDP on full-length GR is the ability to bind to hormone, either by binding to the ligand binding domain or by binding to proteins regulating hormone binding. Hormone binding of GR was determined as a measure of GR folding. SK-N-MC cells were transfected with a GR expression vector and binding of radioactive cortisol was determined in whole cells 24 h after transfection in the presence of increasing concentrations of CDDP during a 1-h incubation. Nonspecific binding was monitored by adding 1000-fold excess of unlabeled cortisol, and specific binding was determined by subtracting the low nonspecific binding from the binding measured in the absence of unlabeled competitor. As shown in Fig. 2, hormone binding by GR was dose-dependently reduced by CDDP. Virtually complete inhibition was reached at 100 μM CDDP. Furthermore, the level of GR protein extracted from the cells remained unchanged throughout the titration, indicating that GR is not degraded after the 1-h incubation, but merely rendered nonfunctional. These results strongly suggest that CDDP inhibits the chaperone-dependent folding of GR, an effect independent of its interaction with DNA.

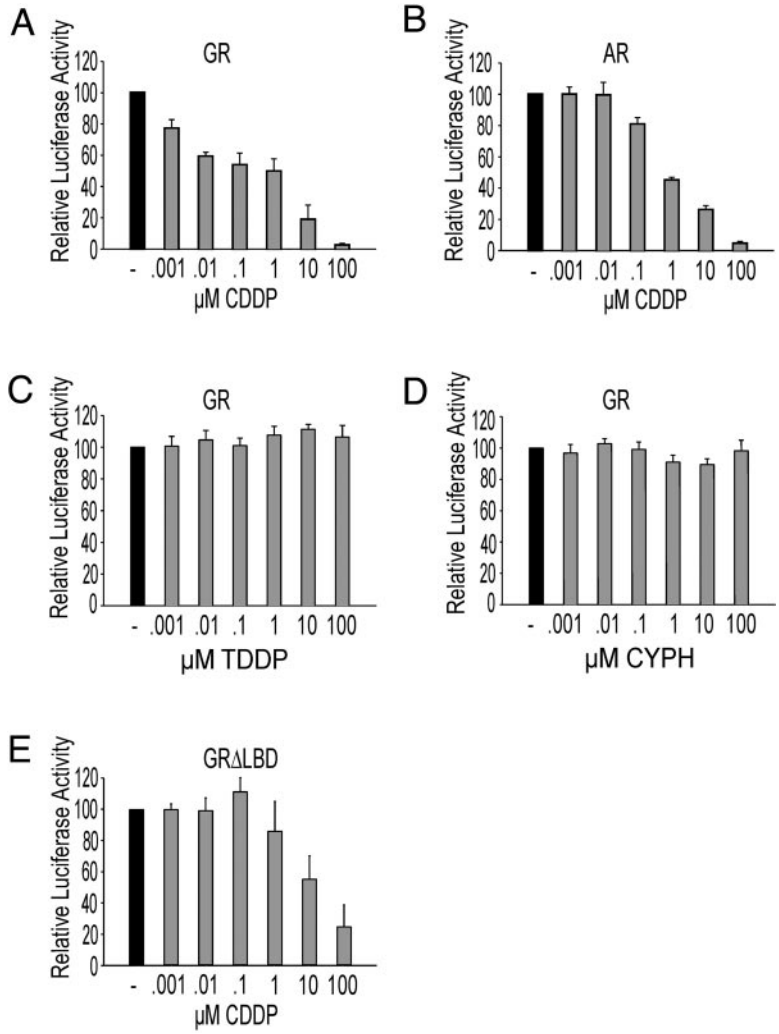

Fig. 1. Inhibition of GR- and AR-Dependent Transactivation by CDDP

Human neuroblastoma SK-N-MC cells were transfected with a GR-dependent reporter plasmid (MTV-Luc) a control plasmid expressing β-galactosidase and either a plasmid expressing full-length GR (A, C, D), AR (B) or GR lacking the ligand binding domain (E, "GRΔLBD"). After transfection, cells were cultivated with either 10 nM cortisol (A, C, D) or 10 nM dihydrotestosterone (B), together with increasing amounts of cisplatin (CDDP) (A, B, E), transplatin (TDDP) (C), or cyclophosphamide (CYPH) (D). Luciferase activities were normalized to the β-galactosidase activities and are presented as percent activity with hormone-stimulated cells set as 100%. Results represent mean values ± SEM of three independent experiments performed in duplicate.

The Effect of CDDP Is Specific for Steroid Receptors and Leads to Proteasome-Dependent Depletion of Cellular GR

A possible target to mediate these DNA-independent effects of CDDP is Hsp90. Other Hsp90-targeting drugs like geldanamycin (GA) and radicicol bind to the N-terminal ATP site and reduce the activity and protein levels of not only steroid receptors, but also several kinases (10, 23, 40–43). Therefore, we addressed two important questions: does prolonged treatment with CDDP affect protein levels of GR and are other Hsp90-dependent proteins also affected?

Figure 3 shows that treatment of GR-transfected SK-N-MC cells with CDDP or GA led to a dose-dependent decrease in the protein level of GR. The degradation observed here after 16 h of incubation with CDDP was clearly preceded by the inactivation of hormone binding by GR, which was observed after 1 h in the absence of degradation (Fig. 2). However, protein levels of the serine/threonine kinase raf-1 (Fig. 3C) and the tyrosine kinase c-src (Fig. 3D), as well as of Hsp90 itself (Fig. 3E), were not affected by CDDP, in contrast to GA. These data support the hypothesis that CDDP interaction with Hsp90 has specific consequences in that it impairs correct folding of GR, which leads to its degradation, but not of the protein kinases raf-1 and c-src.

We also tested whether degradation of GR is proteasome dependent, similar to the GA-induced degradation of other Hsp90 substrate proteins (32, 44, 45). GR-transfected SK-N-MC-cells were treated with

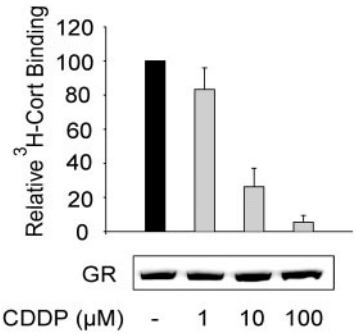

Fig. 2. CDDP Inhibits Hormone Binding of GR *in Vivo*

SK-N-MC cells were transfected with a plasmid expressing GR and binding of radiolabeled cortisol was determined after 24 h in whole cells. Cortisol and CDDP (at the concentrations indicated) were added for 1 h. Hormone binding with cortisol alone was set to 100%. Results represent mean values ± SEM of three independent experiments performed in duplicate. GR levels were assessed by Western blotting.

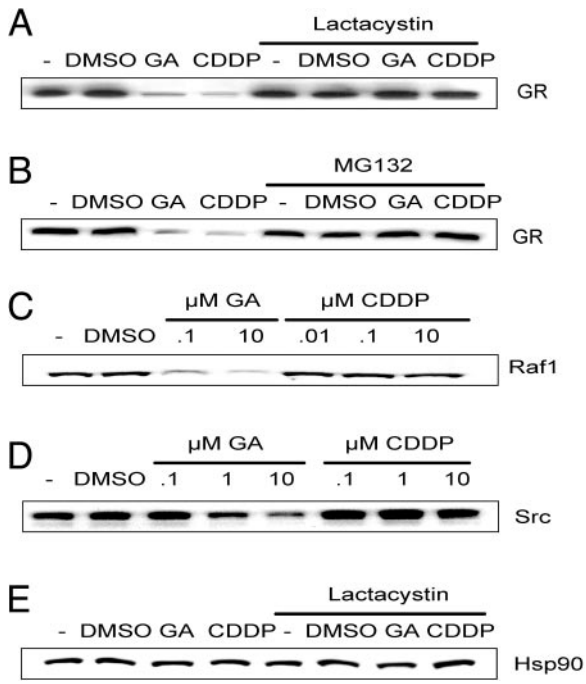

Fig. 3. CDDP Induces Proteasome-Dependent Degradation of GR, But Not of Hsp90-Dependent Kinases

SK-N-MC cells were transfected with a plasmid expressing GR (pRK7GR, 0.75 μg per plate) and treated for 16 h at 37 C with either 1 μM GA or 10 μM CDDP and the proteasome inhibitors lactacystin (100 μM, A) or MG132 (50 μM, B) where indicated. Nontransfected cells were used in C–E and treated with 1 μM GA or 10 μM CDDP (or the concentrations indicated in C and D) and lactacystin. Total cellular protein (100 μg) was fractionated by SDS-PAGE, probed with α-GR- (H-300) (A and B), α-Raf1- (C), α-c-Src- (mAB327) (D), or α-Hsp90 (H114) (E) antibody followed by chemiluminescent detection.

CDDP in combination with the highly specific proteasome inhibitors lactacystin (46) or MG132 (47). As a control, cell cultures were treated with GA instead of CDDP (43). After 16 h incubation in the presence of these drugs, GR levels were assessed by Western blotting of cell extracts. Treatment with lactacystin or MG132 alone had no apparent effect (Fig. 3, A and B), but both efficiently inhibited degradation of GR by GA, in full agreement with a previous report (32). Lactacystin and MG132 also blocked GR degradation induced by CDDP (Fig. 3, A and B), indicating that the same proteolytic pathway is induced.

To corroborate our conclusion that the Hsp90-targeting drug CDDP specifically affects steroid receptors, we analyzed the functions of several Hsp90-dependent proteins. GA and radicicol are known to elicit a cellular stress response by releasing HSF1 from Hsp90, and this response can be monitored by transcription from HSF1-dependent promoters (22). We therefore tested the activity of an HSF1-dependent promoter transfected into SK-N-MC cells with and without exposure to CDDP or GA. Whereas GA clearly showed induction of this promoter, CDDP had no effect (Fig. 4A).

Activation of the Hsp90-regulated kinases lck, which is a member of the src kinase family, and raf-1 is accompanied by their phosphorylation (48). To analyze whether CDDP may influence the activation of these kinases, we pretreated Jurkat cells with 1.8 μM GA or with 100 μM CDDP, followed by stimulation with an anti-CD3 antibody (OKT3). Levels and phosphorylation of lck and raf-1 were detected after cell lysis and Western blotting. As shown in Fig. 4B, GA efficiently reduced the basal protein levels and prevented the activation of both, lck and raf-1. In contrast to GA, CDDP had only a marginal affect on the basal protein levels and phosphorylation of raf-1 and activated lck. It should be noted that GR is efficiently inhibited by CDDP also in Jurkat cells under these conditions (data not shown).

We finally compared the effect of GA and CDDP on ATP-hydrolysis of Hsp90 purified from *Saccharomyces cerevisiae* (Hsp82), which has previously been used to characterize the ATPase activity of Hsp90 (49, 50). CDDP did not inhibit the ATPase activity of the yeast Hsp82 up to a concentration of 1 mM (data not shown), in contrast to GA (51).

From these data, we conclude that the effect of CDDP on Hsp90-dependent processes differs from that of GA in that the effect of CDDP is much more specific, *i.e.* is confined to steroid receptors.

CDDP Disrupts Hsp90-GR Interaction and Prevents Hsp90-GR Complex Formation *in Vitro*

To shed light on the mechanism of CDDP action on Hsp90-GR interaction, we supplemented cell lysates of GR-transfected SK-N-MC cells with 10 μM CDDP. After incubation for 1 h on ice, Hsp90 was immunoprecipitated and blots were probed for Hsp90 and for co-precipitated GR. Whereas GR was readily coimmu-

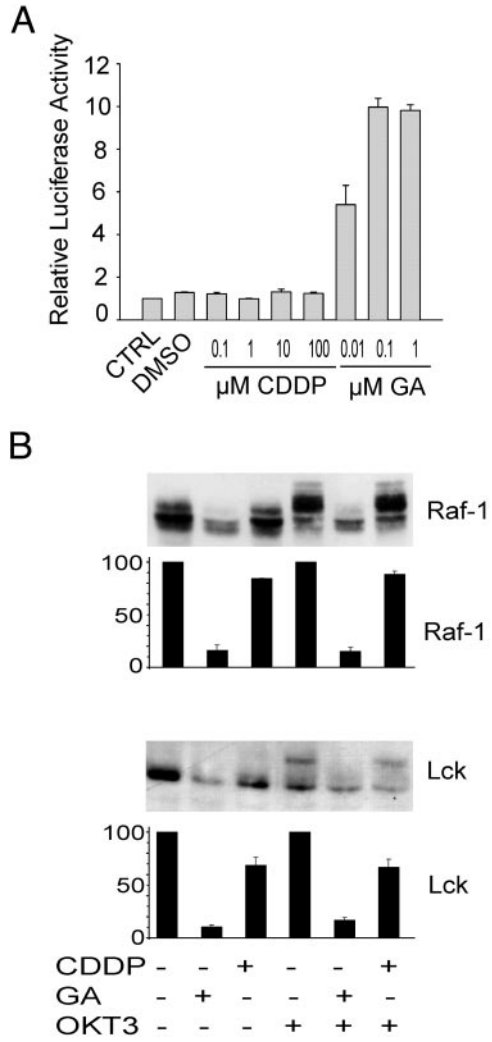

Fig. 4. CDDP Is Permissive for Activation of lck and raf Kinase and Does Not Activate HSF1-Dependent Promoters

A, SK-N-MC cells were transfected with an HSF1-dependent luciferase reporter and a β-galactosidase control plasmid. After transfection, cells were cultivated with or without DMSO as well as with increasing amounts of CDDP or GA as indicated. Luciferase activities were normalized to the β-galactosidase activities and are presented as fold induction with untreated cells as reference. Results represent mean values ± SEM of three independent experiments. B, After pretreatment with 1.8 μM GA or 100 μM CDDP for 16 h, Jurkat cells were stimulated with 5 μg/ml OKT3 antibody, and Raf-1 and Lck proteins were detected by immunoblotting. Shown are representative Western blots and the quantification of three independent experiments.

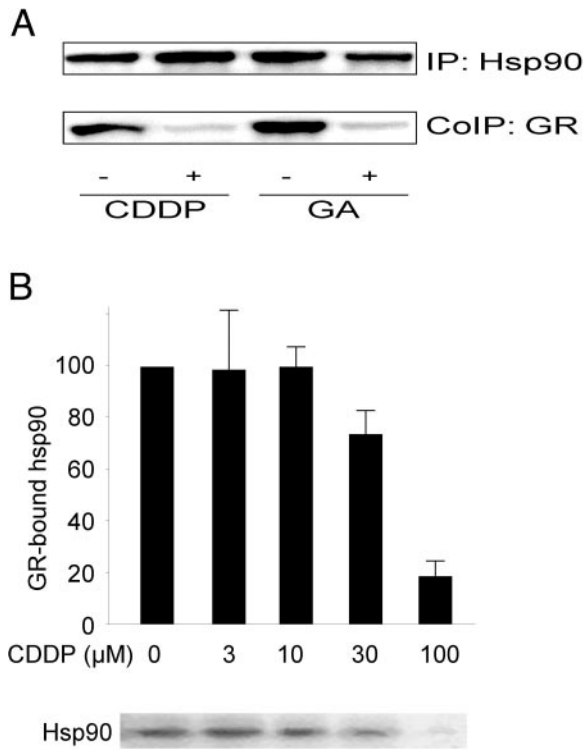

Fig. 5. CDDP Interferes with Association of Hsp90 with GR

A, SK-N-MC cells were transfected with a GR expressing plasmid (pRK7GR). Cell lysates were incubated with or without 10 μM CDDP or 1 μM GA for 2 h at 4 C and then immunoprecipitated with an Hsp90-directed antibody. After SDS-PAGE and transfer to nitrocellulose, blots were probed for Hsp90 and GR. B, Hsp90 was radiolabeled using an *in vitro* transcription/translation system, β-mercaptoethanol removed by gel filtration, treated with CDDP and unreacted CDDP removed by gel filtration. Association with myc-tagged GR ligand binding domain, bound to Sepharose beads via a myc antibody, was allowed for 10 min in the presence of reticulocyte lysate and bound proteins were eluted with high salt buffer. The relative amounts of bound labeled Hsp90 were assessed after gel electrophoresis (*lower panel*). Results represent mean values ± SEM of three independent experiments.

noprecipitated with Hsp90 from untreated cell lysates, preincubation with CDDP or GA caused a marked decrease in the amounts of co-precipitated GR (Fig. 5A).

To further elucidate the molecular mechanism of CDDP action, we investigated the effect of CDDP treatment of Hsp90 on complex formation with GR. Chaperone complexes with GR can be reconstituted in reticulocyte lysate with the GR ligand binding domain alone, and immune-isolated similarly to the full-length receptor (52). We therefore analyzed complex formation between CDDP-treated Hsp90 and the ligand binding domain of GR in reticulocyte lysate. Hsp90 was radioactively labeled by *in vitro* translation in reticulocyte lysate, β-mercaptoethanol was removed and the protein mixture was incubated with different concentrations of CDDP. It was important to then remove unreacted CDDP to ensure that it does not modify the ligand binding domain of GR in the following binding reaction. Labeled, CDDP-reacted Hsp90 was incubated with the myc-tagged ligand binding domain of GR, bound to myc antibodies on Sepharose beads for 10 min. As seen in Fig. 5B, CDDP dose-dependently interfered with complex formation between Hsp90 and GR. Thus, CDDP appears to act by both disrupting preformed Hsp90/GR complexes

(Fig. 5A) and preventing these complexes from forming (Fig. 5B).

CDDP Inhibition of GR Hormone Binding Can Be Rescued *in Vitro* by Reticulocyte Lysates with Intact Hsp90

Whereas our data provide strong evidence that the effect of CDDP on GR involves interaction with Hsp90, the possibility remained that CDDP actually targets GR directly, which also may cause its inactivation and subsequent degradation. Moreover, CDDP is reactive toward sulfhydryl groups, and it has been previously demonstrated that sulfhydryl-targeting agents can inactivate GR (53–55). We asked whether targeting of GR or targeting of an associated chaperone like Hsp90 underlies CDDP-induced inactivation of GR. We treated cells expressing GR with CDDP as in the experiment for Fig. 2. After CDDP treatment, cells were lysed, unreacted CDDP was removed by gel filtration and dose-dependent inhibition of hormone binding by CDDP was confirmed (data not shown). However, when we supplemented the hormone binding reaction with reticulocyte lysate (RL) supplemented with ATP, binding was restored (Fig. 6A). No hormone binding was observed with RL alone. This demonstrates that the CDDP impairment of hormone binding by GR cannot be due to an interaction of CDDP with GR. Moreover, hormone binding of CDDP-treated GR was not restored, when we pretreated RL with GA before gel filtration (Fig. 6B). GA is established as specific inhibitor of Hsp90 used in numerous studies (56). Therefore, this result strongly suggests that it must be the intact Hsp90 from RL that restores hormone binding activity to GR by replacing the CDDP-impaired Hsp90 activity from the cell extract.

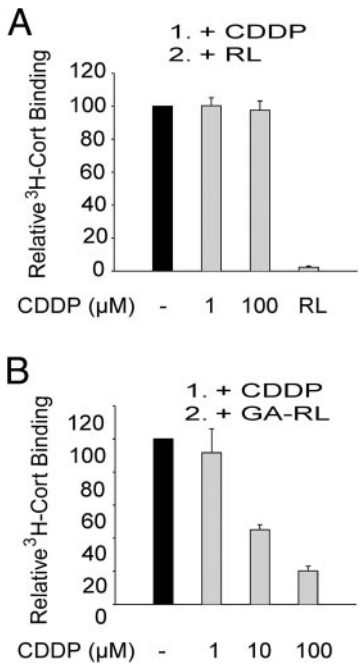

Fig. 6. Hormone Binding of GR Can Be Restored *in Vitro* by Reticulocyte Lysate with Intact Hsp90

Binding of GR expressed in SK-N-MC cells and treated with CDDP (as in Fig. 2) to cortisol was measured. Hormone binding in the absence of CDDP was set to 100%. A, 5% reticulocyte lysate, ATP and Mg^{2+} was added to the extracts from cells treated with1 μM and 100 μM CDDP. B, RL was pretreated with the specific Hsp90-inhibitor geldanamycin (1 μM) before it was added to the CDDP-treated cell extracts. Results represent three independent experiments.

DISCUSSION

Our results establish that CDDP specifically inhibits a subset of Hsp90-dependent processes, in particular the maturation of steroid hormone receptors represented by GR and AR. CDDP apparently interferes with the formation of the Hsp90 complexes required for the folding of these proteins. This discovery has a significant impact on our understanding of both the function of Hsp90 and the physiological consequences of CDDP application.

The most interesting aspect of our results with regard to Hsp90 function is that, unlike GA, CDDP disrupts some functions of Hsp90 but not others. Whereas the activity of the steroid hormone receptors GR and AR was drastically reduced by CDDP, HSF1-dependent transcription was not elicited. In contrast, GA, which targets the N-terminal ATP binding site of Hsp90, inhibits steroid receptors and activates HSF1-dependent promoters (this study and Refs. 22 and 32). Moreover, treatment of cells with CDDP leads to proteasome-dependent degradation of GR, whereas protein level and activation of the Hsp90-dependent protein kinases lck, c-src, and raf-1 were essentially unchanged. Inhibition of Hsp90 by GA, however, results in inactivation and degradation of not only GR, but also lck, c-src, and raf-1 (this study and Refs. 31, 48, and 57).

The inhibitory effect of CDDP on GR-dependent transcription has previously been ascribed to the well-documented interaction of CDDP with DNA (38). Moreover, in the case of the mineralocorticoid receptor induction of oxidative stress by CDDP has been suggested to cause diminished receptor activity (59). At a molecular level, oxidative stress would affect the sulfhydryl groups of proteins and sulfhydryl-targeting drugs have been shown to decrease GR function (53–55). Our observation that a truncated GR lacking the ligand binding domain is also inhibited by CDDP, albeit only at higher concentrations, can be explained by an effect on protein-DNA interactions, either by targeting the DNA or by targeting the cysteines of the zinc fingers in the DNA binding domain of GR. Modification of these cysteines has been shown before to inhibit DNA binding activity of GR (60). We assume that whatever mechanism applies to the inhibition of the truncated GR also contributes to the inhibition of full-

length GR. However, our data with full-length GR strongly suggest that an additional, and possibly the major effect of CDDP on steroid receptors is due to its interaction with Hsp90. Inhibition of hormone binding of GR cannot be explained by interference of CDDP with protein-DNA interactions. Moreover, disruption of the Hsp90 heterocomplex by CDDP, as detected by immunoprecipitation, clearly shows a DNA-independent effect of CDDP. Because we are able to rescue hormone binding of GR after removal of excess CDDP and supplementing with untreated reticulocyte lysate, but not with GA-treated lysate, modification of GR resulting from oxidative stress is most likely not responsible for the effect of CDDP. Furthermore, CDDP not only disrupted existing GR-Hsp90 interactions but also prevented formation of new interactions when reacted only with Hsp90.

The differential effects of the two Hsp90-targeting agents CDDP and GA could be explained by their different interaction site with Hsp90. Crystallographic analysis revealed the ATP-binding pocket of Hsp90 as the binding site of GA (9), whereas CDDP has been reported to bind to the middle to C-terminal part of Hsp90 (8). An additional binding site for ATP in the C-terminal part of Hsp90 has been postulated (61), and CDDP has been proposed to act as a selective nucleotide competitor for this site (62). The significance of this cryptic ATP binding site remains to be elucidated. The other postulated C-terminal ATP competitor novobiocin apparently binds in this region (61) and leads to depletion of Hsp90-dependent proteins including raf1 and v-src (63). This effect is similar to that of GA but different from the effect of CDDP, as we observed no degradation of raf-1 and c-src. Also in contrast to CDDP, novobiocin has been described to inhibit allosterically the N-terminal ATP binding site (62). Thus, we suggest that CDDP and novobiocin interact with the C-terminal region of Hsp90 at different sites and/or in different manners.

With regard to the exact binding site of CDDP on Hsp90, we speculate that it reacts with cysteine residues, most likely to cysteine 596 of the human protein. The corresponding cysteine of rat Hsp90 has been reported to be particularly reactive (64). Although we have not detected significant amounts of covalent Hsp90 dimers in our assays, it is interesting to note that others did so under their experimental conditions (65). These covalent dimers could arise from subsequent reactions of platinated Hsp90.

It should be noted that inhibition of Hsp70 by the SH-targeting drug N-ethylmaleimide has been reported (66). Because Hsp70 participates in folding the GR, it is possible that it is another chaperone target for CDDP, in addition to Hsp90. Reticulocyte lysate treated with GA was unable to restore cortisol binding of GR. Because GA is established as specific inhibitor of Hsp90 (56), Hsp70 was most likely functional in this extract, arguing in favor of Hsp90 being an important, and possibly the major chaperone target of CDDP.

Considering the possible physiological relevance of our findings, we note that CDDP is typically administered at doses of 80–100 mg/m^2 body surface (67, 68). As a rough estimate, the initial concentration in the blood (assuming even distribution throughout the blood) would be 140–180 μM, and about 10–12 μM assuming even distribution throughout the entire body liquid. Depending on the pharmacokinetics (distribution, uptake, degradation) there will be locally and temporally higher and lower concentrations. In any case, at least the initial concentrations are well within those needed to inhibit steroid receptors.

The effect of CDDP on steroid receptors may explain some of its clinical side effects. Delayed nausea and vomiting after application of CDDP remain a significant cause of treatment-related morbidity, because they are inadequately controlled by current therapies. Also newer therapeutics like 5-HT$_3$ receptor antagonists are of insufficient efficacy (69). Corticosteroids make a considerable contribution toward the control of CDDP-induced acute and delayed emesis (70). Two randomized studies demonstrated that the combination of dexamethasone and ondansetron is more efficacious than ondansetron alone (71, 72); ondansetron plus dexamethasone is now considered a standard antiemetic therapy for CCDP-treated patients (67). Also, ACTH has been shown to be effective in the prevention of acute CDDP-induced vomiting (73). The mechanism by which ACTH exerts its effect against emesis are not fully understood. It stimulates the production of corticosteroids, which are known to be active against emesis, but it also acts directly on the brain and modulates behavior.

Another severe side-effect of CDDP treatment is renal failure or renal electrolyte wasting (74, 75). Because this is usually controlled by administration of mineralocorticoid and sodium, it has been suggested that renal responsiveness to mineralocorticoid may be impaired in CDDP-treated patients (59). Although we did not test the mineralocorticoid receptor, in light of our findings we would explain these clinical observations by an effect of CDDP on Hsp90, which leads to reduced function of the mineralocorticoid receptor. Further insight into the molecular mechanisms of CDDP will allow improvements in its important clinical usage.

MATERIALS AND METHODS

Cell Culture and Transfection

Human neuroblastoma SK-N-MC cells (American Type Culture Collection, Manassas, VA; no. HTB-10) were kindly provided by the laboratory of C. Behl (Max Planck Institute of Psychiatry, Munich, Germany). They were cultured in DMEM supplemented with 10% fetal calf serum (FCS), 36 mg/liter sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 0.25 μg/ml amphotericin (all from Life Technologies, Inc.), and 4.5 g/liter glucose at 37 C and 10% CO_2.

Two days before transfection, cells were seeded into medium containing 10% charcoal-stripped, steroid-free FCS.

Dextran T-70 (Pharmacia, Uppsala, Sweden) was used for charcoal-stripping of FCS (76). Jurkat (J32) cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

SK-N-MC cells were harvested at about 60–80% confluency and about 0.5 to 1 $\times$ 10^7 cells were resuspended in 400 μl of electroporation buffer [50 mM K_2HPO_4, 20 mM KAc (pH 7.35)]. A 1.5-μg steroid-responsive luciferase reporter plasmid MTV-Luc (77), 2.5 μg simian virus 40 promoter-driven β-galactosidase expression vector pCH110 (Pharmacia LKB, Freiburg, Germany), and 0.75 μg pRK7GR that expresses human GR (78) from the cytomegalovirus promoter of the vector pRK7 (79) were added and transfection was performed using an electroporation system (Biotechnologies & Experimental Research, San Diego, CA) after determination of the optimal electrical field strength (80). Electroporated cells were replated and cultured for 16 h in fresh medium (containing 10% steroid-free FCS), supplemented with cortisol, dihydrotestosterone, cisplatin, transplatin, geldanamycin, and cyclophosphamide (purchased from Sigma Chemical Co., Deisenhofen, Germany) in the combinations and concentrations indicated in the text and figure legends. Cells without drug were supplemented with the solvent of the respective drug, reference cells without cortisol and drug were supplemented with the solvents of cortisol (*i.e.* ethanol) and the drug.

Luciferase and β-Galactosidase Assay

Luciferase and β-galactosidase assays were as described before (37). Briefly, cells were scraped from the plate in 1 ml of lysis buffer [0.1 M $KHPO_4$ (pH 7.8) and 1 mM dithiothreitol] and cytosolic extracts were made by three freeze and thaw cycles and subsequent centrifugation. Fifty microliters of each supernatant (corresponding to $\sim$1–2 $\times$ 10^5 cells) were transferred to a 96-well plate. One hundred fifty microliters of 33 mM $KHPO_4$ (pH 7.8), 1.7 mM ATP, 3.3 mM $MgCl_2$, and 13 mM Luciferin (Roche Biochemicals, Mannheim, Germany) were added to each sample by the injector of an automatic luminometer (Luminat LB 96, Wallac GmbH, Freiburg, Germany) and light emission was measured for 10 sec. To correct for variations in transfection efficiencies, values of the luciferase assay were normalized using β-galactosidase activities that were measured as follows: 50 μl of cell extract were added to 100 μl galactosidase buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgCl_2$, and 50 mM β-mercaptoethanol) on a 96-well plate. 20 μl of 2 mg/ml ONPG was added and the reaction was incubated at 37 C. After 10–30 min, absorption was measured at 405 nm in a multiphotometer (Dynatech MR5000, Billingshurst, West Sussex, UK). Correcting the luciferase activities by the β-galactosidase activities was important, because a moderate reduction of the general transcription was observed in some experiments at the highest concentrations of cyclophosphamide and CDDP. We also found that fewer cells were attached at the highest concentrations of CDDP and cyclophosphamide. In another control experiment, we found that CDDP did not change the luciferase activity when added to cell extracts before measurement (not shown).

HSF1 Response

For analysis of HSF1-dependent transcription, SK-N-MC cells were cotransfected with 3 μg of an HSF1-driven luciferase reporter plasmid (HSE-Luc) and 3 μg of the β-galactosidase control plasmid. Cells were incubated with or without the indicated amounts of CDDP or GA and harvested after 16 h for analysis of luciferase and β-galactosidase activities as described above.

Hormone Binding of GR *in Vivo* and *in Vitro*

To determine hormone binding of GR *in vivo*, SK-N-MC cells were seeded and transfected with pRK7GR as described above. Twenty-four hours after transfection, cells were incubated with fresh medium containing 10 nM [^{3}H]-cortisol (Amersham, Braunschweig, Germany; specific activity was 62 Ci/mmol) alone or with CDDP at the concentrations indicated. To determine nonspecific hormone binding, a 1000-fold excess of unlabeled cortisol was added in parallel samples. After 1 h, cell plates were put on ice, cells were washed three times with ice-cold PBS, and cells were scraped from the plate in 500 μl of PBS, collected by centrifugation at 2 C and resuspended in 100 μl of PBS. Ninety microliters were used for scintillation counting (Beckman LH 6500, Munich, Germany), the remaining was used to determine the cell number. Counts were divided by the cell number, and nonspecific binding was subtracted from each value.

To determine hormone binding *in vitro*, SK-N-MC cells were seeded out and transfected with pRK7GR and, after 24 h, treated with CDDP for 1 h as described above. Cells were lysed with RIPA buffer [10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1% (vol/vol) deoxycholate, 1% (vol/vol) Triton X-100, 0.1% (wt/vol) sodium dodecyl sulfate, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM aprotinin] at 4 C for 30 min. After centrifugation, residual CDDP which may be present was removed by gel filtration over biospin 6 columns (Bio-Rad, Hercules, CA), which had been preequilibrated with hormone binding buffer [5 mM Tris/HCl (pH 7.4), 1 mM EDTA, 10 mM $Na_2MoO_6x2H_2O$]. At this time, the extracts were divided into aliquots.

To test hormone binding, 10-μl aliquots were either incubated (1 h, 4 C) with 100 nM radiolabeled cortisol alone, or in combination with 1000$\times$ excess (100 μM) unlabeled cortisol to determine nonspecific binding. Bound and free steroids were separated by gel filtration by using Sephadex LH-20 (Pharmacia). Radioactivity was measured in a liquid scintillation counter (Beckman LH 6500) and protein concentration was determined by the Lowry method (81) to normalize the data.

To reverse the inhibition by cisplatin, rabbit reticulocyte lysate (Green Hectares, Oregon, WI) was added (5% of the total volume) to aliquots, along with 2 mM ATP and 6 mM Mg^{2+} and incubated for 10 min at room temperature. Hormone binding was determined as described above. As a control, hormone binding of reticulocyte lysate alone was also determined. To inhibit Hsp90 in the reticulocyte lysate, 1 μM of the Hsp90 inhibitor geldanamycin was added to the lysate, excess was removed by gel filtration (biospin 6) before adding the lysate, together with 2 mM ATP and 6 mM Mg^{2+}, to an aliquot of extract from CDDP-treated cells. Again, hormone binding was determined as described above.

Immunoprecipitation and Western analysis

For immunoprecipitation of Hsp90, SK-N-MC cells were transfected with 0.75 μg pRK7GR as described above and solubilized in 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 1% (vol/vol) deoxycholate, 1% (vol/vol) Triton X-100, 40 mM NaF, 1 mM Na_3VO_4, 0.5 mM PMSF, and 0.5 mM aprotinin. The extract was incubated for 30 min on ice. Lysates were incubated with the Hsp90-specific antibodies and protein A Sepharose (Pharmacia) for 2 h at room temperature. Immunoblot detection of GR and Hsp90 in the immunoprecipitates, as well as of proteins in total cell lysates (100 μg), was performed after SDS-PAGE and electrophoretic transfer of proteins to 0.2-μm nitrocellulose filter (Schleicher & Schuell, Keene, NH). The filters were blocked by 5% nonfat milk in Tris-buffered saline/Tween buffer, specific primary antibodies were added and agitated for 1 h at room temperature. GR and Hsp90 were detected with rabbit polyclonal antibodies (H-300, H-114, Santa Cruz Biotechnology, Inc., Santa Cruz,

CA), Src1 with Mab 327, and raf1 with H-71 (Santa Cruz Biotechnology, Inc.). Washing was three times for 5 min in Tris-buffered saline/Tween buffer. Detection was achieved using appropriate peroxidase-conjugated secondary antibodies and enhanced chemiluminescent (ECL) substrate (Amersham Biosciences, Freiburg, Germany).

Activation of lck and raf

Determination of lck and raf activation was performed essentially as described (48). Jurkat T-cells at a density of 2×10^6/ml were treated with the indicated additions for 16 h. In the last 4 h, serum was deprived to decrease the basal level of growth signals. After pretreatment, T cells were stimulated with 5 μg/ml anti-CD3 antibody (OKT3, gift of Márta Szamel) for 20 min. Cells were washed twice with ice-cold PBS, then lysed using 25 mM Tris-HCl, 100 mM NaCl,1% Brij98, 4 mM EDTA, 1 mM dithiothreitol, 1 mM sodium-vanadate, 10 mM sodium-fluoride, 10 mM β-glycerophosphate, 1 mM PMSF, 5 μg/ml leupeptin, 0.7 μg/ml pepstatin A (pH 7.4), for 30 min. at 4 C. After centrifugation (14,000 $\times$ *g*, 10 min), 30 μg protein of the supernatant were loaded per well on a 7.5% SDS-PAGE, transferred to nitrocellulose, then blots were probed with anti-raf-1 and anti-lck antibodies (kind gift of Attila Steták).

Binding of Hsp90 to the Ligand Binding Domain of GR *in Vitro*

The procedure was adapted from Young and Hartl (52). Briefly, Hsp90 was labeled using an *in vitro* transcription and translation system (Promega). Buffer was changed to buffer B [100 mM KOAc, 20 mM HEPES-KOH (pH 7.5), 5% glycerol] using biospin 6 columns (Bio-Rad). Incubation with or without CDDP was for 30 min, followed by another gel filtration to remove excess CDDP. Binding reaction was with myc-tagged ligand binding domain of GR, which had been bound to anti-myc antibodies on Sepharose beads (52), together with reticulocyte lysate, ATP and Mg^{2+} for 10 min. Unbound material was removed, beads were washed twice with buffer B and proteins were eluted with high salt buffer [20 mM Tris-Cl (pH 7.5), 500 mM NaCl, and 1 mM EDTA]. After gel electrophoresis and autoradiography, the intensity of Hsp90 bands was determined using Scion Image (Scion Corp., Frederick, MD).

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Mini-review

Aging and molecular chaperones

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Abstract

Chaperone function plays a key role in sequestering damaged proteins and in repairing proteotoxic damage. Chaperones are induced by environmental stress and are called as stress or heat shock proteins. Here, we summarize the current knowledge about protein damage in aged organisms, about changes in proteolytic degradation, chaperone expression and function in the aging process, as well as the involvement of chaperones in longevity and cellular senescence. The role of chaperones in aging diseases, such as in Alzheimer's disease, Parkinson's disease, Huntington's disease and in other neurodegenerative diseases as well as in atherosclerosis and in cancer is discussed. We also describe how the balance between chaperone requirement and availability becomes disturbed in aged organisms, or in other words, how chaperone overload develops. The consequences of chaperone overload are also outlined together with several new research strategies to assess the functional status of chaperones in the aging process.

Keywords: Molecular chaperones; Heat shock proteins; Stress proteins; Hsp70; Hsp90; Protein aggregation; Protein denaturation; Chaperone overload

1. Introduction: molecular chaperones

Chaperones are ubiquitous, highly conserved proteins (Hartl, 1996), either assisting in the folding of newly synthesized or damaged proteins in an ATP-dependent active process or working in an ATP-independent passive mode sequestering damaged proteins for future refolding or digestion. Environmental stress leads to proteotoxic damage. Damaged, misfolded proteins bind to chaperones, and liberate the heat shock factor (HSF) from its chaperone complexes. HSF is activated and transcription of chaperone genes takes place (Morimoto, 2002). Most chaperones, therefore, are also called stress or (after the archetype of experimental stress) heat shock proteins (Hsp-s).

2. Aging proteins—proteins of aging organisms

During the life-span of a stable protein, various posttranslational modifications occur including backbone and side chain oxidation, glycation, etc. In aging organisms, the disturbed cellular homeostasis leads to an increased rate of protein modification: in an 80-year old human, half of all proteins may become oxidized (Stadtman and Berlett, 1998). Susceptibility to various proteotoxic damages is mainly increased due to dysfunction of mitochondrial oxidation of starving yeast cells (Aguilaniu et al., 2001). In prokaryotes, translational errors result in folding defects and subsequent protein oxidation (Dukan et al., 2000), which predominantly takes place in growth arrested cells (Ballesteros et al., 2001).

Additionally, damaged signalling networks loose their original stringency, and irregular protein phosphorylation occurs (e.g.: the Parkinson disease-related α-synuclein also becomes phosphorylated, leading to misfolding and aggregation; Neumann et al., 2002).

3. Aging protein degradation

Irreversibly damaged proteins are recognized by chaperones, and targeted for degradation. Proteasome level and function decreases with aging, and some oxidized, aggregated proteins exert a direct inhibition on

Abbreviations: HSF, heat shock transcription factor; Hsp, heat shock or stress protein; Grp, glucose regulated protein (the number refers to the molecular weight in kilodaltons).

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proteasome activity. Chaperones also aid in lysosomal degradation. The proteolytic changes are comprehensively reviewed by Szweda et al. (2002). Due to the degradation defects, damaged proteins accumulate in the cells of aged organisms, and by aggregation may cause a variety of protein folding diseases (reviewed by Sőti and Csermely, 2002a).

4. Aging chaperones I: defects in chaperone induction

Damaged proteins compete with the HSF in binding to the Hsp90-based cytosolic chaperone complex, which may contribute to the generally observed constitutively elevated chaperone levels in aged organisms (Zou et al., 1998; Sőti and Csermely, 2002b). On the contrary, the majority of the reports showed that *stress-induced synthesis* of chaperones is impaired in aged animals. While HSF activation does not change, DNA binding activity may be reduced during aging (Heydari et al., 2000). A number of signalling events use an overlapping network of chaperones not only to establish the activation-competent state of different transcription factors (e.g. steroid receptors), but also as important elements in the attenuation of respective responses. HSF transcriptional activity is also negatively influenced by higher levels of chaperones (Morimoto, 2002). Differential changes of these proteins in various organisms and tissues may lead to different extents of (dys)regulation. More importantly, the cross-talk between different signalling pathways through a shared pool of chaperones may have severe consequences during aging when the cellular conformational homeostasis is deranged (see below).

5. Aging chaperones II: defects in chaperone function

Direct studies on chaperone function in aged organisms are largely restricted to α-crystallin having a decreased activity in aged human lenses (Cherian and Abraham, 1995; Cherian-Shaw et al., 1999). In a recent study, an initial test of passive chaperone function of whole cytosols was assessed showing a decreased chaperone capacity in aged rats compared to those of young counterparts (Nardai et al., 2002).

What can be the mechanism behind these deleterious changes in chaperone function? Chaperones may also be prone to oxidative damage, as GroEL is preferentially oxidized in growth-arrested *E. coli* (Dukan and Nyström, 1999). Macario and Conway de Macario (2002) raised the idea of 'sick chaperones' in aged organisms in a recent review. Indeed, chaperones are interacting with a plethora of other proteins (Csermely, 2001a), which requires rather extensive binding surfaces. These exposed areas may make chaperones a preferential target for proteotoxic damage: chaperones may behave as 'suicide proteins' during aging, sacrificing themselves instead of 'normal' proteins. The high abundance of chaperones (which may constitute more than 5% of cellular proteins), and their increased constitutive expression in aged organisms makes them a good candidate for this 'altruistic courtesy.' It may be especially true for mitochondrial Hsp60, the role of which would deserve extensive studies.

6. Aging chaperones III: defects in capacity, the chaperone overload

Another possible reason of decreased chaperone function is chaperone overload (Csermely, 2001b). In aging organisms, the balance between misfolded proteins and available free chaperones is grossly disturbed: increased protein damage, protein degradation defects increase the amount of misfolded proteins, while chaperone damage, inadequate synthesis of molecular chaperones and irreparable folding defects (due to posttranslational changes) decrease the amount of available free chaperones. Chaperone overload occurs, where the need for chaperones may greatly exceed the available chaperone capacity (Fig. 1).

Under these conditions, the competition for available chaperones becomes fierce and the abundance of damaged proteins may disrupt the folding assistance to other

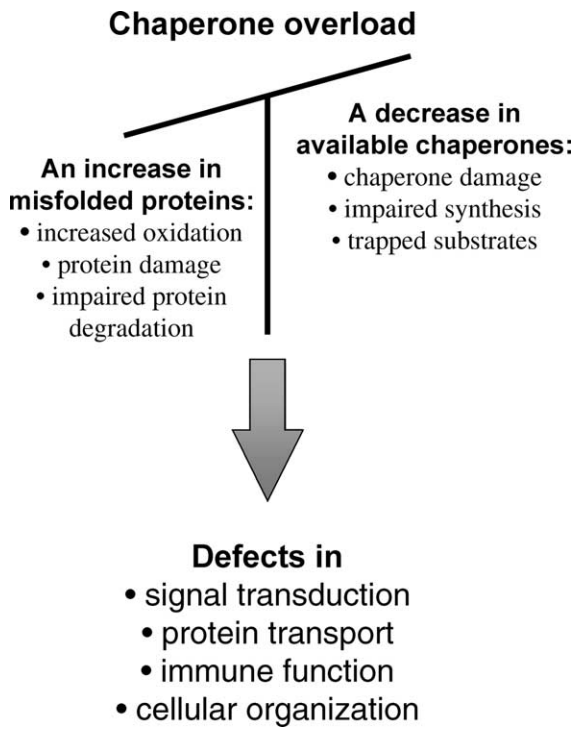

Fig. 1. Chaperone overload: a shift in the balance between misfolded proteins and available free chaperones in aging organisms. The accumulation of chaperone substrates along with an impaired chaperone function may exhaust the folding assistance to specific chaperone targets and leads to deterioration in vital processes. Chaperone overload may significantly decrease the robustness of cellular networks, and compromise the adaptative responses. See text for details.

chaperone targets, such as: (1) newly synthesized proteins; (2) 'constantly damaged' (mutant) proteins; and (3) constituents of the cytoarchitecture (Csermely, 2001a). This may cause defects in signal transduction, protein transport, immune recognition, cellular organization as well as the appearance of previously buffered, hidden mutations in the phenotype of the cell (Csermely, 2001b). Chaperone overload may significantly decrease the robustness of cellular networks, as well as shift their function towards a more stochastic behavior. As a result of this, aging cells become more disorganized, their adaptation is impaired.

7. Senescent cells and chaperones

The involvement of chaperones in aging at the cellular level is recently reviewed (Sőti et al., 2003). Non-dividing-senescent-peripheral cells tend to have increased chaperone levels (Verbeke et al., 2001), and cannot preserve the induction of several chaperones (Liu et al., 1989), similarly to cells from aged animals. Activation and binding of HSF to the heat shock element is decreased in aged cells (Choi et al., 1990). Interestingly, cellular senescence seems to unmask a proteasomal activity leading to the degradation of HSF (Bonelli et al., 2001).

Chaperone induction per se seems to counteract senescence. Repeated mild heat shock (a kind of hormesis) has been reported to delay fibroblast aging (Verbeke et al., 2001), though it does not seem to extend replicative life-span. A major chaperone, Hsp90 is required for the correct function of telomerase, an important enzyme to extend the life-span of cells (Holt et al., 1999).

Mortalin (mtHsp70/Grp75), a member of the Hsp70 family, produces opposing phenotypic effects related to its localization. In normal cells, it is pancytoplasmically distributed, and its expression causes senescence. Its upregulation and perinuclear distribution, however, is connected to transformation, probably via p53 inactivation. Mortalin also induces life-span extension in human fibroblasts or in *C. elegans* harboring extra copies of the orthologous gene (Kaul et al., 2002).

8. Aging organisms and chaperones: age-related diseases

Unbalanced chaperone requirement and chaperone capacity in aged organisms helps the accumulation of aggregated proteins, which often cause folding diseases, mostly of the nervous system, due to the very limited proliferation potential of neurons. Over expression of chaperones often delays the onset or diminishes the symptoms of the disease (Sőti and Csermely, 2002b).

Other aging diseases, such as atherosclerosis and cancer are also related to chaperone action. Here space limitation precludes a detailed description of these rapidly developing fields, however, numerous recent reviews were published on these subjects, where the interested readers may find a good summary and several hints for further readings (Ferreira and Carlos, 2002; Neckers, 2002; Sarto et al., 2000; Wick and Xu, 1999).

9. Chaperones and longevity

Increased chaperone induction leads to increased longevity (Tatar et al., 1997). Moreover, a close correlation exists between stress resistance and longevity in several long-lived *C. elegans* and *Drosophila* mutants (Lithgow and Kirkwood, 1996). As the other side of the same coin, damaged HSF has been found as an important gene to cause accelerated aging in *C. elegans* (Garigan et al., 2002). Caloric restriction, the only effective experimental manipulation known to retard aging in rodents and primates (Ramsey et al., 2000), restores age-impaired chaperone induction, while reversing the age-induced changes in constitutive Hsp levels (see Sőti and Csermely, 2002a,b). These examples confirm the hypothesis that a better adaptation capacity to various stresses greatly increases the chances to reach longevity.

10. Conclusions and perspectives

Aging can be defined as a multicausal process leading to a gradual decay of self-defensive mechanisms, and an exponential accumulation of damage at the molecular, cellular and organismal level. The protein oxidation, damage, misfolding and aggregation together with the simultaneously impaired function and induction of chaperones in aged organisms disturb the balance between chaperone requirement and availability. There are several important aspects for future investigation of this field:

- the measurement of active chaperone function (i.e. chaperone-assisted refolding of damaged proteins) in cellular extracts does not have a well-established method yet;
- we have no methods to measure free chaperone levels;
- among the consequences of chaperone overload, changes in signal transduction, protein transport, immune recognition and cellular organization have not been systematically measured and/or related to the protein folding homeostasis of aging organisms and cells.

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MINIREVIEW

Apoptosis, necrosis and cellular senescence: chaperone occupancy as a potential switch

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Summary

Chaperone function plays a key role in repairing proteotoxic damage and in the maintenance of cell survival. Here we compare the regulatory role of molecular chaperones (heat shock proteins, stress proteins) in cellular senescence, apoptosis and necrosis. We also review the current data on chaperone level and function in aging cells, and list some possible therapeutic interventions. Finally, we postulate a hypothesis, that increasing chaperone occupancy might be an important event which forces cells out of the normal cell cycle towards senescence. In the case of severe stress, this may lead to apoptosis or, following lethal stress, to cell necrosis.

Key words: apoptosis; chaperones; heat shock proteins; necrosis; senescence; stress proteins.

Introduction: molecular chaperones

Chaperones are ubiquitous, highly conserved proteins, and are key elements of the maintenance of the conformational homeostasis of proteins in our cells (Hartl, 1996; Bukau & Horwich, 1998). Classes of major chaperones are listed in Table 1. Chaperones either assist in the folding of newly synthesized or damaged proteins in an ATP-dependent, active process, or work in an ATP-independent, passive mode, sequestering damaged proteins for future refolding or proteasome-mediated degradation. Chaperones participate in signalling, protein traffic and many more cellular functions, and therefore they are vital for our cells during their whole lifetime. However, the demand increases after environmental stress, leading to proteotoxic damage. In stressed cells ATP levels drop significantly, and thus several chaperones may become ATP-independent 'holders' of damaged proteins, preventing their fatal aggregation. After the cell has been recovered, and the ATP level is increased, chaperones, which have a rather low affinity for ATP, regain their ATP-dependent mode, and are converted to 'folders' helping in the refolding, transport and/or ATP-dependent degradation of sequestered, damaged proteins.

Various targets of molecular chaperones include (1) newly synthesized proteins, (2) 'constantly damaged' (mutant) proteins and (3) newly damaged proteins. Chaperones also bind to numerous other proteins, such as other chaperones, co-chaperones, constituents of the cytoskeleton, etc. These targets and other chaperone-associated proteins might easily compete with each other (Csermely, 2001a,b). Chaperones may neutralize the conformational consequences of several mutations, and therefore 'buffer' their potential phenotypical changes and make them phenotypically silent (Rutherford & Lindquist, 1998; Roberts & Feder, 1999; Fares *et al*., 2002; Queitsch *et al*., 2002). An increased amount of damaged proteins may cause the phenotype of these mutations to re-appear by a competition for the chaperone-buffer. As we will discuss later, this phenomenon may occur in aging organisms and in cultured cells (Csermely, 2001b). Chaperone occupancy emerges as an integrator of cellular, organismal and populational responses.

Senescence, apoptosis and necrosis

Several cell types, such as human diploid fibroblasts, endothelial cells, T lymphocytes, epidermal keratinocytes, adrenocortical cells, smooth muscle cells, glial cells, lens epithelial cells and human pancreatic β-cells, exhibit only a limited number of replications in cell culture. Morphological and functional properties change until the cell reaches a non-dividing – senescent – state (Hayflick & Moorhead, 1961; Hayflick, 1965; Smith & Pereira-Smith, 1996). Continued proliferation of human cell cultures beyond the 'Hayflick limit' (e.g. by inactivation of the p53 and retinoblastoma growth inhibitory pathways) results in critical telomere erosion culminating in a period of massive cell death termed 'cellular crisis' (Wright & Shay, 1992). It was thought that the replicative capacity of senescent cells decreases with the age of the donor. However, later studies, including that of Cristofalo *et al*. (1998), found no significant correlation between the proliferative potential of various cell lines and the age of donors (Rubin, 2002).

However, cellular senescence is not a general phenomenon: certain rodent stem cells, human astrocytes, rodent glial cells and rodent oligodendrocyte precursor cells seem to have an indefinite lifespan in cell culture, while maintaining a normal phenotype (Rubin, 2002). Ramirez *et al*. (2001) reported that inadequate cell growth conditions may also lead to premature

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Table 1 Major classes of molecular chaperones

Most important eukaryotic representatives[a]	Recent reviews
Hsp25[b], Hsp27, crystallins, small heat shock proteins	Arrigo (2001); VanMontfort *et al*. (2002)
Hsp60, chaperonins	Bukau & Horwich (1998); Hartl (1996); Thirumalai & Lorimer (2001)
Hsp70, Hsc70, Grp78	Bukau & Horwich (1998); Hartl (1996); Mayer *et al*. (2002)
Hsp90, Grp94	Csermely *et al*. (1998); Pearl & Prodromou (2002); Pratt & Toft (2002); Richter & Buchner (2001); Young *et al*. (2001)
Hsp104	Porankiewicz *et al*. (1999)

[a]Neither the co-chaperones (chaperones which help the function of other chaperones listed), nor the so-called folding catalysts, the peptidyl-prolyl isomerases (immunophilins) and protein disulphide isomerases were included in this table, albeit almost all of these proteins also possess a 'traditional' chaperone activity in their own right. Several chaperones of the endoplasmic reticulum (e.g. calreticulin, calnexin, etc.), which do not belong to any of the major chaperone families, as well as some heat shock proteins (e.g. ubiquitin), which do not possess chaperone activity were also not mentioned.
[b]The abbreviations 'Hsp' and 'Grp' refer to heat shock proteins, and glucose-regulated proteins, chaperones induced by heat shock or glucose deprivation, respectively. Numbers refer to their molecular weight in kDa.

growth arrest. These findings emphasized the importance of the selection process of primary cells during biopsy and initial culturing, and raised the possibility that the 'stress history' of primary cells both in the donor organism and during biopsy/culture may also significantly influence the replicative lifespan of cultured primary cells. Premature senescence can be induced by a large number of stressors, e.g. increased oxygen, hydrogen peroxide, *tert*-butylhydroperoxide, γ-radiation, UV light, DNA strand breaks and pro-inflammatory cytokines (e.g. TNF-α or IL-1; Toussaint *et al.*, 2002), if the overall stress level is moderate. As an additional example, UV-B or oxidative stress induced replicative senescence of normal and telomerase-immortalized human foreskin fibroblasts (DeMagalhaes *et al*., 2002). Severe stress, however, causes cell death.

Cells typically die either by apoptosis or necrosis. These two forms of cell death are probably much closer to each other than previously thought (Proskuryakov *et al*., 2002). Both necrosis (where the cell membrane is ruptured, and the released cell content causes a massive inflammatory response) and apoptosis (where the cell content remains 'well-packed' in the apoptotic bodies, and inflammation does not occur) can be (1) caused by the same pathophysiological exposures, (2) prevented by anti-apoptotic mechanisms and (3) transformed from one form to the other by chemical interventions.

Senescent cells can stay viable at this state for several years with regular renewal of the medium (Bree *et al*., 2002). Senescent fibroblasts are resistant to programmed cell death (Wang, 1995), are unable to undergo p53-dependent apoptosis, and are shifted to necrosis upon DNA damage (Seluanov *et al*., 2001). However, apoptosis-resistance is not a general feature of senescent cells, which may also be apoptosis prone depending on the cell type and apoptotic stimuli, e.g. porcine pulmonary artery endothelial cells show an enhanced apoptosis when cultured for a prolonged period of time (Zhang *et al*., 2002). Senescent fibroblasts promote carcinogenesis of neighbouring cells by secreting tumorigenic factors (Krtolica *et al*., 2001). Therefore, the accumulation of senescent cells may contribute to the age-dependent dramatic increase of cancer incidences. Before elucidating the role of chaperones in cellular senescence, here we give a brief survey of the most important aspects of their multiple roles in apoptotic and necrotic processes.

Role of chaperones in apoptosis and necrosis

In agreement with the cytoprotective role of molecular chaperones it has been shown that they generally inhibit apoptosis (Samali & Orrenius, 1998). As an example, overexpression of Hsp70 in mice led to leukaemia due to a massive decrease of T-cell apoptosis in the thymus (Seo *et al*., 1996). Hsp70 and many other heat shock proteins can overcome both caspase-dependent and caspase-independent apoptotic stimuli and confer immortality in various human cell types (Nylandsted *et al*., 2000; Verbeke *et al*., 2001b). Hsp70 inhibits stress kinases, inducing a block in Bid activation and in all the consecutive steps of cytochrome c release, caspase-3 activation and poly ADP-ribose polymerase cleavage in human cell lines (Gabai *et al*., 1998, 2002). As a general anti-apoptotic effect, small heat shock proteins and Hsp70 protect against oxidative stimuli and thus block an important initiation factor of apoptotic processes (Su *et al*., 1999; Arrigo, 2001). Besides this, small Hsp-s also prevent cytochrome c release in Jurkat cells (Samali *et al*., 2001) and the activation of caspases in U937 cells (Garrido *et al*., 1999). As an additional mechanism, both Hsp70 and Hsp90 block the formation and activation of the Apaf-1 complex and, consequently, the activation of caspase-9 (Bree *et al*., 2002). Hsp90 is also a chaperone for PDK1 and Akt, the downstream members of the PI3K–PDK–Akt anti-apoptotic pathway (Sato *et al*., 2000; Fujita *et al*., 2002), and helps in the overexpression of the anti-apoptotic protein, Bcl-2 (Dias *et al*., 2002) in various human cell lines.

On the other hand, there are examples for the positive involvement of stress proteins in apoptotic signalling (Punyiczki & Fésüs, 1998). Hsp90 and its 75-kDa homologue Hsp75 participate in the signalling of tumour necrosis factor in human and rat cells (TNF-α; Song *et al.*, 1995; Galea-Lauri *et al*., 1996). The capacity of stress proteins may be exhausted due to a robust stress response resulting in protein misfolding and aggregation. Chaperone overload initiates either cell cycle arrest or apoptosis by two mechanisms: by proteasomal inhibition (which blocks cyclin degradation; Bence *et al*., 2001), and by induction of the JNK-dependent pathway suppressing the JNK-inhibitor, Hsp70 (Gabai *et al*., 1998, 2002) in human cell lines. Hsp90 inhibition also results in exaggerated apoptosis of TNFα-treated human cells (Lewis *et al*., 2000). Cytoplasmic translocation of mitochondrial Hsp60 is one of the pro-apoptotic signals, which (together with that of cytochrome c) promotes the activation of cytoplasmic caspases, when mitochondrial integrity becomes compromised in Jurkat T lymphocytes (Samali *et al*., 1999; Xanthoudakis *et al*.,

1999). Several peptidyl-prolyl *cis-trans* isomerase cyclophilins, which are also apoptosis-activated nucleases, are potential direct elements in the apoptotic machinery in HeLa cells (Montague *et al*., 1997). One of the major endoplasmic reticulum chaperones, calreticulin, promotes both Ca-dependent apoptosis and necrosis pathways in several *C. elegans,* rat and human model systems (Nakamura *et al*., 2000; Xu *et al*., 2001; Kageyama *et al*., 2002). However, calreticulin protects neuroblastoma and renal epithelial cells from apoptosis upon cell differentiation as well as iodoacetamide treatment, respectively (Liu *et al*., 1997; Johnson *et al*., 1998).

In contrast to the necrosis-promoting effect of calreticulin in *C. elegans* (Xu *et al*., 2001), heat shock proteins generally protect cells against necrosis. In the absence of Hsp27 and Hsp70 the lethal stress increases ceramide generation, which induces cell necrosis in human fibroblasts (Verbeke *et al*., 2001b). In addition, Hsp70 helps to convert the ATP-depletion-induced cell necrosis to apoptosis (Vayssier & Polla, 1998).

During cell necrosis, cells release various chaperones, such as calreticulin, Hsp10, Hsp70, Hsp90, etc., which serve as a 'danger signal' (Basu *et al*., 2000). Extracellular chaperones provoke inflammation and induce the necrosis of nearby endangered human cells (Proskuryakov *et al*., 2002).

Involvement of chaperone function in cellular senescence

Several chaperones have a direct effect on cellular senescence (Table 2). Overexpression of Hsp27 in bovine arterial endothelial cells leads to an accelerated growth and senescence (Piotrowicz *et al*., 1995). Bag-1, a co-chaperone of Hsp70, slows down cell proliferation by impaired activation of the necessary Raf kinase, when Hsp70 levels are high enough, i.e. after stress (Song *et al*., 2001). When a senescence-promoting factor, mot-1, was isolated from cytoplasmic extracts of senescing (mortal) fibroblasts, it turned out to be a member of the Hsp70 chaperone family. Anti-mortalin antibodies rescued cells from senescence and induced cell proliferation. In contrast, overexpression of mot-2, another member of the mortalin family, in human fibroblasts permitted their escape from senescence (Wadhwa *et al*., 2002).

The previous examples may lead to the conclusion that chaperones promote cellular senescence. However, chaperone induction *per se* may counteract senescence, since repeated mild heat shock (a kind of hormesis) has been reported to delay fibroblast aging (Rattan, 1998), though it does not seem to extend replicative lifespan. Hsp90 is required for the correct function of telomerase, a major enzyme involved in determining the lifespan of cells (Holt *et al*., 1999). Up-regulation of telomerase activity in transformed prostate epithelial cells is completely due to an increased assembly by up-regulated chaperones (Harvey *et al*., 2002). It is reasonable to assume that the proliferative potential of telomerase-positive stem cells may correlate with their available chaperone capacity. Besides telomerase activity, protection of shortened/altered telomeres (Karlseder *et al*., 2002) might be another important element of chaperone-mediated senescence-delay. Interestingly, in contrast to the senescence-promoting effect of extra copies of Hsp27, when a mutant, non-phosphorylated form of Hsp27 was expressed in bovine arterial endothelial cells, cellular senescence was hindered (Piotrowicz *et al*., 1995). Hsp90 also chaperones cyclin-dependent kinases, and when Hsp90 level is diminished, even a mild heat shock induces cell cycle arrest (Nakai & Ishikawa, 2001).

Little is known about the action of chaperones in non-dividing, senescent cells. Apolipoprotein J (clusterin), an extracellular chaperone, has been described as a biomarker of senescence, and is implicated in the prevention/delay of apoptosis (Petropoulou *et al*., 2001; Trougakos & Gonos, 2002). As another example, impaired Hsp90 function leads to the activation of HSF-1, restoration of the heat shock response and slower chronological aging of non-dividing *Saccharomyces cerevisiae* (Harris *et al*., 2001). On the other hand, impaired Hsp70 induction (and probably function) of senescent cells leads to their reduced thermotolerance due to a loss of an Hsp70-mediated inhibitory control of stress kinase signalling (Volloch *et al*., 1998). Increased chaperone occupancy may significantly contribute to decreased p53 stability in senescent cells, causing an enhanced cell necrosis (Seluanov *et al*., 2001). Exhausted chaperones may thus lead to a reversal of the general apoptosis resistance of senescent cells.

Changes of chaperone function in cellular senescence

Like cells from aged animals (Sõti & Csermely, 2002) senescent cells tend to have increased chaperone levels: as one of the

Table 2 Role of molecular chaperones in cellular senescence

Chaperone	Change	References
Increased senescence		
Hsp27	increased senescence of endothelial cells decreased cell proliferation by decreased	Piotrowicz *et al*. (1995)
Bag-1	Raf activation after stress	Song *et al*. (2001)
cytoplasmic mortalin-1 (a Hsp70 homologue)	senescent fibroblast phenotype	Wadhwa *et al*. (2002)
Decreased senescence		
non-phosphorylatable Hsp27	senescence inhibition of endothelial cells	Piotrowicz *et al*. (1995)
mortalin-2 (a Hsp70 homologue)	malignant transformation of NIH 3T3 fibroblasts	Wadhwa *et al*. (2002)
Hsp90 (Hsp70, p23, Hsp40, Hop)	telomerase activation: extended proliferative lifespan	Holt *et al*. (1999); Harvey *et al*. (2002)

examples, chondrocytes have an elevated Hsp27 level (Pfeuty & Gueride, 2000). Senescing fibroblasts possess higher Hsp70 as well as lower Hsp90 and Hsp27 levels. Repeated mild heat shock partially prevented the decrease in Hsp27 level, while making the changes in Hsp70 and Hsp90 levels more pronounced (Verbeke *et al*., 2001a).

Senescing fibroblasts are defective in the induction of several chaperones, such as Hsp70 and Hsp90 as well as the collagen-specific Hsp47 (Liu *et al*., 1989; Miyaishi *et al*., 1995; Bonelli *et al*., 1999). The exact mechanism of the defective Hsp-activation is not known. As with aged animals (Sõti & Csermely, 2002), a decreased activation and binding of heat shock factor-1 (HSF-1) to the respective DNA-element, HSE was reported in senescing cells (Choi *et al*., 1990). Lu *et al*. (2000) found a decreased trimerization of HSF-1 after hypo-osmotic shock in senescent fibroblasts. However, Bonelli *et al*. (1999) identified mRNA processing right before RNA translocation from the nucleus to the cytoplasm as the defective step of Hsp70 synthesis in senescent human fibroblasts. As another mechanism, the increased amount of damaged proteins in senescent cells may occupy Hsp70 and Hsp90, which may cause the release of HSF-1 from the Hsp70Hsp90HSF-1 complex (Morimoto, 1998). This would explain both the increase in the basal levels of major chaperones (an adaptive response) and also the defect in their further induction (a partially non-responsive state).

Possible therapeutic applications

Induction of the senescent state may be an important step in preventing malignant transformation. However, the elimination of senescent cells might be the ultimate goal in various forms of cancer, since senescent cells themselves play an important role in the initiation of malignant transformation (Wang, 1995; Krtolica *et al*., 2001). Low doses of Hsp90-antagonists inhibit telomerase without affecting the growth rate of tumour cells. Based on this finding it was postulated that inhibition of Hsp90 can provoke senescence by the inhibition of telomerase (Harvey *et al*., 2002). Hence, chaperone inhibition is a promising tool to decrease cytoprotection and to initiate apoptosis or necrosis of senescent cells. There are several drug candidates which inhibit chaperone function (Table 3). All these drug candidates may be used to induce senescence and, later, to eliminate senescent cells in patients.

On the other hand, either improving the function of post-replicative cells may be useful in several other disease states, such as in neurodegenerative diseases (Alzheimer's, Parkinson's, etc.). A class of drugs known as chaperone co-inducers have a well-established cytoprotective role (Vígh *et al*., 1997), and may help to preserve the functional reserve capacity of differentiated/senescent cells.

The protein homeostasis hypothesis of senescence

Senescence runs in parallel with an accumulation of damage at the molecular–cellular level. The attenuation of molecular chaperone inducibility and the simultaneous accumulation of damaged proteins raises the possibility that preservation of protein homeostasis is a major determinant in the occurrence and duration of cellular senescence. This concept leads to our 'protein homeostasis hypothesis of senescence' (Fig. 1), a key element of which is the balance between the amount of damaged (denatured) proteins and the available capacity of molecular chaperones to refold/repair them. When the balance is:

1 in favour of available chaperones (compensated stress): proliferative signals are transduced and cells may be temporarily arrested, but finally they divide normally again;

2 in favour of denatured proteins (stress, senescence): proliferative signals stop, cells become irreversibly arrested and enter into a prolonged senescent phase;

3 strongly in favour of denatured proteins (lethal stress): chaperones become unavailable, stress kinases are activated and proteasome inhibition leads to apoptosis;

4 very strongly in favour of denatured proteins (supralethal stress): complete loss of chaperone availability, ceramide accumulation and ATP depletion leads to necrosis.

Two intriguing consequences arise from the above hypothesis:

A When denatured proteins are just slightly, but permanently higher than the level of available chaperones (case 2, remitting in senescent state), silent mutations may be exposed, contributing to polygenic diseases, such as atherosclerosis, diabetes, cancer, etc. (Csermely, 2001b).

B In agreement with the assumption of Toussaint *et al*. (2002), the above hypothesis raises the possibility that many forms of cellular senescence are induced by the effects of prolonged stress on primary cells, when transferring and maintaining them

Table 3 Drug candidates influencing molecular chaperones

Drug candidate	Major effect	Company and web-site	References
Geldanamycin analogues	Hsp90 inhibition	Conforma Inc. (www.conforma.com)	Neckers (2002)
Geldanamycin-testosterone	Specific Hsp90 inhibition in tumours	Kosan Bioscience (www.kosan.com)	Harvey *et al*. (2002)
Radicicol	Hsp90 inhibition	Kyowa Hakko Kogyo Ltd. (www.kyowa.co.jp)	Soga *et al*. (1998)
Purine-scaffold Hsp90 binders, PU3	Hsp90 inhibition		Chiosis *et al*. (2002)
?	Hsp90 inhibition	RiboTargets Co. (www.ribotargets.com)	
Deoxyspergualine	Hsp70 inhibition	Nippon Kayaku Co. (www.nipponkayaku.co.jp)	
Arimoclomol, Iroxanadine	Chaperone co-induction	Biorex R & D Co. (www.biorex.hu)	Vígh *et al*. (1997)

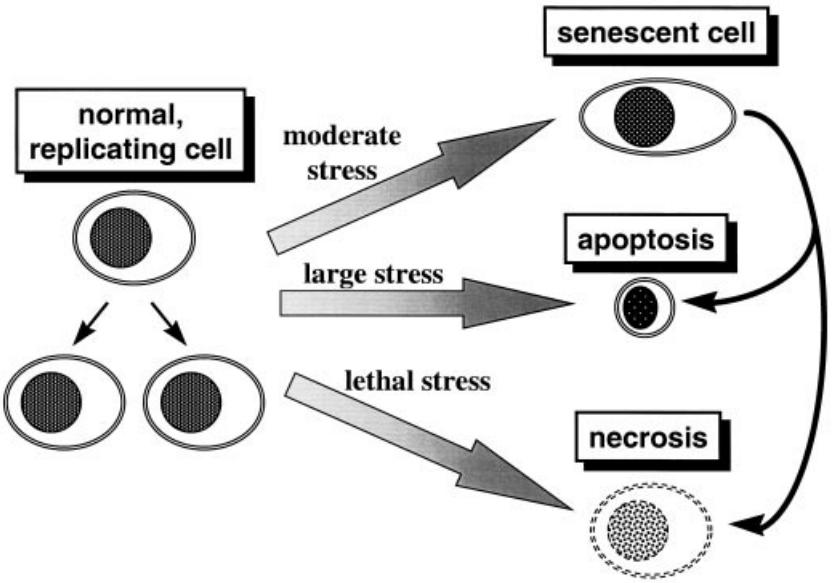

Fig. 1 Hypothetical relationships between chaperone occupancy (chaperone overload), cell proliferation, cellular senescence, apoptosis and necrosis. If there is no chaperone overload, i.e. the amount of damaged proteins does not exceed the amount of available chaperone capacity for a longer time period, cells proliferate normally. Chaperone overload may arise from a constant and large elevation in the amount of damaged proteins and/or a prolonged inefficiency of the cell to produce enough heat shock proteins and other chaperones to repair damaged proteins or a sudden discrepancy between protein damage and chaperone induction (such as a proteotoxic insult combined with the impaired chaperone induction). A modest chaperone overload makes the cells very sensitive to the senescent state. If chaperone overload becomes robust, cells become very sensitive to apoptosis. In the case of a complete, extreme chaperone overload, the possibility of cell necrosis is highly increased.

in culture. In agreement with the above hypothesis, in certain rodent stem cells, human astrocytes, rodent glial cells and rodent oligodendrocyte precursor cells, the use of appropriate culture media allows the cells to escape senescence while remaining untransformed (Rubin, 2002). In light of the above findings (which still have to be extended to more senescence-prone human cells), it is an exciting question to ask whether and how senescent cells exist *in vivo*. Our hypothesis would imply that a low level of persistent environmental stress is an important factor in inducing replicative senescence in living organisms by exhausting their capacity for chaperone-induction.

As a general conclusion, chaperones may not only constitute the most ancient defence mechanism of our cells, but also behave as direct sensors of their functional competence. Various levels of chaperone overload may make an important contribution to the signals directing the cell to senescence, apoptosis or necrosis.

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Comparative analysis of the ATP-binding sites of Hsp90 by nucleotide affinity cleavage: a distinct nucleotide specificity of the C-terminal ATP-binding site

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The 90-kDa heat shock protein (Hsp90) is a molecular chaperone that assists both in ATP-independent sequestration of damaged proteins, and in ATP-dependent folding of numerous targets, such as nuclear hormone receptors and protein kinases. Recent work from our lab and others has established the existence of a second, C-terminal nucleotide binding site besides the well characterized N-terminal, geldanamycin-sensitive ATP-binding site. The cryptic C-terminal site becomes open only after the occupancy of the N-terminal site. Our present work demonstrates the applicability of the oxidative nucleotide affinity cleavage in the site-specific characterization of nucleotide binding proteins. We performed a systematic analysis of the nucleotide binding specificity of the Hsp90 nucleotide binding sites. N-terminal binding is specific to adenosine nucleotides with an intact adenine ring. Nicotinamide adenine dinucleotides and diadenosine polyphosphate alarmones are specific N-terminal nucleotides. The C-terminal binding site is much more unspecific—it interacts with both purine and pirimidine nucleotides. Efficient binding to the C-terminal site requires both charged residues and a larger hydrophobic moiety. GTP and UTP are specific C-terminal nucleotides. 2′,3′-*O*-(2,4,6-trinitrophenyl)-nucleotides (TNP-ATP, TNP-GTP) and pyrophosphate access the C-terminal binding site without the need for an occupied N-terminal site. Our data provide additional evidence for the dynamic domain–domain interactions of Hsp90, give hints for the design of novel types of specific Hsp90 inhibitors, and raise the possibility that besides ATP, other small molecules might also interact with the C-terminal nucleotide binding site *in vivo*.

Keywords: alarmones; Hsp90; molecular chaperone; NAD; nucleotide analogs.

The 90-kDa heat shock protein (Hsp90) is a cytoplasmic chaperone that helps the folding of nuclear hormone receptors and various protein kinases [1–4]. Hsp90 is an ATP-binding chaperone [5,6] and ATP binding induces a conformational change in Hsp90 [7,8]. Assembly of the Hsp90-organized chaperone machinery, the foldosome, with target proteins requires ATP [9,10]; moreover, ATP binding and hydrolysis are essential for the *in vivo* function of Hsp90 [11,12].

Crystallization of the N-terminal domain uncovered a Bergerat-type ATP-binding fold [13], which can also be occupied by geldanamycin (GA) [14] and radicicol [15,16]. These natural antitumor antibiotics abolish Hsp90-dependent folding of immature client proteins, and direct them to proteolysis [17,18].

Recent communications have reported a second ATP-binding site in the C-terminal domain of Hsp90 [19–21]. Our studies demonstrated that the C-terminal nucleotide binding site becomes accessible only after the occupancy of the N-terminal site and is sensitive to cisplatin [20].

The characterization of the nucleotide binding properties of Hsp90 has been hindered for quite a while by the low affinity interactions of nucleotides with this protein, which required the development of new experimental techniques and approaches. More than a decade ago it was been shown by us that Hsp90 has a low affinity ATP/GTP-binding site(s) and is able to autophosphorylate itself using both nucleotides [5]. Later, David Toft and coworkers analyzed the nucleotide specificity of full-length Hsp90 by means of γ-phosphate-linked ATP–Sepharose affinity chromatography. They showed a competition with soluble ADP and ATP, but not with GTP up to 5 mM [9]. On the contrary, recent experiments on N-terminally truncated Hsp90 constructs suggested that GTP, indeed, may bind to the C-terminal domain [19]. Using different fluorescent ATP analogs, including N^6-etheno-ATP, Scheibel *et al.* [6] could not detect a high affinity ATP-binding to Hsp90. However, they could see a weak binding to an ATP-analog spin-labeled on the ribose hydroxyls [6]. Unfortunately, the question, whether GA inhibited this interaction was not addressed. Another study demonstrated that CTP and

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Abbreviations: AMP-PNP, adenyl-5′-yl-imidodiphosphate; ATPγS, adenosine 5′-[γ-thio]-triphosphate; FSBA, 5′-[*p*-(fluorosulfonyl) benzoyl]-adenosine; GA, geldanamycin; GMP-PNP, guanyl-5′-yl-imidodiphosphate; Hsp, heat shock protein; Hsp90, 90 kDa heat shock protein; OMFP, *o*-methylfluorescein phosphate; TNP-nucleotides, 2′,3′-*O*-(2,4,6-trinitrophenyl)-nucleotides.

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NAD affected the tertiary–quaternary structure of the Hsp90 homolog of *Neurospora crassa* [22].

Since the available data in the literature is rather sporadic, and previous experiments obviously could not take into account the existence of the second ATP-binding site on Hsp90, which has been uncovered just recently [19–21], in the present study we undertook a systematic and comparative analysis of the nucleotide specificity of both the N-terminal and C-terminal Hsp90 nucleotide binding sites. In this study we demonstrate that oxidative nucleotide affinity cleavage is a useful technique to characterize the nucleotide binding sites of Hsp90. Using this approach we show that the N-terminal site is fairly specific for adenine nucleotides with an intact adenine ring. On the contrary, the C-terminal site is much more unspecific—it binds both purine and pirimidine nucleotides. Nicotinamide adenine dinucleotides and diadenosine polyphosphate alarmones are specific N-terminal nucleotides, while GTP and UTP are specific C-terminal nucleotides. Our data provide additional evidence for the dynamic domain–domain interactions of Hsp90, help the design of more site-specific Hsp90 inhibitors, and raise the possibility that besides ATP other small molecules might also interact with the C-terminal nucleotide binding site *in vivo*.

Materials and methods

Chemicals

The chemicals used for PAGE, protein determination, blotting membranes, Q2 FPLC and Econo-Pac HTP cartridges were from Bio-Rad. Butyl-Sepharose 4B and DEAE-Sepharose Fast Flow were from Pharmacia LKB Biotechnology Inc. GA was from Gibco-BRL. TNP-nucleotides and etheno-ATP were from Molecular Probes. The ECL bioluminescence kit was from New England Nuclear. The K3725B anti-(C-terminal Hsp90) Ig [23] was a kind gift of T. Nemoto (Department of Oral Biochemistry, Nagasaki University, Nagasaki, Japan), H. Iwanari and H. Yamashita (Institute of Immunology Ltd, Tokyo, Japan). The K41218 anti-(N-terminal Hsp90) Ig [23] was purchased Institute of Immunology Ltd. The PA3-012 anti-(N-terminal Hsp90) Ig was from Affinity Bioreagents (Golden, CO, USA). γ-Phosphate-linked ATP–Sepharose was prepared according to [24]. All the other chemicals used were from Sigma Chemicals Co. Fluka AG.

Purification of Hsp90

Hsp90 was purified from rat liver using consecutive chromatography steps on ButylSepharose 4B, DEAE–Sepharose Fast Flow, Econo-Pac HTP and mono-Q FPLC as described previously [25]. The purity of the final Hsp90 preparations was >95% as judged by silver staining of SDS polyacrylamide gels [26]. Protein concentrations were determined according to Bradford [27].

Oxidative nucleotide affinity cleavage

Affinity cleavage was performed as described by Alonso and Rubio [28], according to the details given in Sőti *et al.* [20]. Briefly, 2 μg purified rat liver Hsp90 was preincubated in the absence or presence of 36 mM GA for 1 h on ice in 20 mM Hepes, 50 mM KCl pH 7.4. Different nucleotides or analogs were added at a final concentration of 1 mM, if not otherwise indicated, and after an additional incubation of 15 min at 37 °C affinity cleavage was induced by the addition of 0.5 mM $FeCl_3$ and 30 mM ascorbate and completed by an additional incubation of 30 min at 37 °C. Hsp90 fragmentation was assessed by sequential immunoblotting with anti-(C-terminal) and anti-(N-terminal) Igs.

Quantification of nucleotide binding

Quantitative determinations were performed as described earlier [20]. Blots were analyzed by densitometry of the most prominent fragments. The N-terminally cleaved 70-kDa fragment (C70) was taken as a representative of N-terminal nucleotide binding, the C-terminally cleaved 46-kDa fragment (N46) represented the C-terminal nucleotide binding, respectively.

ATP–Sepharose binding

Between 3 and 5 μg rat Hsp90 was preincubated on ice for 1 h in 200 μL of a buffer consisting of 20 mM Hepes, 50 mM KCl, 6 mM $MgCl_2$, 0.01% NP40 pH 7.5. In the case of ATP competition, samples contained an ATP regeneration system (10 mM creatine phosphate and 20 $U \cdot mL^{-1}$ creatine kinase). Finally, 25 μL ATP–Sepharose was added and tubes were incubated at 37 °C for 30 min with frequent agitation, then the resin was pelleted, washed three or four times with the above buffer and analyzed by SDS/PAGE.

Results

γ-Phosphate-linked ATP–Sepharose binds Hsp90 via both its N- and C-terminal ATP-binding sites

In our previous experiments, we analyzed the N-, and C-terminal nucleotide binding sites of Hsp90 using two independent techniques. The oxidative nucleotide affinity cleavage was successfully applied to Hsp90 in our previous work [20]. γ-Phosphate-linked ATP–Sepharose binding has been used as the first biochemical assay for the unambiguous identification of Hsp90 as an ATP-binding protein by Grenert *et al.* [9]. Though C-terminal fragments of Hsp90 also bound to the resin [19], and we demonstrated that Hsp90 was able to bind in the presence of a saturating concentration of the N-terminal inhibitor, GA [20], others could not detect binding under these circumstances [29]. We were intrigued by this apparent contradiction, and made an additional attempt to resolve the discrepancy.

Using the affinity cleavage the hydroxyl radicals generated by the oxidation of iron tethered to the polyphosphate moiety of ATP resulted in two major cleavage products in Hsp90: a 70-kDa major Hsp90 fragment (C70) at the N-terminal binding site, and a 46-kDa major fragment (N46) at the C-terminal Hsp90 nucleotide binding site ([20] and Fig. 1A, lane 3). The C-terminal site became accessible only if the N-terminal site was occupied and not cleaved—in our case with the N-terminal specific inhibitor GA (Fig. 1A, lane 4) [13,19]. Performing the cleavage reaction on Hsp90 bound to the γ-phosphate-linked ATP–Sepharose resin showed that Hsp90 is bound to the ATP–Sepharose

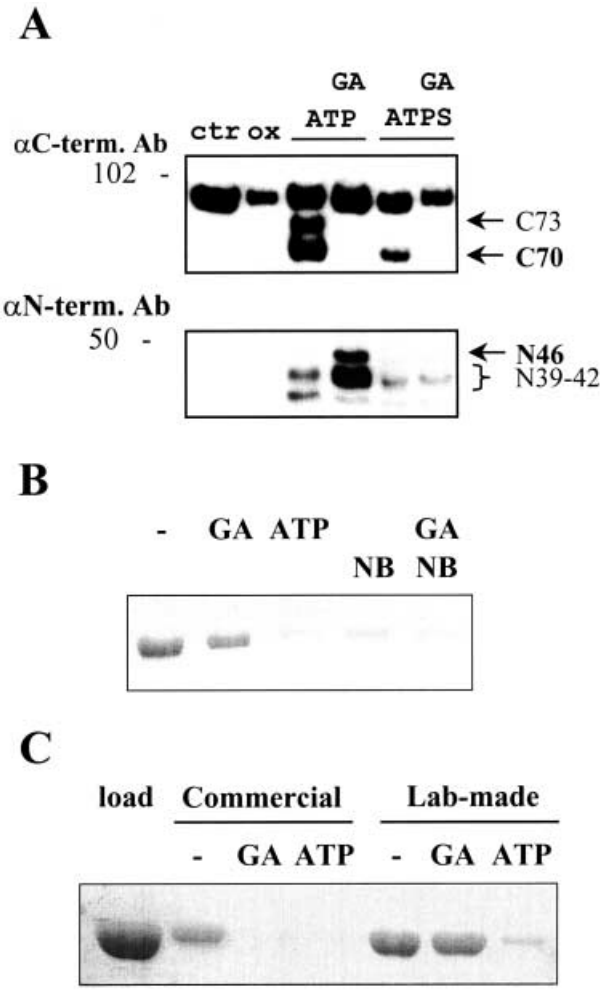

Fig. 1. γ-Phosphate-linked ATP–Sepharose binds Hsp90 via both its N- and C-terminal ATP-binding sites. (A) Affinity cleavage on γ-phosphate-linked ATP–Sepharose. Ctr, Untreated protein; ox, protein incubated with redox system. In lanes 5 and 6 (ATPS), 25 μL γ-phosphate-linked ATP–Sepharose was added instead of ATP. C70 and N46 denote the major N- and C-terminal ADP/ATP-fragments, respectively. Similarly, C73 and N39-42 indicate the major N- and C-terminal ATP fragments, respectively. (B) Novobiocin inhibits γ-phosphate-linked ATP–Sepharose binding. Hsp90 was preincubated in the absence or presence of 36 μM geldanamycin (GA) and/or 10 mM novobiocin (NB). (C) Different γ-phosphate-linked ATP-Sepharose resins interact differently with the C-terminal nucleotide binding domain of Hsp90. Binding of Hsp90 to the commercially available and 'lab-made' ATP-Sepharose resins was analyzed as described in Materials and Methods. Figures are representatives of three independent experiments.

through both nucleotide binding domains (lane 5; C70 and N46), and in the presence of GA, only the C-terminal site is cleaved (lane 6; N46). Unbound Hsp90 in the supernatant did not undergo any ATP-dependent cleavage (data not shown). Fig. 1A also shows that the fragments characteristic of the γ-phosphate (C73 and N39-42) appear neither at the N- nor the C-terminal site, respectively. Instead, the 39-kDa fragment present at the C-terminal site is produced by the diphosphate moiety of ADP [20]. The reason for this may be that the ATP-bound resin may impose a steric hindrance to the binding of the terminal phosphate, therefore Hsp90 adopts an 'ADP-conformation' [20] on the resin. This may explain how the C-terminal binding site could escape attention, where the affinity towards ATP is higher than to ADP [19,20].

Independent evidence for the involvement of both ATP-binding sites in Hsp90/ATP–Sepharose interactions comes from the application of different Hsp90 inhibitors (Fig. 1B). While binding of Hsp90 was not prevented by the N-terminal-specific GA (lane 3) or radicicol (data not shown), novobiocin inhibited binding completely (lanes 5 and 6). This experiment gave further evidence that Hsp90 is also bound to the ATP–Sepharose *via* its C-terminal nucleotide binding site, and confirmed our previous observation that novobiocin, which binds to the C terminus of Hsp90 [19] allosterically inhibits the N-terminal binding site [20]. It has to be noted, that using several lots of commercially available ATP–Sepharose the C-terminal binding was not always detected, especially when the assay was conducted under more stringent conditions (e.g. three washes, data not shown).

Comparative analysis of the nucleotide specificity of Hsp90 nucleotide binding sites

After demonstrating that these techniques may be used to study the biochemistry of the nucleotide binding domains, we performed a comparative analysis of the nucleotide specificity of Hsp90 nucleotide binding sites. Fig. 2A shows that the N-terminal nucleotide binding site prefers adenine nucleotides (ATP and dATP). Binding of CTP was slightly permitted, while GTP and UTP did not bind to this site. We observed no significant binding of dGTP and dUTP, or UDP-glucose to the N-terminal binding site (data not shown). The C-terminal domain allowed binding of all kinds of nucleotides tested. We would like to note that the anti-(C-terminal) Ig K3725B, and the anti-(N-terminal) Ig PA3-012 used in most of our experiments were both Hsp90β-specific antibodies. However, analysis of silver stained gels, as well as the repetition of few selected experiments with the K41218 anti-(N-terminal) Ig, which recognizes both Hsp90 isoforms, revealed no significant differences between the nucleotide-binding specificities of Hsp90α and Hsp90β (data not shown).

As an additional proof, we analyzed the competition of these nucleotides with ATP–Sepharose binding, in the absence (N- and C-terminal binding), and in the presence (only C-terminal binding) of GA. Fig. 2B shows that these experiments yielded similar results. ATP and CTP competed with both sites, while GTP and UTP exhibited a C-terminal preference (Fig. 2B). These experiments provided evidence for the applicability of the nucleotide affinity cleavage technique to study the specificity of the nucleotide binding sites. Since GTP is a C-terminal-specific nucleotide, we further analyzed the properties of Hsp90 nucleotide binding sites using affinity cleavage with different ATP- and GTP-derivatives.

Interactions of nonhydrolyzable nucleotides with Hsp90

In agreement with the specificity profile of the previous experiments, the N-terminal domain bound the

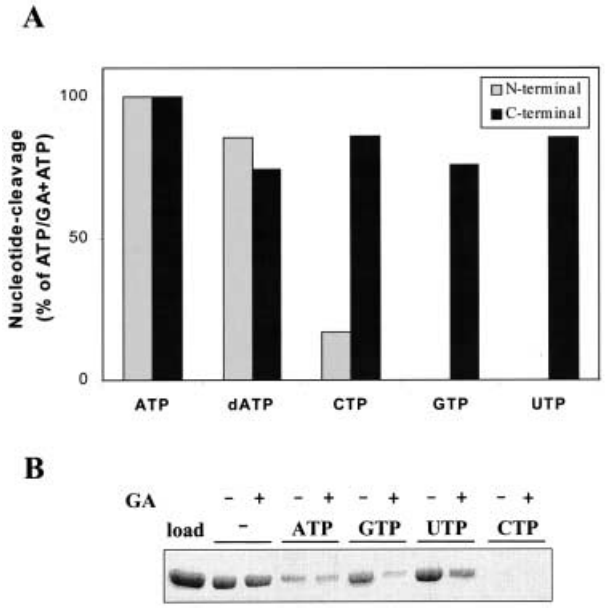

Fig. 2. The Hsp90 N- and C-terminal nucleotide-binding sites display divergent nucleotide specificities. (A) Affinity cleavage assay. Hsp90 was affinity-cleaved in the presence of various nucleotides at a concentration of 1 mM. Nucleotide binding was determined in the absence (N-terminal) or in the presence (C-terminal) of 36 μM GA. Blots were analyzed by densitometry of the N-terminally cleaved 70-kDa (C70) or the C-terminally cleaved 46-kDa (N46) major fragments for N- and C-terminal nucleotide binding, respectively. Data were normalized to the cleavage-efficiency of ATP and GA + ATP in N- and C-terminal nucleotide binding, respectively, and are the means of two independent experiments. (B) ATP–Sepharose competition. Hsp90 was preincubated with 20 mM nucleotides as indicated, then ATP–Sepharose binding was tested. Note that the ATP–Sepharose has a ligand density of 10–15 μmol·mL^{-1}. The figure represents one of two experiments with similar results.

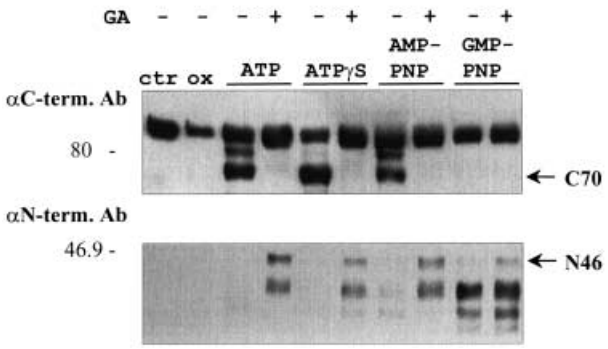

Fig. 3. Interactions of nonhydrolyzable nucleotides with Hsp90. Hsp90 was preincubated in the absence or presence of 36 μM GA, affinity-cleaved using 2 mM ATP, 1 mM ATPγS, adenyl-5′-yl-imidodiphosphate (AMP-PNP) or guanyl-5′-yl-imidodiphosphate (GMP-PNP) and cleavage products were assessed. Western blots are representative of three independent experiments.

poorly hydrolyzable ATP analog, adenosine 5′-[γ-thio]-triphosphate (ATPγS), and the unhydrolyzable adenyl-5′-yl-imidodiphosphate (AMP-PNP), but not guanyl-5′-yl-imidodiphosphate (GMP-PNP, Fig. 3). Binding of both ATPγS and AMP-PNP could be prevented by GA. Binding of these nucleotides to Hsp90 is in agreement with several previous reports (reviewed in [3]). ATPγS usually contains enough ADP to saturate the N-terminal nucleotide binding site, which has a 10- to 20-fold lower affinity to ATP than to ADP [9,13]. ATPγS produced an N-terminal fragmentation resembling that of ADP (see the absence of the C73 γ-phosphate binding fragment in lane 5) [20], but the application of an ATP regeneration system restored the usual ATP cleavage pattern (data not shown).

The C-terminal domain bound each nonhydrolyzable nucleotides tested. GMP-PNP produced a strong fragmentation at the C-terminal domain, seen in blots developed with either anti-(N-terminal) or anti-(C-terminal) Ig (Fig. 3 and data not shown). Interestingly, GMP-PNP could interact with the middle-C-terminal domain in the absence of GA (Fig. 3).

Differently substituted nucleotide analogs bind better to the C-terminal than to the N-terminal domain of Hsp90

It has been reported that Hsp90 cannot bind strongly to adenine-modified nucleotide analogs, but interacts with ribose-modified ATP with an affinity comparable to that of unmodified ATP [6]. Therefore we studied the interaction of differently substituted nucleotides with Hsp90. N^6-etheno-ATP, and the 2′,3′-trinitrophenyl ATP derivative, TNP-ATP displayed a much weaker binding to the Hsp90 N terminus than ATP (Fig. 4). GA competed with the N-terminal binding of both nucleotides. In agreement with no binding of GTP and GMP-PNP to the N terminus (Figs 2 and 3) N-terminal binding of TNP-GTP was not detected (Fig. 4). The C-terminal domain bound each of these nucleotide analogs. TNP-nucleotide binding was possible without GA, though the characteristic N46 band was stronger in the presence of GA. Similarly to GNP-PNP, TNP-nucleotides produced stronger fragmentation at the C-terminal domain, seen in blots developed with either anti-N- or anti-C-terminal Igs (Fig. 4 and data not shown).

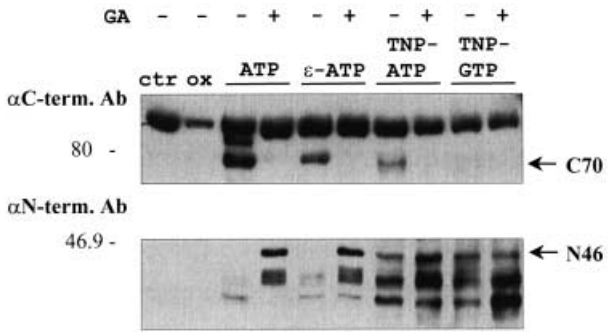

Fig. 4. Differently substituted nucleotide analogs bind better to the C-terminal than to the N-terminal domain of Hsp90. After a preincubation in the absence or presence of 36 mM GA, Hsp90 was affinity-cleaved using 1 mM of ATP, N^6-etheno-ATP (ε-ATP), 2′,3′-O-(2,4,6-trinitrophenyl)-ATP or 2′,3′-*O*-(2,4,6-trinitrophenyl)-GTP (TNP-ATP and TNP-GTP, respectively) and cleavage products were analyzed. Western blots are representative of three independent experiments.

Binding of TNP-nucleotides was also confirmed by fluorescence measurements, but the small increase in quantum yield made detailed analysis impossible (data not shown).

Nicotinamide-adenine dinucleotides bind to the N-terminal, but not to the C-terminal domain of Hsp90

After an earlier prediction of Callebaut *et al.* [30] Garnier *et al.* [21] also proposed the C-terminal ATP binding site to be a Rossmann fold. Following these suggestions we became interested to measure if nicotinamide adenine dinucleotides bind to Hsp90. Here we could utilize the diphosphate structure as a good chelator of Fe^{2+} ions allowing an oxidative cleavage reaction similar to that with nucleoside triphosphates or nucleoside diphosphates. To our surprise, it was the N-terminal domain of Hsp90, which bound both NAD^+ and NADH + H^+. GA competed with both nucleotides efficiently (Fig. 5). Similar to our results with the ribose-substituted nucleotide analog, TNP-ATP, the esterification of ribose-2′-OH both in $NADP^+$ and NADPH strongly inhibited their binding (Fig. 5). On the contrary, none of the nicotinamide adenine dinucleotides displayed a significant interaction with the C-terminal nucleotide binding site. ATP + GA, as a positive control, induced the appearance of the N46 in the presence of all nucleotides (Fig. 5).

Binding of alarmones to Hsp90

Diadenosine polyphosphates and diguanosine polyphosphates serve as alarmones both in prokaryotic and eukaryotic organisms [31]. Moreover, their interaction with the Hsp70 homologue molecular chaperone, DnaK, has been shown [32,33]. We were interested whether these alarmones bind to Hsp90. Fig. 6 shows that indeed, all of the diadenosine polyphosphates bound to the N-terminal site of Hsp90 at 1 mM, and binding could be inhibited by GA. However, none of the diadenosine polyphosphates tested displayed a significant binding to the C-terminal site of Hsp90, and they did not bind to the N-terminal site at a final concentration of 2 μM. Half-maximal binding of diadenosine polyphosphate (AP_4A) to the N-terminal domain occurred above 200 μM (which is the highest physiological concentration; Fig. 6 and data not shown). Interestingly,

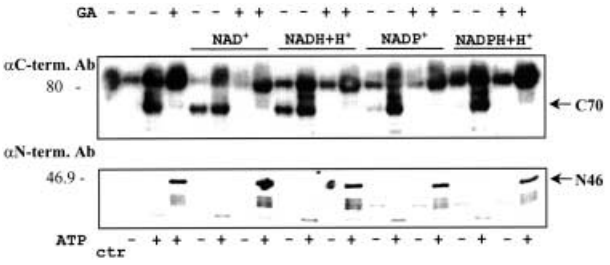

Fig. 5. Nicotinamide adenine dinucleotides bind to the N-terminal, but not to the C-terminal domain of Hsp90. After a preincubation in the absence or presence of 36 μM GA, Hsp90 was affinity-cleaved in the presence of ATP, and/or different nicotinamide adenine dinucleotides at final concentrations of 1 mM, as indicated. Western blots are representative of two independent experiments.

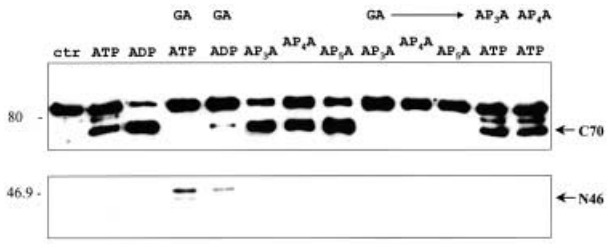

Fig. 6. Binding of diadenosine polyphosphates to Hsp90. After a preincubation in the absence or presence of 36 μM GA, or 1 mM diadenosine polyphosphates as indicated, Hsp90 was affinity-cleaved in the presence of ATP, ADP or diadenosine polyphosphates at a final concentration of 1 mM. Western blots are representative of three independent experiments.

alarmones induced a stronger cleavage than ATP (Fig. 6), which is not due to their higher binding efficiency to Hsp90 as the characteristic alarmone cleavage pattern could be 'diminished' (i.e. competed) by the addition of equimolar ATP (compare the second vs. the last two lanes of Fig. 6). The results show that the cleavage efficiency of the β-phosphate-linked Fe^{2+} is weaker with ATP than with ADP and ADP-like compounds such as alarmones. ATP may induce a different conformation of Hsp90 than ADP or alarmones, probably because Hsp90 should adopt a thermodynamically less favored conformation to capture the ATP-γ-phosphate. Diguanosine polyphosphate (GP_4G) displayed a very weak binding, which was exclusive to the C terminal domain (data not shown). Based on our data Hsp90 does not seem to be a specific alarmone-binding protein *in vitro*.

Binding of noniron-chelating nucleotide analogs and pyrophosphate to Hsp90

We were interested whether a common structural element of the many nucleotide polyphosphates tested, pyrophosphate, is able to induce a specific cleavage pattern of Hsp90 in our oxidative cleavage assay. Indeed, pyrophosphate bound weakly to the N- and much stronger to the C-terminal

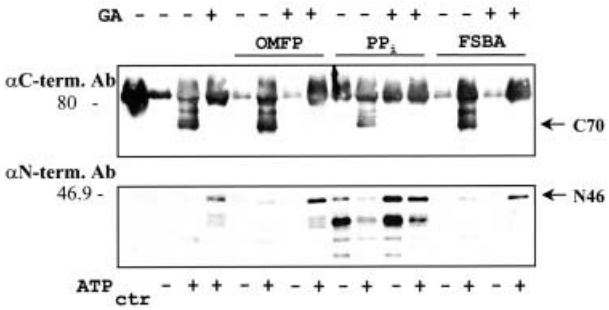

Fig. 7. Binding of noniron-chelating nucleotide analogs and pyrophosphate to Hsp90. After a preincubation in the absence or presence of 36 μM GA, Hsp90 was affinity-cleaved in the presence of 1 mM ATP, and/or 0.1 mM *o*-methylfluorescein-phosphate (OMFP), 1 mM sodium-pyrophosphate (PP_i) and 1 mM fluorosulfonyl-benzoyl-adenosine (FSBA), as indicated. Western blots are representative of two independent experiments.

domains in the absence of GA (Fig. 7). Binding to the N-terminal domain was inhibited by GA. Pyrophosphate cleavage was much less specific than that of the nucleotides, since pyrophosphate induced a strong, GA-independent fragmentation of both the C-terminal and the middle domain of Hsp90 (Fig. 7 and data not shown). ATP inhibited the pyrophosphate-induced C-terminal cleavage (Fig. 7).

o-Methylfluorescein phosphate (OMFP) was a good substrate of the Hsp90-associated ATPase in our previous experiments and competed well with ATP in the 'regular' assays of the Hsp90-associated ATPase [34]. Fluorosulfonyl-benzoyl-adenosine (FSBA) has been used to label and identify ATP-binding sites [35,36] and also weakly labeled Hsp90 (data not shown). Therefore we wanted to know if the hydrolyzable 'ATP-analog' OMFP as well as FSBA [35,36], interact with the oxidative affinity cleavage assay despite the fact that they do not efficiently chelate iron. Nevertheless, in our experiments they displayed a weak binding to the N-terminal domain (Fig. 7). Neither OMFP, nor FSBA could compete with ATP at their maximal concentration of 0.1 and 1 mM, respectively. However, they opened the C-terminal nucleotide-binding domain in the absence of GA, and induced ATP-binding and the appearance of the specific N46 fragment (Fig. 7). Fluorescein isothiocyanate behaved similarly to OMFP and FSBA (data not shown).

Discussion

Nucleotide affinity cleavage as a tool to characterize the specificity of nucleotide binding domains

Using the well characterized N-terminal nucleotide binding site and the ATP–Sepharose assay we could demonstrate for the first time that nucleotide affinity cleavage is a useful technique to study the biochemical properties of nucleotide binding domains. It may be especially important in case of: (a) multiple nucleotide binding sites, because they can be distinguished; (b) low affinity interactions; and (c) 'stringent' site structure, where, e.g. fluorophore or other substitution is not well tolerated. Though it has not yet been shown, the Fe(II)–ATP complex may display a different binding affinity, or even the orientation (therefore the cleavage) of the iron-polyphosphate moiety might differ form that of the biologically predominant magnesium–ATP. Furthermore, the susceptibility of neighboring peptide bonds may differ from protein to protein, resulting in different cleavage efficiency. Further studies are needed to investigate the general applicability of this technique in nucleotide-binding proteins.

Nucleotide binding to the N-terminal domain of Hsp90

The N-terminal nucleotide binding site of Hsp90 is fairly specific. It binds ATP and 2′-deoxy-ATP with similar efficiency (Fig. 8). On the contrary, it does not show a significant interaction with GTP, pirimidine nucleotides, and nucleotides in which the ribose-2′-OH position has been substituted (TNP, ribose-attached resin; phosphate in NADP). The integrity of the adenine ring is also important for binding, since Hsp90 does not bind to C8-linked ATP-resins under stringent conditions ([6,13]; Cs. Sőti and P. Csermely, unpublished observations), and a substitution at the 6-adenine position (e.g. etheno-ATP) disrupts binding as well.

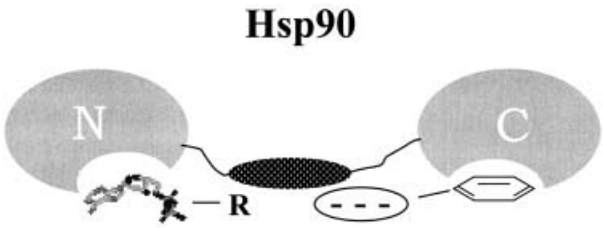

Fig. 8. Nucleotide specificity of the N- and C-terminal nucleotide binding sites of Hsp90. N-terminal domain (N) requires adenine nucleotides with an intact adenine ring; the stick model is the structure of the kinked ADP in the Hsp90 crystal, the phosphates pointing out of the domain; R stands for phosphates (ATP) or other moieties as in NAD or adenosine alarmones. γ-Phosphate is anchored in the middle domain (black). C-terminal domain (C) needs a larger hydrophobic moiety (labeled by the aromatic ring) connected to charged residues (phosphates, like pyrophosphate; labeled by negative charges). The large hydrophobic domain allows the binding of a variety of purine and pirimidine nucleotides. The charged residues bind to a region close to the N-terminal γ-phosphate binding motif.

The Hsp90 N-terminal nucleotide binding site binds NAD and adenosine polyphosphate alarmones. It is worth noting that NAD binding of Hsp90 may interfere with some ATPase measurements based on coupled assays at low ATP concentrations [12]. However, Hsp90 does not show a NADPH : quinone oxidoreductase activity [37], and its alarmone binding efficiency is fairly low. Alarmone binding gives another evidence that the γ-phosphate should point out of the nucleotide binding cleft, and the bulky second adenine should protrude far from the domain reinforcing the notions made by the γ-phosphate-linked ATP–Sepharose [9,20].

Nucleotide binding to the C-terminal domain of Hsp90

Nucleotide binding to the C-terminal nucleotide binding site is fairly unspecific. This site binds both purine and pirimidine nucleotides, when the N-terminal site is already occupied (Fig. 8). UTP and GTP are C-terminal-specific nucleotides. Based on the demonstration that autophosphorylation of Hsp90 is insensitive to high concentrations of GA, but inhibited by novobiocin, a recent report [38] suggested that the C-terminal ATP-binding site may be responsible for Hsp90 autophosphorylation. In light of these data our earlier finding that Hsp90 autophosphorylation can be achieved by GTP [5] gives an additional support for the C-terminal specificity of GTP.

Our experiments showed that the C-terminal site also interacts with ribose-modified nucleotides with affinities comparable to unsubstituted ATP, which may shed new light on earlier findings [6]. The C-terminal site (unlike the N-terminal site) does not interact with nicotinamide adenine dinucleotides and alarmones. This is in contrast with the predictions of Garnier *et al.* [21], who proposed the C terminus as a NAD-binding site.

Binding to the C-terminal site demands both charged groups and a large, hydrophobic moiety (e.g. ATP can inhibit pyrophosphate binding). The negligible alarmone binding suggests that the C-terminal site is more restricted with respect to the phosphate positioning, since another nucleoside weakens the affinity. On the other hand, our previous assumptions [20] indicated that the γ-phosphate binding site is beyond the C-terminal domain. It is still an interesting open question how much the C-terminal nucleotide binding site overlaps with the C-terminal dimerization domain and with the C-terminal binding sites for substrates and for Hsp90-interacting cochaperones.

As an important finding of our present studies, some nucleotide analogs, such as TNP-nucleotides and pyrophosphate bind to the C-terminal nucleotide binding site without the requirement for previous occupancy of the N-terminal site. The structural means by which these nucleotide analogs release the N-terminal site-mediated block of C-terminal binding need to be clarified in further experiments.

As another interesting outcome, experiments shown in Fig. 7 provide additional evidence for the domain–domain interactions of Hsp90: N-terminal ATP-binding and cleavage inhibit pyrophosphate-dependent cleavage of the C-terminal domain (Fig. 7, lane 10 bottom panel). On the other hand, noniron binding N-terminal ATP agonists unlock the C-terminal domain and permit ATP binding and fragmentation (Fig. 7, lanes 6 and 14, bottom panel).

In conclusion, the present studies provide the first systematic and detailed characterization of the nucleotide binding specificity of the N- and C-terminal nucleotide binding sites of the 90-kDa molecular chaperone, Hsp90. Our data also provide additional evidence for the domain–domain interactions of Hsp90 and help the design of new Hsp90 inhibitors, which would be highly useful both in uncovering the physiological function and mechanism of Hsp90 action and also in clinical practice.

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Modelling the actions of chaperones and their role in ageing

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Abstract

Many molecular chaperones are also known as heat shock proteins because they are synthesised in increased amounts after brief exposure of cells to elevated temperatures. They have many cellular functions and are involved in the folding of nascent proteins, the re-folding of denatured proteins, the prevention of protein aggregation, and assisting the targeting of proteins for degradation by the proteasome and lysosomes. They also have a role in apoptosis and are involved in modulating signals for immune and inflammatory responses. Stress-induced transcription of heat shock proteins requires the activation of heat shock factor (HSF). Under normal conditions, HSF is bound to heat shock proteins resulting in feedback repression. During stress, cellular proteins undergo denaturation and sequester heat shock proteins bound to HSF, which is then able to become transcriptionally active.

The induction of heat shock proteins is impaired with age and there is also a decline in chaperone function. Aberrant/damaged proteins accumulate with age and are implicated in several important age-related conditions (e.g. Alzheimer's disease, Parkinson's disease, and cataract). Therefore, the balance between damaged proteins and available free chaperones may be greatly disturbed during ageing. We have developed a mathematical model to describe the heat shock system. The aim of the model is two-fold: to explore the heat shock system and its implications in ageing; and to demonstrate how to build a model of a biological system using our simulation system (biology of ageing e-science integration and simulation (BASIS)).

Keywords: Chaperones; Stress response; Mathematical model; Ageing

1. Introduction

Many chaperones are also known as heat shock proteins (Hsps), because they are synthesised in increased amounts after brief exposure of cells to an elevated temperature. However, they are present in cells at all times and are upregulated by a variety of other stresses such as irradiation, hyperoxia, viral infection and oxidative stress. They range in molecular mass from ~15 to 110 kDa and are divided into groups based on both size and function, and include Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and the small HSPs (Kregel, 2002). They are present in the cytosol, mitochondria, ER and nucleus with different family members functioning in different organelles. They have an affinity for the exposed hydrophobic patches on incompletely folded proteins and hydrolyse ATP, often binding and releasing their protein with each cycle of ATP hydrolysis.

Chaperones have many cellular functions. They are involved in the folding of nascent proteins, the refolding of denatured proteins, the prevention of protein aggregation, assisting the targeting of proteins for degradation by the proteasome and lysosomes, have a role in apoptosis, and are involved in modulating signals for immune and inflammatory responses.

Many newly synthesized proteins need assistance from chaperones in order to fold correctly. 10–20% of new proteins associate with chaperones (Wickner et al., 1999). For example, the Hsp70 machinery acts early in the life of many proteins, binding to a string of about seven

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hydrophobic amino acids before the protein leaves the ribosome. Once a protein has been correctly folded, it can later become misfolded as a result of a variety of stresses. Misfolded proteins have exposed hydrophobic surfaces which will clump together and then precipitate out of solution. Chaperones play a role in dissolving protein aggregates and precipitates (Glover and Lindquist, 1998). They can also prevent aggregation of misfolded proteins from taking place by binding to the hydrophobic surface and then assisting in protein refolding. Hsp60-like proteins form a large barrel-shaped structure into which misfolded proteins are fed, preventing their aggregation and providing them with a favourable environment in which to attempt to refold.

If a denatured protein cannot be refolded, then chaperones assist in its degradation. Chaperones and proteolytic systems are often co-ordinately regulated (Mathew and Morimoto, 1998). The ubiquitin–proteasome machinery is the major route of removal of proteins. Ubiquitination is mediated by a cascade of enzyme activities. Ubiquitin is expressed constitutively in all cells and expression is upregulated upon exposure to stress. The fate of proteins that accumulate during exposure of cells to stress is dependent on the interplay of chaperones and the ubiquitin–proteasome system.

Chaperones generally inhibit apoptosis (Samali and Orrenius, 1998). Hsp27, Hsp70 and Hsp90 are predominantly anti-apoptotic. For example Hsp70 and many other Hsps can overcome both caspase-dependent and caspase-independent apoptotic stimuli (Nylandsted et al., 2000; Verbeke et al., 2001). On the other hand, there are examples of positive involvement of Hsps in apoptotic signalling (Punyiczki and Fesus, 1998).

The ‘‘protein homeostasis hypothesis of senescence’’ is based on the balance between the amount of damaged (denatured) proteins and the available capacity of molecular chaperones to refold/repair them (Sőti et al., 2003).

When the balance is

1. In favour of available chaperones (compensated stress): cells may be arrested temporarily but divide normally again after repair/refolding takes place.
2. In favour of damaged proteins (stress, senescence): cells become irreversibly arrested. Strongly in favour of damaged proteins (lethal stress): proteasome inhibition leads to apoptosis.
3. Very strongly in favour of damaged proteins (supralethal stress): complete loss of chaperone availability, ATP depletion leads to necrosis (See Fig. 1 of Sőti et al., 2003.)

The level of chaperones in the cell increases after conditions of stress. Stress-induced transcription requires activation of heat shock factor (HSF) that binds to the heat shock promoter element (HSE) (Morimoto, 1998). There are four known HSFs but only HSF1 is found in vertebrates. Under normal growth conditions, HSF1 activity is repressed and exists either in the cytosol or nucleus in an inert monomeric state. HSF1 is maintained as a non-DNA-binding inactive complex both by internal coiled-coil interactions and by stoichiometric binding with Hsp90, Hsp70 and other chaperones. The synergistic interaction between these chaperones modulates HSF1 activity by feedback repression. During and after stress, the cellular proteins undergo denaturation and/or polyubiquitination and sequester the chaperones bound to HSF1. The inactive HSF1 becomes free and translocates into the nucleus if it was previously in the cytosol (Verbeke et al., 2001). Activation of HSF1 is accompanied by the transition from an inert monomeric state to a transcriptionally active trimer. The active trimers bind to the DNA heat shock element (HSE) and stimulate transcription of heat shock proteins.

In this paper we describe a model of the heat shock system and the interaction of molecular chaperones with denatured proteins. The model is then used to explore the cellular conditions under which protein homeostasis can be maintained and the conditions that lead to protein aggregation. This model is one of a number of sub-models which are currently being developed for the virtual ageing cell as part of the biology of ageing e-science integration and simulation (BASIS) system (Kirkwood et al., 2003). Further details can be found on our web-site (www.basis.ncl.ac.uk), where this model is available for users to run their own simulations and to change parameter values.

2. Molecular chaperones and ageing

There is an accumulation of damaged and denatured proteins with age, especially in post-mitotic cells such as neurons. In neurons, damaged protein may aggregate and become resistant to degradation causing neurodegeneration. Neuronal chaperones have been found to be localised in neuronal plaques and fibrillary tangles and it has been suggested that they were probably involved in an attempt to sequester the β-amyloid and other damaged proteins (Sőti and Csermely, 2000; Sőti and Csermely, 2003).

Chaperones have many exposed binding surfaces and so are themselves very susceptible to oxidative damage which may result in their functional decline. This is especially true of long-lived chaperones such as crystalline. Although levels of chaperones within cells do not decline with age, it has been shown that the induction of heat shock protein after stress is decreased in aged animals (Sőti and Csermely, 2003). The level of HSF1 does not change with age but a decrease in the activation and binding of its DNA binding site has been observed in aged animals (Heydari et al., 2000). However, levels of Hsp90 may also increase with age which would suppress the activation of HSF1. There is also a decline in proteasome activity with age which results in a further increase in the amount of damaged proteins within cells. The decline in Hsp induction and the increase in denatured protein, including damage to chaperones, all

contribute to the overall decline in chaperone capacity with age. According to the hypothesis of Sőti et al. (2003) this will lead to an increase in cellular senescence, apoptosis or necrosis, depending on the degree of damage and the balance between damaged protein and available free functional chaperones.

3. Elements of the model

We have developed a model of protein turnover and the role of Hsp90 in guarding protein homeostasis. The main components of the model are described below.

3.1. Hsp90 and heat shock factor-1 (HSF1)

Hsp90 is a 90 kDa molecular chaperone and is one of the most abundant cytosolic proteins in eukaryotes, constituting about 1–2% of total protein (Jakob and Buchner, 1994). Hsp90 is a phosphorylated dimer containing 2–3 covalently bound phosphates per monomer. It forms multichaperone complexes that bind to so-called client proteins such as steroid receptors and protein kinases, under nonstress conditions. Clients are inherently unstable multidomain proteins with hydrophobic interaction surfaces. In these interactions, Hsp90 stabilises the clients in a partially folded, functional state in an ATP-dependent active process, to prevent their collapse and degradation. Hsp90 also interacts with actin and tubulin, constituents of the cytoskeleton, and stabilizes the integrity of the cellular network (Sreedhar et al., 2003). However, during stress, it works in an ATP-independent passive mode sequestering damaged proteins for future refolding or proteasome-mediated degradation.

Hsp90 is not able to refold denatured proteins by itself but requires other chaperones to complete the task, in particular Hsp70 (Csermely et al., 1998). In our current model, we assume that there is always sufficient Hsp70 in the cell for refolding to take place and do not include Hsp70 as a separate element. The reason for this assumption is to keep the model as simple as possible, but we have built the model in such a way that it will be relatively straightforward to add Hsp70 (and other chaperones) to the model at a future date. Although the cellular concentration of Hsp90 is high, most of this protein is bound to other proteins or complexes and so there is a need for an increase in Hsp90 levels when there is a rise in denatured proteins in the cell. Denatured proteins sequester Hsp90 that is bound to heat shock factor-1 (HSF1) and so in our model we will only consider the Hsp90–HSF1 complex as a source of Hsp90 for denatured proteins and ignore all other complexes containing Hsp90. Even under normal cellular conditions, a small proportion of protein becomes denatured and is constantly being reformed with the aid of chaperones. So we assume that there is enough free Hsp90 within the cell to form complexes with this low level of denatured protein.

Heat shock factor-1 (HSF1) contains a DNA-binding motif in the amino terminus and adjacent hydrophobic heptad repeats which mediates subunit trimerisation. Hsp90 forms complexes with HSF1 in unstressed cells (Nadeau et al., 1993). During stress, e.g. elevated temperatures, the level of denatured proteins rises and then competes for Hsp90, causing Hsp90 to dissociate from HSF1. HSF1 then forms trimers, a reaction which is favoured at increased temperature (Zou et al., 1998) and becomes transcriptionally active. Trimerisation is a reversible reaction in vivo but not in vitro (Zou et al., 1998). Trimers bind to DNA at the HSE which results in the transcription of heat shock proteins. For example, at elevated temperatures, the concentration of Hsp90 increases several folds. These findings suggest that Hsp90 is a repressor of HSF1 activation under normal conditions (Zou et al., 1998).

3.2. Protein

Proteins are continually being turned over in a cell: degraded if they are damaged or when no longer needed by the cell; and newly synthesized as required. Newly synthesized proteins have to fold into their correct three-dimensional conformation. Many proteins are able to fold without any assistance but about 10–20% of newly-synthesized proteins are found to be associated with chaperones.

The protein that we initially chose for our model was citrate synthase (CS), an enzyme composed of two identical 48 kDa subunits, which catalyses the reaction of oxaloacetic acid and acetyl-CoA to form citric acid and CoA. It is a fairly stable protein having a half-life of about 6–7 days in skeletal muscle (Booth and Holloszy, 1977). It is inactivated and aggregates rapidly upon incubation at 43 °C (Jakob et al., 1995). Jakob et al. (1995) showed that Hsp90 binds transiently to unfolding intermediates of thermal unfolding CS and that these intermediates are able to rapidly refold to their native state upon release from Hsp90.

We chose a protein which is fairly stable, but we can also use our model to examine how protein stability affects protein homeostasis. If we know the half-life of the protein then we can adjust the parameters that affect protein turnover. This will be further explained in the next section.

3.3. Reactive oxygen species

Reactive oxygen species (ROS) are produced by mitochondria as a by-product of normal metabolism. Levels of ROS within the cell are normally kept at low levels by the action of antioxidants. However, if a cell is stressed the levels of ROS can increase considerably. There is also a gradual increase in ROS levels during ageing. For example, the number of damaged mitochondria may increase with age and so more ROS are produced, or there may be a decrease in

the efficiency of anti-oxidant systems. High levels of ROS cause damage to DNA, protein and lipids. In particular, there may be an increase in protein misfolding.

3.4. ATP

Hsp90 has an ATP/ADP binding site in its N terminal domain. The passive, stress-induced chaperone activity of Hsp90 does not require ATP. The stabilisation of client proteins by Hsp90 is ATP-dependent but we do not include this process in our model. The process of refolding damaged proteins requires ATP (in fact, it is Hsp70 which uses ATP for the refolding of damaged proteins but for simplicity we omit Hsp70 from the refolding step in our model at the present time). Degradation of proteins by the ubiquitin system also requires ATP. If the level of damaged proteins within a cell reaches a critical threshold, the cell may become senescent, undergo apoptosis or undergo necrosis. The fate of the cell is partly determined by the ATP level.

4. Description of the model

New proteins need to be synthesized in order to replace the damaged proteins which are degraded. The rate of synthesis depends on the half-life of the protein. Once synthesized, they have to be folded into their native state. For simplicity we model this as one reaction. In this model we assume that proteins are either in their native form, i.e. correctly folded (NatP) or are misfolded (MisP).

We assume that Hsp90 is present in its active dimeric state. Although Hsp90 is very abundant in the cell, we only consider the pool of Hsp90 which is available for binding with HSF1 and for binding to a low level of misfolded protein (5% of total protein). Initially the majority of HSF1 is bound to Hsp90 and the concentration of HSF1 is too low to form trimers. This low level prevents further transcription of the chaperone. Any misfolded protein may be either refolded (with the help of a chaperone), degraded (by the ubiquitin–proteasome system) or form aggregates (AggP). Both refolding and degradation require ATP. There are two ways in which a misfolded protein can form an aggregate: either two misfolded proteins bind together or a misfolded protein binds to a previously formed aggregate. An increase in the number of misfolded proteins leads to Hsp90 disassociating from HSF1 and binding to MisP. HSF1 is then free to form dimers (DiH), and then trimers (TriH), with both reactions being reversible. A trimer can bind to HSE to form a complex (HSETriH) which then activates the transcription of Hsp90 leading to an increase in the level of Hsp90 in the cell. This increases the chance of any misfolded protein being correctly refolded.

We build our model by constructing a set of biochemical reactions to describe the processes outlined above. The reactions are described below in Section 4.1. The network diagram of reactions is shown in Fig. 1. We use mass-action kinetics for all the reactions, i.e. we assume that the instantaneous rate of a reaction is directly proportional to the concentration or each reactant raised to the power of its stoichiometry. We also assume that all the reactions take place within one compartment, namely the cell, and do not consider subcellular localization in this model. However, it is intended that we will modify the model in the future to include different compartments within the cell such as the nucleus, the cytosol, and the mitochondria. We would then need to add further reactions to represent translocation from one compartment to another. For example, in our model we would add a reaction to represent the translocation of HSF1-trimers from the cytosol to the nucleus.

The model is encoded using the systems biology markup language (SBML) (Hucka et al., 2003). SBML is a computer-readable format for representing models of biochemical reaction networks. Once a model has been encoded, it can then be simulated using one of a number of software tools. Our BASIS system provides one such tool, where models can be simulated, results plotted and parameters can be changed (www.basis.ncl.ac.uk).

The model described here is too complex to reason about directly and so it is necessary to carry out simulations to see how the system changes with time. We used stochastic modelling rather than a deterministic analysis, since it is important to include the inherently stochastic behaviour of the intra-cellular processes. Also some of the species in the model are present in small numbers, and so it would be incorrect to use a deterministic approach.

The model was simulated using the STOCKS simulator, developed by Andrzej Kierzek and colleagues (Puchalka and Kierzek, 2004), which combines the Gibson and Bruck (2000) algorithm with the τ-leap method of Gillespie (Gillespie, 2001).

4.1. Reactions in the model

The following set of reactions are shown in Fig. 1a.

New proteins are synthesized and fold into their native state at rate k_1: $\xrightarrow{k_1}$NatP.

Normal proteins (NatP) become misfolded, depending on the level of ROS in the cell, at a rate k_2: NatP + ROS $\xrightarrow{k_2}$MisP + ROS. This also includes nascent proteins which do not fold correctly.

Misfolded proteins (MisP) are bound by Hsp90 at rate k_3, competing with HSF1: MisP + Hsp90$\xrightarrow{k_3}$MCom, where MCom represents the complex of misfolded protein with Hsp90.

Misfolded proteins bound to Hsp90 dissociate at rate k_4: MCom$\xrightarrow{k_4}$MisP + Hsp90.This represents unsuccessful refolding.

The misfolded protein which is bound to Hsp90 (MCom) is refolded to form NatP and released at rate k_5: MCom + ATP$\xrightarrow{k_5}$NatP + Hsp90 + ADP. This reaction requires ATP.

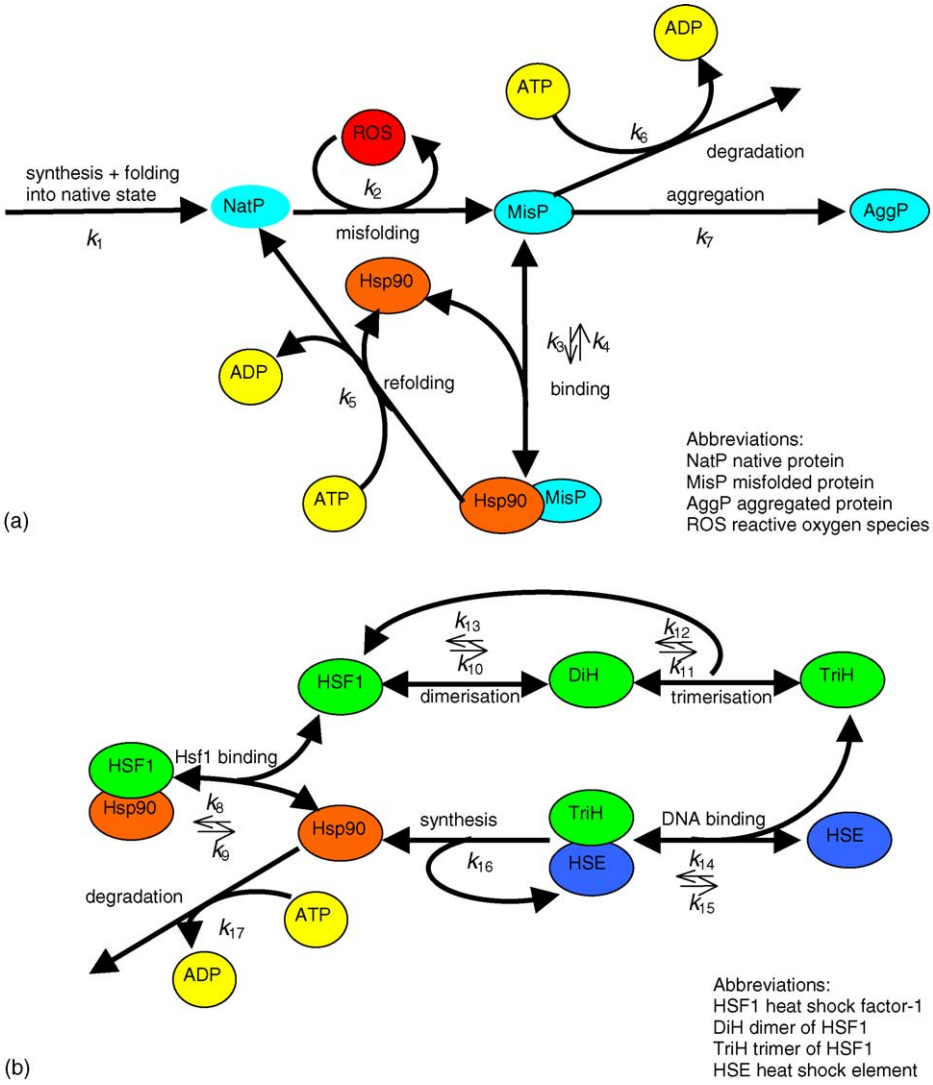


Fig. 1. Network diagram of the model. (a) Protein turnover. (b) Autoregulation of Hsp90.

If refolding is unsuccessful, misfolded proteins are degraded at rate k_6: $\mathrm{MisP} + \mathrm{ATP} \xrightarrow{k_6} \mathrm{ADP}$. This reaction requires ATP.

Misfolded proteins which are neither refolded nor degraded form aggregates (AggP) at rate k_7: $2\mathrm{MisP} \xrightarrow{k_7} \mathrm{AggP}$.

The next set of reactions are shown in Fig. 1b.

Hsp90 binds to HSF1 to form a complex at rate k_8: $\mathrm{Hsp90} + \mathrm{HSF1} \xrightarrow{k_8} \mathrm{HCom}$, where HCom represents the complex of Hsp90 and HSF1.

Hsp90–HSF1 complexes dissociate at rate k_9: $\mathrm{HCom} \xrightarrow{k_9} \mathrm{HSF1} + \mathrm{Hsp90}$.

HSF1 forms dimers at rate k_{10} : $2\mathrm{HSF1} \xrightarrow{k_{10}} \mathrm{DiH}$. HSF1 reacts with dimers to form trimers at rate k_{11}: $\mathrm{HSF1} + \mathrm{DiH} \underset{k_{12}}{\overset{k_{11}}{\rightleftharpoons}} \mathrm{TriH}$. These reactions are reversible with the reverse reactions proceeding at rate k_{12} and k_{13}, respectively.

The trimer (TriH) binds to HSE at rate k_{14}: $\mathrm{TriH} + \mathrm{HSE} \underset{k_{15}}{\overset{k_{14}}{\rightleftharpoons}} \mathrm{HSETriH}$, and dissociates at rate k_{15}.

Bound HSE activates transcription of Hsp90 at rate k_{16}: $\mathrm{HSETriH} \xrightarrow{k_{16}} \mathrm{HSETriH} + \mathrm{Hsp90}$.

Hsp90 degrades at rate k_{17}: $\mathrm{Hsp90} + \mathrm{ATP} \xrightarrow{k_{17}} \mathrm{ADP}$, a reaction which requires ATP.

The following set of reactions are not shown in Fig. 1.

ATP is generated by the mitochondria in the cell from ADP at rate k_{18}: $\mathrm{ADP} \xrightarrow{k_{18}} \mathrm{ATP}$.

Other cellular processes, apart from refolding, consume ATP to form ADP at rate k_{19}: $\mathrm{ATP} \xrightarrow{k_{19}} \mathrm{ADP}$.

Reactive oxygen species (ROS) are produced by the mitochondria at rate k_{20}: $\xrightarrow{k_{20}} \mathrm{ROS}$.

ROS is scavenged by antioxidants at rate k_{21}: $\mathrm{ROS} \xrightarrow{k_{21}} \varnothing$.

4.2. Setting the parameter values and initial conditions

Table 1 lists the species and initial conditions. We assume that there are 6×10^6 molecules of native protein initially. Since we assume that under normal conditions, 5%

Table 1
Initial conditions for the model

Species	Initial value (number of molecules)	Comments
Native protein	6000000	Only one type of protein is considered
Hsp90–HSF1 complex	5900	The majority of HSF1 is bound to Hsp90
Hsp90	300000	Assume that there is sufficient free Hsp90 to bind to 5% of native protein
HSF1	100	Assume ratio of bound to free HSF1 is 59:1 initially
ROS	100	This value can be varied during the simulation
ATP	10000	Assume only a small proportion of cellular ATP is available for the refolding reaction
ADP	1000	Ratio of ATP:ADP is 10:1 under normal cellular conditions
HSE	1	One inducible gene on chromosome 14 (Csermely et al., 1998)

All species not shown in Table 1 have their initial amount set to zero.

of native protein is misfolded and complexed to Hsp90, we set the initial level of Hsp90 to 300,000. We assume that there are 6000 molecules of HSF1 but that the majority of it is initially complexed to Hsp90. The levels of ROS, ATP and ADP are initially set at 100, 100,000 and 1000, respectively.

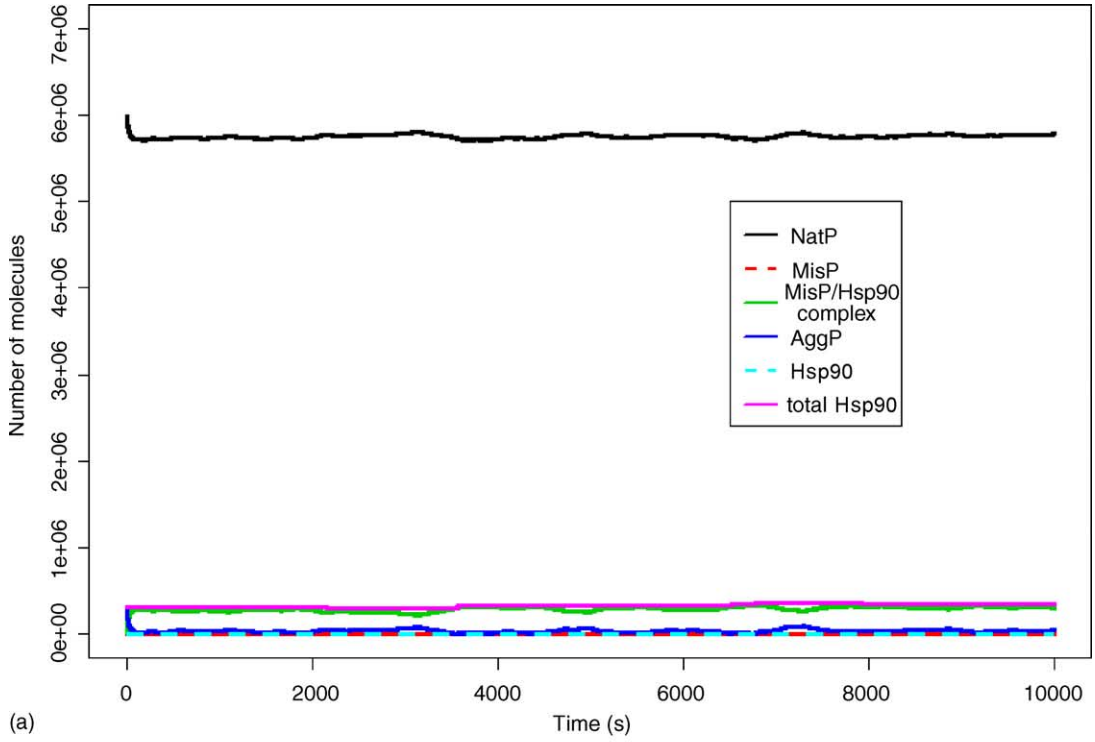
Table 2 lists the default parameter values and also some of the assumptions made in estimating the values. It is not always possible to obtain exact parameter values as experimental data are often qualitative rather than quantitative. For example, it is known that Hsp90 has a strong binding affinity for HSF1, as it is a client protein, and a much weaker affinity for a misfolded protein. Therefore, we set the values of k_3 and k_8, the rates for binding of Hsp90 to misfolded protein and HSF1, respectively, so that k_8 is 10 times greater than k_3.

In some cases we have quantitative data, for example the half-life of a protein, but it is not always possible to use the values directly. It would seem straightforward to calculate the degradation rates and synthesis rates of protein from the

Table 2
Default parameter values of the model

Reaction	Parameter	Default value	Units	Assumptions made
Protein synthesis	k_1	10.0	mol s^{-1}	Half-life of 6–7days (Kawanaka et al., 1997)
Misfolding	k_2	0.00002	mol^{-1} s^{-1}	Ratio of native:misfolded proteins is 19:1 under normal conditions (Lodish et al., 2000)
Binding of misfolded protein by Hsp90	k_3	50.0	mol^{-1} s^{-1}	The binding affinity of misfolded protein to Hsp90 is less than that of HSF1
Dissociation of misfolded protein complex	k_4	0.00001	s^{-1}	The rate of unsuccessful refolding is low compared to refolding under normal conditions
Re-folding	k_5	4.0×10^{-6}	mol^{-1} s^{-1}	Rapid reaction when bound to Hsp90 (Jakob et al., 1995) if ATP levels are high
Protein degradation	k_6	6.0×10^{-7}	mol^{-1} s^{-1}	Half-life of 6–7days (Kawanaka et al., 1997)
Protein aggregation	k_7	1.0×10^{-7}	mol^{-1} s^{-1}	This is a slow reaction unless high levels of misfolded protein
Binding of HSF1 and Hsp90	k_8	500.0	mol^{-1} s^{-1}	The affinity of HSF1for Hsp90 is 10 times stronger than that of misfolded proteins
Dissociation of HSF1 complex	k_9	1.0	s^{-1}	Under normal conditions most of HSF1 is complexed to Hsp90
Dimerisation of HSF1	k_{10}	0.01	mol^{-1} s^{-1}	This reaction is rapid only when levels of unbound HSF1 are high
Trimerisation of HSF1	k_{11}	100.0	mol^{-1} s^{-1}	This is a fast reaction once dimers are formed
Dissociation of HSF1-trimers	k_{12}	0.5	s^{-1}	This is a slow reaction
Dissociation of HSF1-dimers	k_{13}	0.5	s^{-1}	This is a slow reaction
Binding of HSE and HSF1-trimers	k_{14}	0.05	$mol^{-1}s^{-1}$	This reaction only proceeds when trimers are available
Dissociation of HSE and HSF1-trimers	k_{15}	0.08	s^{-1}	If all HSF1 forms trimers, the ratio of the forward to reverse reaction is about 1000:1
Hsp90 transcription	k_{16}	1000.0	s^{-1}	This is fast when HSE is bound
Hsp90 degradation	k_{17}	8.02×10^{-9}	s^{-1}	Half-life of 1 day
ATP formation	k_{18}	12.0	s^{-1}	Assume that ratio of ATP:ADP is 10:1 under normal conditions
ADP formation	k_{19}	0.02	s^{-1}	
ROS production	k_{20}	0.1	mol s^{-1}	Assume constant production level of ROS
ROS removal	k_{21}	0.001	s^{-1}	Rate of removal depends on level of ROS

mol: number of molecules.



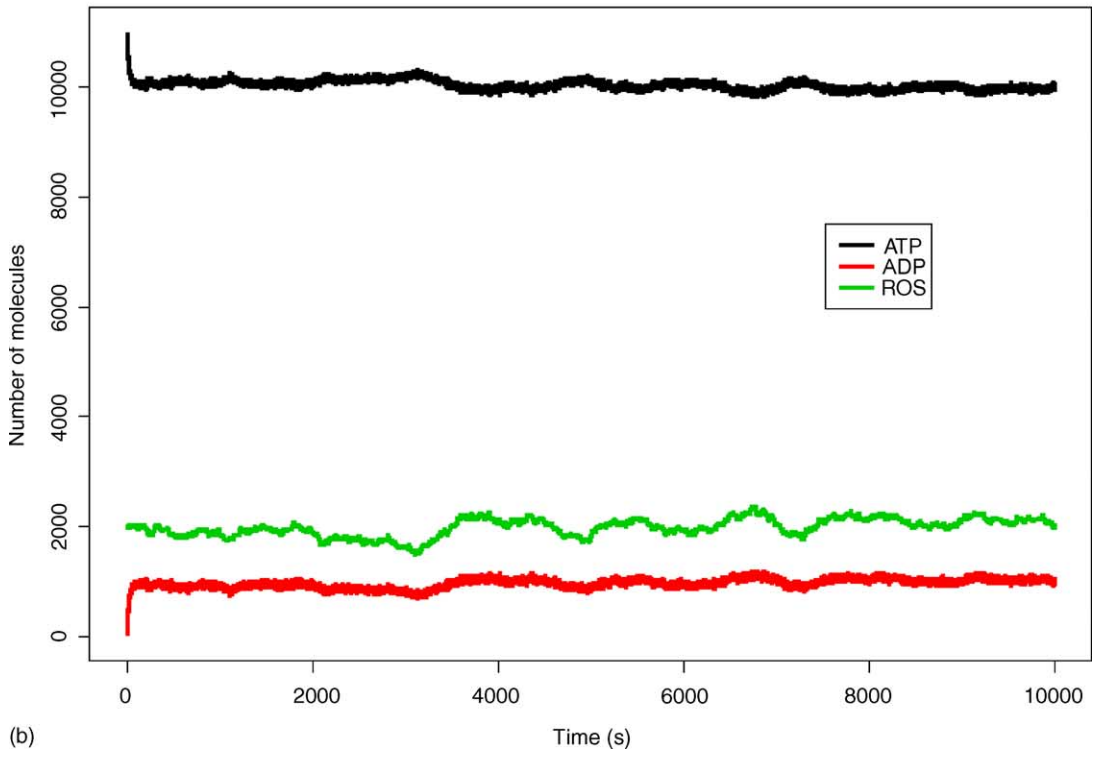


Fig. 2. Simulation results for a normal unstressed cell. (a) Levels of native protein (NatP), misfolded protein (MisP), misfolded protein complexed with Hsp90, aggregated protein (AggP) and unbound Hsp90. (b) Levels of ATP, ADP and ROS (ROS was scaled ×20 for clearer visualisation). Initial conditions and parameters as in Tables 1 and 2, respectively.

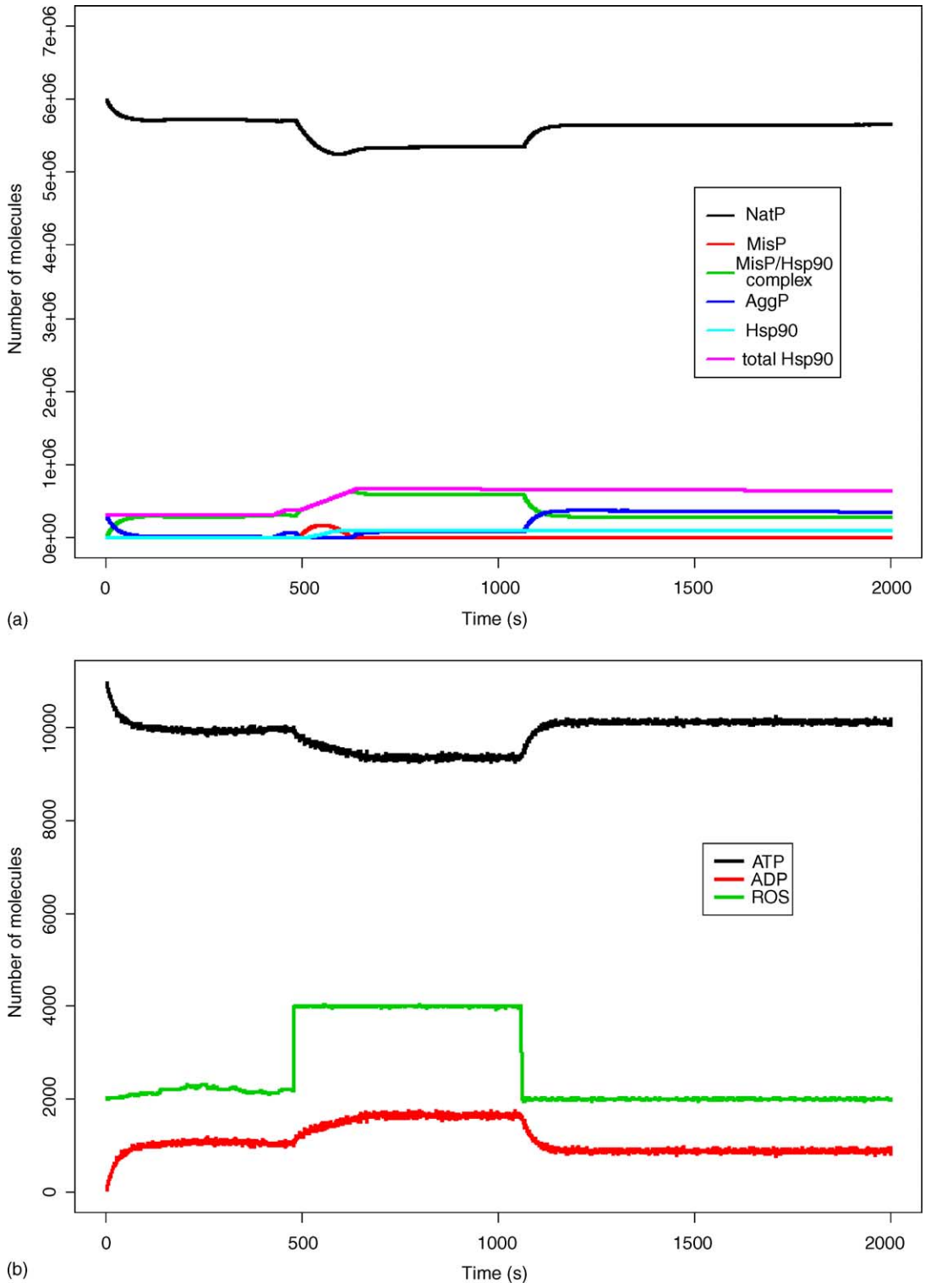


Fig. 3. Simulation results for cell undergoing period of stress. In this simulation the level of ROS was increased two-fold for 10 min. (a) Levels of native protein (NatP), misfolded protein (MisP), misfolded protein complexed with Hsp90, aggregated protein (AggP) and unbound Hsp90. (b) Levels of ATP, ADP and ROS (ROS was scaled $\times 20$ for clearer visualisation). Initial conditions and parameters as in Tables 1 and 2, respectively.

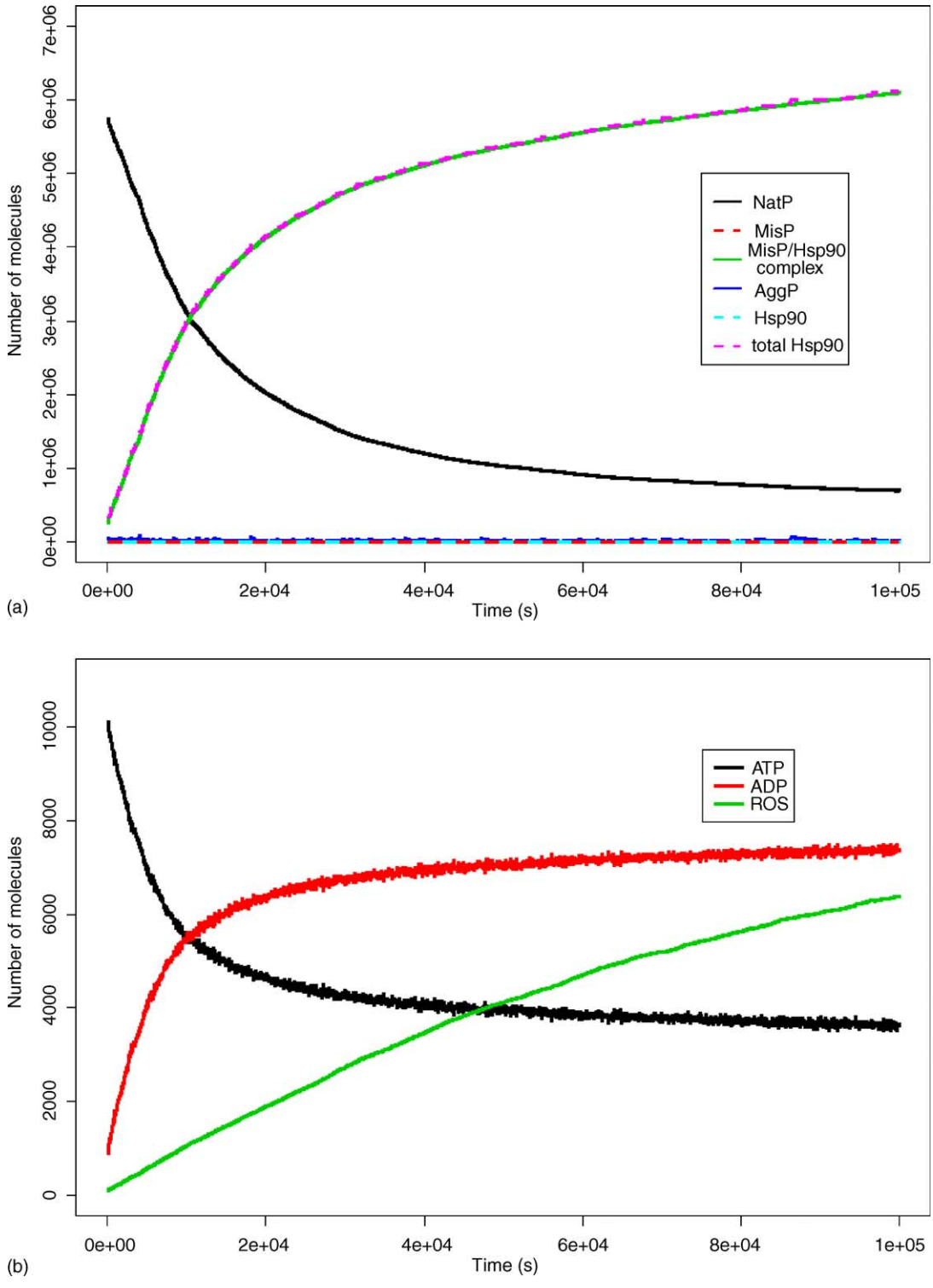


Fig. 4. Simulation results for a cell experiencing increased levels of ROS with time. (a) Levels of native protein (NatP), misfolded protein (MisP), misfolded protein complexed with Hsp90, aggregated protein (AggP) and unbound Hsp90. Total Hsp90 increases in step with misfolded protein complexed to Hsp90, native protein corresponding decreases, all other species are close to zero. (b) Levels of ATP, ADP and ROS. Initial conditions as in Table 1. $k_{21} = 0.00001$, all other parameters as in Table 2.

given half-life, if we assume that the protein level remains constant. However, we have more than one step to degradation which means that further information is needed. In our model we have chosen a protein with a half-life of 6 days. Using the formula $\lambda = -\ln(0.5)/t_{1/2}$, where λ is the degradation rate, gives $\lambda = 1.34 \times 10^{-6}\,s^{-1}$. Since the native protein has to be misfolded before it can be degraded, k_6 needs to be higher than this value. If all misfolded protein was degraded then we could simply set $k_6 = \lambda \times 19$ (since the ratio of native to misfolded protein is 19:1). However, misfolded protein can also be refolded or form aggregates. So to find the value of k_6 which results in the half-life of native proteins being equal to 6 days, we set the synthesis rate to zero and ran the simulation for various values of k_6 until we found a value which gave the correct half-life. Using this procedure we obtained a value of $k_6 = 6.0 \times 10^{-7}$.

In order to calculate the synthesis rate k_1, we assume that the level of protein remains constant. We set the initial value of native protein as 6,000,000 molecules so at steady state $k_1 = 6 \times 10^6 \times 1.34 \times 10^{-6} = 8.04$. We can also calculate the misfolding rate k_2, using the observation that 95% of protein is in its native form under normal conditions (Lodish et al., 2000).

We set the parameter describing the binding affinity of Hsp90 to HSF1 (k_6), so that it is ten times greater than the binding affinity of Hsp90 to misfolded protein (k_3). In reality, the ratio of these parameters should be in the region of 100–1000. The reason for this discrepancy is that we have only included a subset of protein in our model. If we had included total cellular protein, then there would be a larger amount of misfolded protein competing with HSF1 for Hsp90.

The key benefit of a mathematical model is that it allows us to explore the effects of varying any of the default parameter values. For example, we varied the rate of ROS removal k_{21}, so that the level of ROS increased with time (see Section 5.3).

5. Results

5.1. Unstressed cell

Using the initial conditions and parameter values listed in Tables 1 and 2, we carried out a simulation for 10,000 s. (Note: time here and in subsequent results refers to simulation time within the biological context of the model, not the actual run-time of the simulation on the computer.) Fig. 2 shows a typical simulation. If we repeated the simulations many times and averaged the results we would obtain smoother curves. However, even from a single simulation we can see that the species quickly reach a steady state. About 95% of the total protein is in its native form with the remaining 5% being misfolded and complexed to Hsp90. There is no aggregated protein present.

The levels of ROS, ATP and ADP remain fairly constant throughout the simulation with values 100, 10000, and 1000, respectively (see Fig. 2b).

5.2. Cell exposed to transient stress

We next examined the effect of increasing the ROS level two-fold for 10 min, after the simulation had been running for about 8 min. Fig. 3 shows the results of a simulation run for 2000 s. We can see a sudden decrease in native protein as it denatures and rapidly forms complexes with Hsp90. There is also an increase in aggregated protein. When the ROS level returns to normal, some of the denatured protein is refolded. However, the aggregated protein persists because we have assumed that it cannot be degraded and that aggregation is not reversible. This results in the level of native protein being lower than its initial level. The level of Hsp90 remains at a higher level as it is a relatively stable protein but we would expect it to return slowly to normal levels.

5.3. Increase in ROS with time

In this simulation we decreased the rate of ROS removal by changing the parameter k_{21} from 0.001to 0.00001. This causes a gradual increase in ROS over time. As shown by Fig. 4, the model predicts that the level of native protein rapidly declines and there is a corresponding increase in the denatured protein which forms complexes with Hsp90. There is a corresponding decrease in the level of ATP as ATP is needed for both the refolding and degradation of misfolded protein. However, note that the linear increase in ROS leads to a non-linear decrease in ATP. This is due to the initial steep decrease in the level of native protein, which is then followed by a more gradual decline. The level of Hsp90 increases and is able to deal with the increase in denatured protein so that the level of aggregated protein remains close to zero. The decline in ATP results in the misfolded protein remaining in complex with Hsp90 as both the capacity for refolding and degradation is reduced. Note that we have so far assumed that there is no decline in the

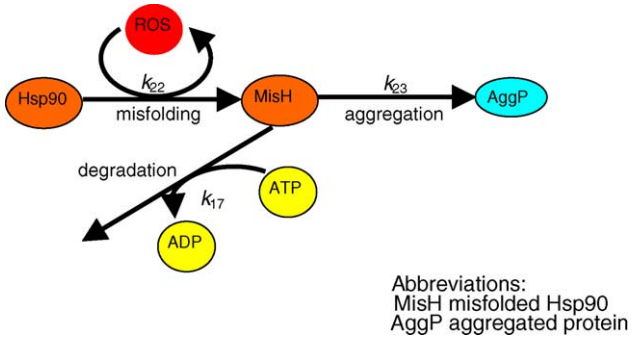
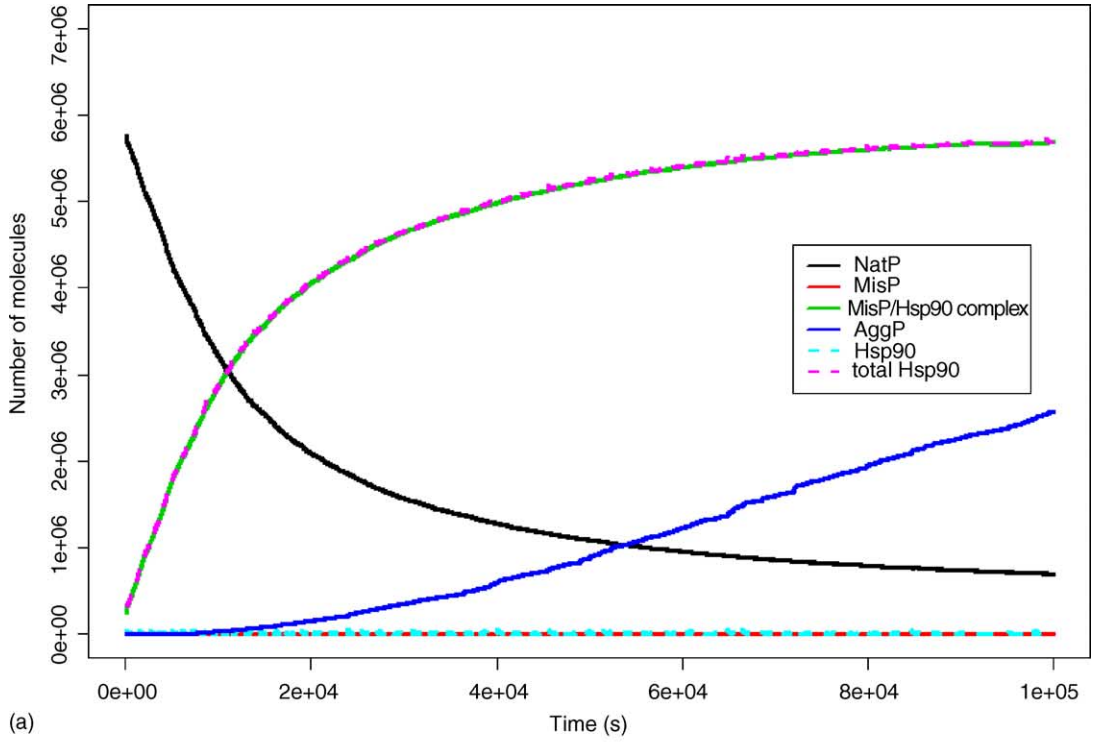


Fig. 5. Extending the model. Hsp90 becomes misfolded and then either is degraded or forms aggregates.



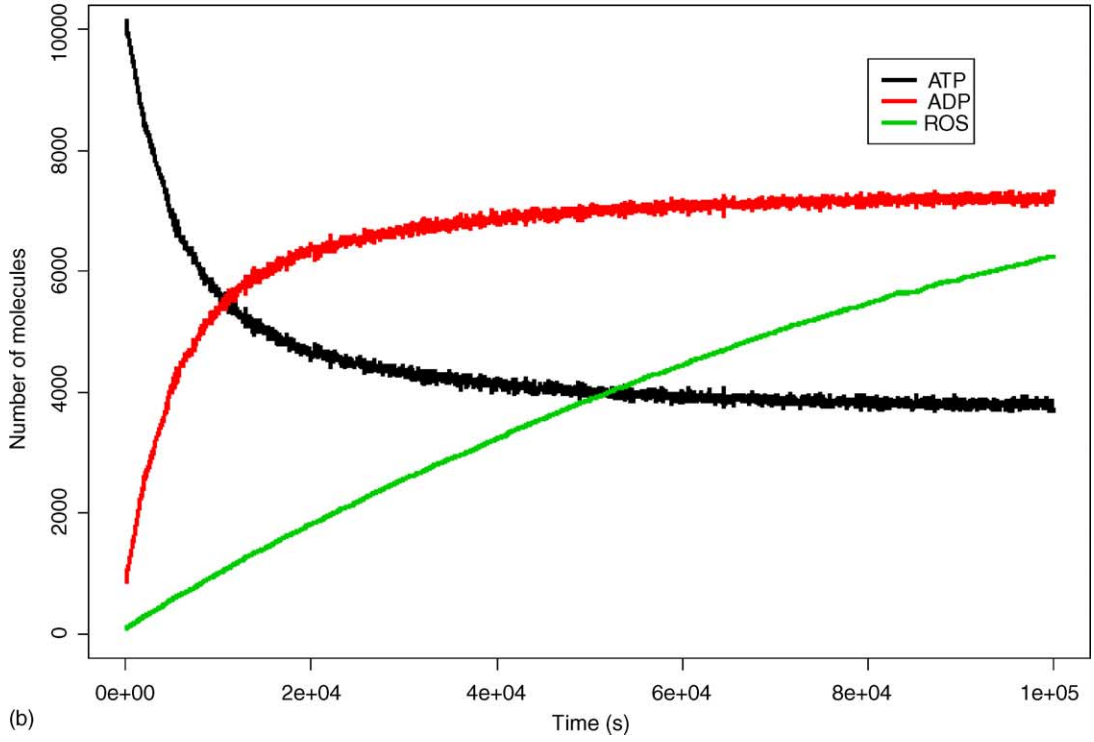


Fig. 6. Simulation results for the extended model with increasing levels of ROS. (a) Levels of native protein (NatP), misfolded protein (MisP), misfolded protein complexed with Hsp90, aggregated protein (AggP) and unbound Hsp90. (b) Levels of ATP, ADP and ROS. Initial conditions as in Table 1. $k_{17} = 1.52 \times 10^{-7}$, $k_{21} = 0.00001$, $k_{22} = 10^{-6}$, $k_{23} = 10^{-7}$, all other parameters as in Table 2.

capacity of Hsp90. In the next section we modify this assumption.

5.4. Extensions to the model

One of the advantages of building our models using the network approach is that it is straightforward to extend the model as more information becomes available or new hypotheses are formed. For example, in our basic model we modelled the degradation of Hsp90 by one reaction (see Fig. 1b). The rate of this reaction depended on the level of Hsp90 and ATP. However, Hsp90 is also subject to damage and as it has many hydrophobic regions, it will also form aggregates when it is not in its correct conformation. So we modified the model so that Hsp90 can become misfolded, and that once misfolded, it can either be degraded or form aggregates. Fig. 5 shows the additional reactions. For simplicity the figure shows the reaction of aggregation of misfolded Hsp90 by a single arrow. However, we actually model this as three different reactions, either two misfolded Hsp90 molecules can aggregate, a misfolded Hsp90 can aggregate with a misfolded protein other than Hsp90, or a misfolded Hsp90 can bind to previously formed aggregated protein. The result of adding this detail to the model, is that the level of aggregated proteins begins to rise with time due to the decline in functional Hsp90 as can be seen by comparing Fig. 6 with Fig. 4.

6. Discussion

We have developed a mathematical model of the chaperone system, with particular focus on the role of Hsp90 in guarding protein homeostasis. Our model shows that when the level of denatured protein rises as a result of increased stress, Hsp90 is upregulated and can prevent the formation of aggregated protein, provided that the stress is not too severe or long-lasting. As levels of oxidative stress increase with age, we see that eventually there comes a point when the levels of Hsp90 cannot increase enough to form complexes with the increasing numbers of denatured proteins, and as a consequence, aggregated protein begins to form. If the chaperones themselves also become damaged and the degradation machinery becomes less efficient with time, then the formation of aggregates is further increased. Since ATP is required for both refolding and degradation, a decline in ATP with age makes the situation worse.

The models described here are available on our web-site (www.basis.ncl.ac.uk) and we encourage the reader to carry out their own simulations and to change parameter values of interest. The levels of native protein, misfolded protein and aggregated protein can then be compared with Fig. 2.

It is also possible to change the initial number of each species in the model, for example the initial amount of native protein. However, when changing initial levels of species it may also be necessary to change parameter values that affect the synthesis or degradation of the relevant species. For example, if we change the initial amount of native protein it is also be necessary to change the protein synthesis rate, otherwise the level of native protein would steadily decline and eventually reach the original level. An on-line companion document explaining how to explore the chaperone model using BASIS is available at this web-site.

The simulations were carried out under a Linux Debian environment on a standard PC. The runtime required was relatively short, for example, Fig. 3 was generated in under 4 min. For the interested reader, simulations can be carried out using the BASIS system, which is accessible through a web-browser.

Our next step in developing this model will be to include the different cellular outcomes: temporary arrest, cellular senescence, apoptosis and necrosis as given by the ‘‘protein homeostasis hypothesis’’ (Sőti et al., 2003). The outcome will depend on the levels of denatured protein, aggregated protein, Hsp90 and ATP. We will then be able to obtain the distribution of cells in each state at different time points and will also be able to compare the distributions obtained for different sets of parameter values.

We set the levels of ROS, ATP and ADP in this model and put in reactions to represent ROS production and scavenging and general reactions of ATP/ADP. However, we are also currently developing models of the mitochondria where the ROS and ATP are produced. Future developments will link the chaperone model to the mitochondria model by taking out the general reactions of ROS and ATP and replacing the initial levels of these species with the levels output from the mitochondria model.

The model that we have described can be extended in many ways. For example, we could include the function of other chaperones such as the role of Hsp100 in dissolving protein aggregates. Further detail could also be added to many of the reactions described such as protein synthesis, Hsp90 synthesis and the degradation steps.

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Molecular chaperones as regulatory elements of cellular networks

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Molecular chaperones help hundreds of signaling molecules to keep their activation-competent state, and regulate various signaling processes ranging from signaling at the plasma membrane to transcription. Besides these specific regulatory roles, recent studies have revealed that chaperones act as genetic buffers stabilizing the phenotypes of various cells and organisms. This may be related to their low affinity for the proteins they interact with, which means that they represent weak links in protein networks. Chaperones may uncouple protein, signaling, membrane, organelle and transcriptional networks during stress, which gives the cell additional protection. The same networks are preferentially remodeled in various diseases and aging, which may help us to design novel therapeutic and anti-aging strategies.

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Introduction

The term 'molecular chaperone' denotes a large family of abundant, ubiquitous proteins that form an ancient defense system in our cells. Chaperones promote cell survival by sequestering damaged proteins and preventing their aggregation. During stressful conditions, such as elevated temperature, they prevent protein aggregation by facilitating the refolding or elimination of misfolded proteins. The stress-induced response to damaged proteins is helped by a sophisticated regulatory system, which shuts down most cellular functions and, in parallel, induces the synthesis of several chaperones and other survival-promoting proteins. Therefore, many of the chaperones are also called stress or 'heat shock' proteins in reference to the archetype of cellular stress, heat shock. Besides their role during stress, chaperones have multiple roles under normal conditions. They promote the transport of macromolecules (e.g. proteins or RNA) and participate in almost every remodeling event involving larger protein complexes, including signaling, transcription, cell division, migration, differentiation, etc [1–3]. The multiple roles of chaperones have inflated the term, which is now used to describe almost any protein (or RNA) that transiently accompanies other molecules and promotes their transport or assembly to larger complexes. Thus chaperones for RNAs, copper and lipids have also been described. Certain chaperones are specialized to a single protein or to a small class of proteins, like the chaperones of catenin, collagen, the major histocompatibility complex, myosin and others. The term 'intramolecular chaperone' has been coined for protein segments (usually residing in the N terminus) that help the folding of the rest of the protein. Moreover, small compounds can be termed 'chemical chaperones', and are used in clinical practice to cure protein folding diseases. Space limitations restrict this review to the 'original' chaperones: those protein chaperones that have multiple protein substrates.

Chaperones mostly form low-affinity, dynamic, temporary interactions (weak links) in cellular networks. Given that chaperones generally have a large number of partners, they behave like hubs in protein–protein interaction networks. Moreover, many chaperone effects (e.g. cell survival or changes in the phenotype diversity) are typical integrative properties, which can rarely be understood by studying the individual chaperone–client interactions exclusively. Thus the network approach is a promising tool to explain some key aspects of chaperone function [3,4••,5•,6•]. We will highlight several potential connections between the individual chaperone–protein contacts and cellular networks, and will explain how some aspects of the network approach can be used to understand the integrative properties of chaperone-mediated regulation. Finally, we will show how the network approach is linked to chaperone-related therapeutic and anti-aging strategies.

Chaperones and cellular networks

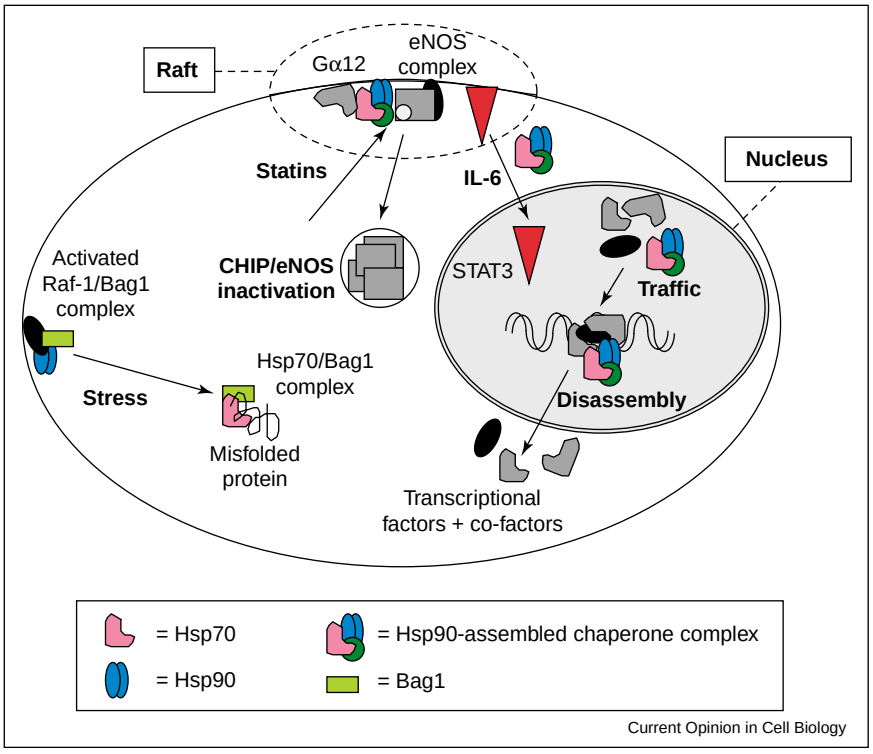
Chaperones form large complexes and have a large number of co-chaperones to regulate their activity, binding properties and function [1–3]. These chaperone complexes regulate local protein networks, such as the mitochondrial protein transport apparatus [7] and the assembly [8] and substrate specificity [9•] of the major cytoplasmic proteolytical system, the proteasome.

Chaperones and the signaling, membrane and organelle networks

The major chaperone-regulated cellular networks are related to signaling, membrane structure and transcription. Though the network approach has been worked out only for segments of the whole signaling network or 'signalome' [10], chaperones may be important elements in the promotion of cross-talk between various signaling processes. The Hsp90 chaperone complex promotes the maturation of >100 kinase substrates including several members of the Raf-1-related signaling pathway. The antiapoptotic protein Bag1 (Bcl-2-associated athanogene protein 1) activates this pathway. Under stress, Bag1 is associated with another chaperone, Hsp70 (70-kDa heat shock protein), which leads to the attenuation of Bag1-mediated Raf-1 activation (Figure 1). Thus, the Bag1/Raf-1 interaction may contribute to the mechanism underpinning how stress shuts down cell proliferation [11].

Another well-known chaperone-mediated signaling pathway, the activation of nitric oxide synthases, gives us an example of chaperone effects on various membranes. The endothelial nitric oxide synthase (eNOS) is activated if assembled to a raft-associated complex containing Hsp90 (90-kDa heat shock protein), the Akt kinase (protein kinase B) and calmodulin. The formation of this complex is helped by statins, the widely used anti-atherosclerotic drugs [12]. A co-chaperone of Hsp70 and Hsp90, CHIP (carboxyl terminus of Hsc70-interacting protein), redirects the maturating eNOS, which usually follows a Golgi-to-plasma-membrane route, into an insoluble cellular compartment, leading to its inactivation [13]. Both Hsp90 and Hsp70 are raft-associated chaperones [14]. Besides its role in eNOS trafficking, Hsp90 helps the GTP-binding protein $G_{\alpha 12}$ to associate with membrane rafts [15] and promotes the traffic of STAT3 (signal transducer and activator of transcription protein 3) from membrane rafts to the cell nucleus after interleukin-6 stimulation [16] (Figure 1). Rab3A, a key player in Ca-dependent exocytosis, is also regulated by the Hsp90/Hsp70/cysteine string protein chaperone complex in synaptic membranes [17]. Finally, studies of *Vígh et al.* showed that chaperones may have a general role in membrane stabilization [18]. All these examples link chaperones to the membrane network of the cell, which integrates the plasma membrane, the endoplasmic reticulum (ER), various vesicles, the nuclear membrane and

Figure 1



Molecular chaperones in the regulation of signaling: a few recent advances. Chaperones play an essential role in the maturation and activation of hundreds of protein kinases. Bag1, the co-chaperone of Hsp70, can activate the Hsp90-dependent Raf-1 kinase. Sequestration of Bag1 by Hsp70 during stress may provide a mechanism for how stress shuts down cell proliferation. Chaperones participate in raft-dependent signaling of eNOS, G-proteins, and STATs. Chaperones also help the subnuclear traffic and disassembly of transcriptional factors and related complexes. (Please note that members of the Hsp90-associated chaperone complex vary in the different pathways and are shown using the same symbol only for clarity.)

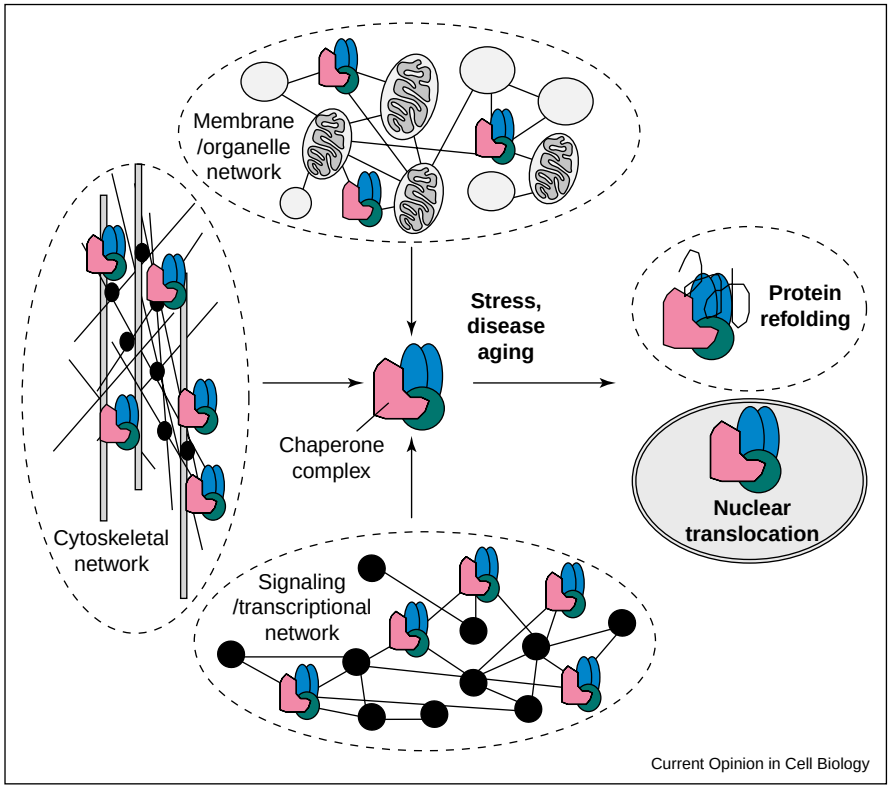
mitochondria [19–21]. Moreover, chaperones may facilitate cytoplasmic traffic [3,22–25]. Links between the ER, the mitochondria and the cytoplasm have already been shown to signal messages of cellular stress between these compartments [26–28]. Chaperones may emerge as stabilizers and regulators of the connectivity and traffic of these important networks.

The connections between elements of the mitochondrial network, the ER, nuclear membranes and the cytoplasmic meshwork may be key points of cellular integrity and information transfer, while de-coupling of these segments may be an efficient protection against any cellular damage. One might expect that chaperones are needed for an efficient coupling of these cellular networks. De-coupling of network elements and modules is a widely used method to stop the propagation of damage [4••,6•]. In case of stress, the increased occupancy of chaperones by damaged proteins together with the stress-induced translocation of chaperones to the nucleus [1–3,29] might lead to an 'automatic' de-coupling of network elements and modules, providing the cell periphery with an additional safety measure (Figure 2).

Chaperones and the transcriptional network

Chaperones are well known to protect the cell nucleus after stress. As a novel version of this role, Hsp70 was shown to drive damaged nuclear proteins to the nucleolus, clearing other nuclear components of misfolded proteins and decreasing the danger of their widespread aggregation [29]. In agreement with these findings, chaperones promote the transport of ribosomal subunits [30] and the mobility of steroid receptors inside the nucleus [31]. Molecular chaperones regulate both the activation [32–34] and the disassembly of numerous transcriptional complexes [35,36] (Figure 1). Thus, chaperones emerge

Figure 2



Chaperones as regulators of cellular networks. Chaperones emerge as integrative regulators of the signaling/transcriptional, cytoskeletal and membrane/organelle networks of the cell. Modification of chaperones as well as a change in the extent to which they are required in various networks may affect most of the other connected cellular functions. As an example, stress (disease or aging) may induce a chaperone-mediated de-coupling of cellular integrity, severing the connections between organelles (e.g. mitochondria, ER, the nuclear membrane and vesicles) as well as preventing cytoplasmic traffic. Signaling and transcriptional regulation are also likely to be impaired. Stress is accompanied by the translocation of chaperones to the nucleus, where they work to maintain the remodeling capacity in the nucleus while promoting the temporary fragmentation of all networks in the cell periphery. The residual or newly formed links between network members are typically weaker than the original connections were, which may decrease cellular noise and provide an additional level of system stabilization [6•]. Thick and thin lines denote strong and weak links, respectively. (Please note that link strengths change continuously in the cell; therefore, the clear discrimination between strong and weak links, as well as the identity of all chaperone complexes, are for clarity only.) Black circles denote protein elements of the cytoskeletal and signaling/transcriptional networks. Dotted lines demarcate various networks from each other. Obviously all these networks overlap in the cellular context.

as regulators of the transcriptional network [37]. Stress-induced nuclear translocation of chaperones may preserve nuclear remodeling capacity during environmental damage, and thus protect the integrity of DNA.

Emergent properties of the chaperone-regulated cellular networks

The previous examples showed that chaperones are involved in the regulation of signaling, organelle, membrane, cytoskeletal and transcriptional networks (Figure 2). However, relatively little is known about the chaperone-mediated, emergent properties of cellular functions. One of the most important advances in this area came from Susan Lindquist and her co-workers when they discovered that Hsp90 acts as a buffer of genetic changes in *Drosophila* [38] and in *Arabidopsis* [39]. A recent paper suggests that this effect might originate epigenetically from Hsp90-induced heritable changes in the chromatin structure [40•].

Chaperone overload

Chaperone-induced genetic buffering is diminished during stress, which causes the sudden appearance of the phenotype of previously hidden mutations, thereby promoting population survival by providing a possible molecular mechanism for fast evolutionary changes [38,39]. On the other hand, the stress-induced appearance of genetic variation at the level of the phenotype cleanses the genome of the population by allowing the disappearance of disadvantageous mutations by natural selection. Chaperones are highly conserved proteins [1–3], so similar mechanisms might operate in humans. Moreover, the tremendous advance of medicine in the last two hundred years has significantly reduced the effects of natural selection and potentially increased the accumulation of hidden mutations in the human genome. However, chaperones may become occupied by the damaged proteins in aged organisms (half of cellular proteins of 70–80 years old humans may be already oxidized), resulting in a chaperone overload. As a consequence the protein products harboring the 'hidden mutations' may be released and may contribute to the development of civilization diseases, such as cancer, atherosclerosis and diabetes [41–43]. This effect may be negligible today, although it will increase with each generation. Still, we probably have many hundreds of years to think about a possible solution.

Chaperones as weak links

Recent findings [44•,45•] raised the idea that not only chaperones but a large number of other proteins may also regulate the phenotypic diversity of the population. Though a relatively small number of other regulators have been uncovered so far, it seems unlikely that a common molecular mechanism, such as involvement in signaling or in modifications to the chromatin structure, can explain all the effects observed. If a general explanation is sought, it is more likely to be related to the network properties of the cell. In this context, chaperones are typical weak linkers, providing low-affinity, low-probability contacts with other proteins (Figure 2). Weak links are known to promote system stability in a large variety of networks from macromolecules to social networks and ecosystems, which suggests that this may be a general network-level phenomenon explaining many of the genetic buffering effects of chaperones [6•].

Chaperone therapies

Cellular networks are remodeled under stress [46] and in various diseases. Effective interventions to push the equilibrium towards the original state may not be limited to single-target drugs with a well-designed, high affinity interaction with one of the cellular proteins. In agreement with this general assumption, several examples show that multi-target therapy may be superior to the usual single-target approach [47]. The best known examples of multi-target drugs include Aspirin, Metformin or Gleevec as well as combinatorial therapy and natural remedies. Because of the multiple regulatory roles of chaperones, chaperone modulators provide additional examples of multi-target drugs. Indeed, chaperone substitution (in the form of chemical chaperones [48]), the pharmacological help of chaperone induction by stress, termed chaperone co-induction [49•], and chaperone inhibition [50•] are all promising therapeutic strategies. Both chaperone co-inducers and chaperone inhibitors, including geldanamycin analogues and other Hsp90 inhibitors, have recently completed successful clinical trials.

Conclusions

Chaperones regulate cellular functions at two levels. In several cases they interact with a specific target protein and help it to fold after synthesis, or re-fold after stress. These strong interactions make chaperones important parts of the central scaffold of cellular networks, such as the protein net, the signaling network, the membrane and organelle network and the transcriptional network. However, in most cases chaperones have only a low-affinity, temporary, weak interaction with most of their targets (Figure 2). Changes to these interactions do not affect the general behavior of the whole network, the cell. However, inhibition of these weak interactions might lead to a rise in cellular noise and the destabilization and disintegration of the whole network and by promoting an 'error catastrophe' help us to combat cancer [50•]. In contrast, chaperone activation might decrease cellular noise, and consequent cell-stabilization might give an additional, indirect help to prevent protein folding diseases including various forms of neurodegeneration, such as amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease and Huntington's disease [48,49•]. Besides slowing or reversing the development these diseases, chaperone-based therapies may also generally benefit the aging organism by stabilizing its cells and functions. Thus properly working chaperones may be key

players to help us achieve improved life conditions at an advanced age. The assessment of the multiple roles of chaperones in the context of cellular networks is just beginning.

Update

Recently a promising model was developed to integrate various chaperone-dependent and other elements in the signaling network leading to the activation of heat shock factor-1 and the consequent synthesis of molecular chaperones [51]. Hsp90 was shown to act as a molecular switch of the Erb-B2 oncogenic tyrosine kinase signaling network by regulating the heterodimer formation between Erb-B2 and various other kinases [52]. This extends the membrane-dependent remodeling effects of Hsp90 to a novel field. As a theoretical contribution to chaperone therapies, the efficiency of multi-target drugs over single target drugs has been summarized, and a new drug-design paradigm was proposed in a recent publication [53].

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REVIEW

Heat shock proteins as emerging therapeutic targets

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Chaperones (stress proteins) are essential proteins to help the formation and maintenance of the proper conformation of other proteins and to promote cell survival after a large variety of environmental stresses. Therefore, normal chaperone function is a key factor for endogenous stress adaptation of several tissues. However, altered chaperone function has been associated with the development of several diseases; therefore, modulators of chaperone activities became a new and emerging field of drug development. Inhibition of the 90 kDa heat shock protein (Hsp)90 recently emerged as a very promising tool to combat various forms of cancer. On the other hand, the induction of the 70 kDa Hsp70 has been proved to be an efficient help in the recovery from a large number of diseases, such as, for example, ischemic heart disease, diabetes and neurodegeneration. Development of membrane-interacting drugs to modify specific membrane domains, thereby modulating heat shock response, may be of considerable therapeutic benefit as well. In this review, we give an overview of the therapeutic approaches and list some of the key questions of drug development in this novel and promising therapeutic approach.
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Abbreviations: 17AAG, 17-allylamino-17-demethoxy-geldanamycin; HSF, heat shock factor; Hsp, heat shock protein; PKC, protein kinase C

Molecular chaperones

Molecular chaperones (1) protect other proteins against aggregation, (2) solubilize initial, loose protein aggregates, (3) assist in folding of nascent proteins or in refolding of damaged proteins, (4) target severely damaged proteins to degradation and (5) in case of excessive damage, sequester damaged proteins to larger aggregates. Chaperones are ubiquitous, highly conserved proteins, which utilize a cycle of ATP-driven conformational changes to refold their targets, and which probably played a major role in the molecular evolution of modern enzymes (Hartl, 1996; Csermely, 1997; 1999; Thirumalai & Lorimer, 2001). Due to the significant overlap in their functions, the major classes of molecular chaperones are best classified by their molecular weights, thus, for example, the abbreviation Hsp90 refers to the 90 kDa heat shock protein (Hsp).

Cellular stress leads to the expression of Hsp's. Stress can be any sudden change in the cellular environment, to which the cell is not prepared to respond, such as heat shock. However, almost all types of cellular stress induce Hsp's. Owing to the generality of this phenomenon, Hsp's are often called stress proteins. The rationale behind this phenomenon is that after stress there is an increased need for the chaperone function of Hsp's, which triggers their induction. This need is caused by the increased amount of damaged proteins, by the inhibition of their elimination *via* the proteasome as well as by the damage of the chaperones themselves. Hsp induction might help to renature chaperones and, therefore, Hsp induction might lead to a 'cascading amplification' of available chaperone activity.

Hsp synthesis is induced by the activation of the heat shock factor (HSF)-1. In resting cells several chaperones, most importantly Hsp90, were shown to bind to HSF-1 and keep it in an inactive form. During stress, these repressing chaperones become occupied by misfolded proteins, which results in the dissociation of the cytoplasmic chaperone/HSF-1 complex. Dissociation of HSF-1 from Hsp90 uncovers the nuclear localization signal of this transcription factor and allows its translocation to the cell nucleus. During this process, the trimerization and phosphorylation of HSF-1 occurs (Morimoto, 2002). Though the exact sequence of these events has not been clearly established, recent studies uncovered the polo-like kinase 1 as an important actor in the phosphorylation and consequent nuclear translocation of HSF-1 at the Ser-419 residue (Kim *et al*., 2005). However, other studies (Guettouche *et al*., 2005) found Ser-326 but not Ser-419 as an important site of activation-related phosphorylation of HSF-1. Part of the nuclear HSF-1 is assembled to heat shock granules, which may modify the chromatin structure (Jolly *et al*., 2004). Binding of HSF-1 to the heat shock elements of the heat shock-inducible genes unlocks the RNA polymerase, which becomes arrested ('pauses') in most of these genes after transcribing the initial segment of the mRNA in the absence of HSF-1. HSF-1 is released from the DNA by a nuclear complex of Hsp90, which is probably followed by its retrotranslocation to the cytoplasm. The details of the nuclear translocation of Hsp90 during stress,

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their complex formation in the nucleus and consequent recruitment to the DNA-bound HSF-1 are not entirely known. All elements of the HSF-1 activation and downregulation cascade, such as additional proteins of the cytoplasmic or nuclear HSF-1/Hsp90 complexes, for example, the Ral-binding protein 1 and tubulin in the cytoplasmic HSF-1/Hsp90 complex (Hu & Mivechi, 2003) or p23 in the nuclear HSF-1/Hsp90 complex (Guo *et al*., 2001), are of great interest as potential drug targets (Figure 1).

Hsp's never work alone. They are always forming a complex with each other and recruit various smaller proteins, called cochaperones, which regulate their ATP-ase cycle, therefore the speed of Hsp-assisted refolding. A central chaperone complex of the cytoplasm is assembled around Hsp90 and is called foldosome (Pratt & Toft, 2003).

Therapeutic potential of Hsp90 inhibition

Inhibition of Hsp90 as an efficient tool for anticancer therapies

Hsp90 is one of the most abundant proteins of eukaryotic cells, comprising 1–2% of total proteins under nonstress conditions. It is evolutionarily conserved among species, and is essential for cell survival. Hsp90 exerts its chaperone activity together with a number of cochaperones, playing an important role in the folding of at least 200 specific proteins of various signaling pathways, and in the refolding of denatured proteins after stress (Csermely *et al*., 1998; Buchner, 1999; Pratt & Toft, 2003).

Hsp90 is an ATP-dependent chaperone. The N-terminal domain of Hsp90 contains a rather unique ATP-binding site, the Bergerat-fold, characteristic of only some bacterial gyrases, topoisomerases and histidine-kinases besides Hsp90 (Prodromou *et al*., 1997; Stebbins *et al*., 1997). The unique ATP-binding site allowed the development of specific Hsp90 inhibitors. Recently, it was shown that Hsp90 contains a second nucleotide-binding site at its C-terminal domain, which may open new possibilities for the inhibition of this chaperone (Marcu *et al*., 2000; Garnier *et al*., 2002; Soti *et al*., 2002).

Hsp90 interacts with and stabilizes a growing list of various kinases including several key members of malignant transformation, such as the ErbB2, Src, Abl or Met tyrosine kinases, or the Raf, Akt and cyclin-dependent serine kinases. Besides these, Hsp90 is necessary for the maturation of several transcription factors, like the nuclear hormone receptors and the hypoxia-inducible factor-1. Additionally, Hsp90 binding has been shown to contribute to the accumulation of mutant forms of the tumor suppressor transcription factor p53. Hsp90 is associated with nitric oxide synthases, the antiapoptotic protein, Apaf-1, etc. (Csermely *et al*., 1998; Buchner, 1999; Pratt & Toft, 2003; Zhao *et al*., 2005).

The above examples show that a large number of proteins need the help of molecular chaperones to maintain their activation-competent conformation. 'Conventional' inhibitors interact with their target, directly inhibiting its function. However, chaperone-based inhibitors do not interact with the effector proteins, but inhibit the ability of the associated chaperone(s) to maintain their activation-competent conformation. As a result, the client proteins became degraded by the proteasome (Schulte *et al*., 1997). In contrast to most direct inhibitors, which are often fairly specific for a given protein, chaperone-based inhibitors diminish the level of many protein targets in parallel (Sreedhar *et al*., 2004b; Neckers & Neckers, 2005). Thus, chaperone inhibitors behave as typical multi-target drugs, which were suggested to be more efficient than highly selective single-target drugs in many applications (Csermely *et al*., 2005).

The most important Hsp90 inhibitors are geldanamycin (Whitesell *et al*., 1994), its less toxic analog, 17-allylamino-17-demethoxy-geldanamycin (17AAG) (Schulte & Neckers, 1998)

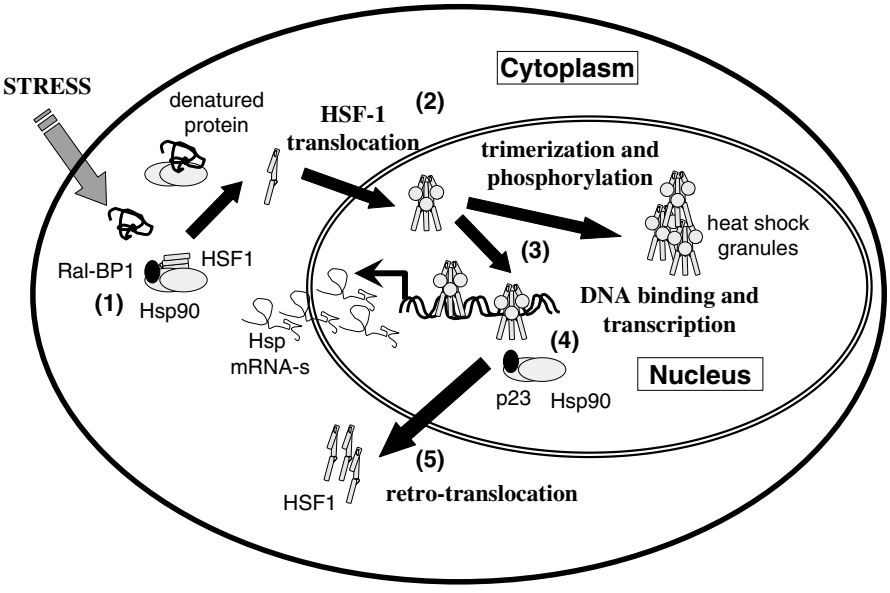

Figure 1 Elements of HSF-1 activation: potential drug targets. The figure contains the major elements of the activation of HSF-1, the major transcription factor leading to the induction of Hsp synthesis. The potential drug targets are the following: (1) cytoplasmic complex of HSF-1 and Hsp90; (2) HSF-1 translocation to the nucleus; (3) intranuclear distribution of HSF-1; (4) nuclear complex of HSF-1 and Hsp90; (5) retrotranslocation of HSF-1 to the cytoplasm. RalBP-1, Ral-binding protein-1; p23, cochaperone of Hsp90.

as well as radicicol, and its more stable oxime derivatives (Soga *et al*., 1998; Agatsuma *et al*., 2002; Ikuina *et al*., 2003), which have a higher affinity for Hsp90 than geldanamycin (Roe *et al*., 1999). Recently, new geldanamycin analogs (Hargreaves *et al*., 2003) as well as a third class of tumor-specific Hsp90 inhibitors, the purine-scaffold inhibitors, were developed (Chiosis *et al*., 2002; Vilenchik *et al*., 2004), and there are ongoing efforts to synthesize even more Hsp90-interacting drug candidates (Banerji *et al*., 2003; Maloney *et al*., 2003; Workman, 2004; Dymock *et al*., 2005; Kreusch *et al*., 2005) (Table 1). Hsp90 inhibition was achieved in an unusual way, using the antitumor agent histone deacetylase inhibitors. These compounds, such as Trichostatin-A, induce the acetylation and consequent inhibition of Hsp90 (Yu *et al*., 2002).

The tumor specificity of Hsp90 inhibitors is helped by an unusual mechanism (Figure 2; Kamal *et al*., 2003). Hsp90 is largely in a latent, uncomplexed state in normal cells. However, Hsp90 becomes activated, forming a large complex with various cochaperones in tumor cells. 17AAG binds to the tumor-specific, complex form of Hsp90 with a 100-fold higher affinity than to the latent form (Kamal *et al*., 2003), raising the possibility that active Hsp90 behaves as a tumor-selective catalyst to convert geldanamycin derivatives to their active conformation (Lee *et al*., 2004). The increased affinity of Hsp90 inhibitors towards tumor-specific Hsp90 is also true for purine-based inhibitors (Vilenchik *et al*., 2004).

Inhibition of Hsp90 induces apoptosis of various tumor cells (Sreedhar & Csermely, 2004). Hsp90 inhibition also leads to a defect in a number of proliferative signals, including the Akt-dependent survival pathway (Munster *et al*., 2001; 2002; Basso *et al*., 2002). Hsp90 inhibition may also sensitize tumor cells against various attacks by helping their lysis under hypoxia, complement attack or mild detergent treatment (Sreedhar *et al*., 2003; 2004a).

Importantly, there are additional drugs which interact with Hsp90, such as the widely used chemotherapeutic agents, cisplatin (Itoh *et al*., 1999), taxol (Byrd *et al*., 1999), as well as the antibactericid novobiocin (Marcu *et al*., 2000). Cisplatin was recently shown to bind to a novel nucleotide-binding site of Hsp90 at its C-terminus (Soti *et al*., 2002), while novobiocin binds to several domains of Hsp90 (Marcu *et al*., 2000; Soti *et al*., 2002). The C-terminal nucleotide-binding pocket has a unique nucleotide-binding specificity (Soti *et al*., 2003), as well as a differential effect on Hsp90 client proteins (Soti *et al*., 2002), which gives hopes that selective inhibitors against this segment of Hsp90 can be developed, showing novel properties in various anticancer protocols. This can be an important approach all the more, since recently a new Hsp90 isoform, Hsp90N, has been reported, which lacks the N-terminal domain; thus, the tumor cells which accumulate this chaperone are resistant against any conventional Hsp90 inhibitors (Grammatikakis *et al*., 2002).

Hsp90 inhibitors enter clinical trials

Though the very first Hsp90 inhibitor geldanamycin showed clear antitumor effects, it encountered difficulties in clinical trials due to its high hepatotoxicity in some of the human tumor models (Supko *et al*., 1995). Thus, a search for new classes of Hsp90 inhibitors with lower toxicity began, and was successful in developing the analog 17AAG. 17AAG possesses all the Hsp90-related characteristics of geldanamycin (Schulte & Neckers, 1998) with lower toxicity (Brunton *et al*., 1998; Chiosis *et al*., 2003; Workman, 2003) and completed five Phase I clinical trials and enters to phase II trials soon (Banerji *et al*., 2005; Neckers & Neckers, 2005). Both geldanamycin and 17AAG can be metabolized by NADH quinone oxidoreductase 1 (DT-diaphorase), which is known to potentiate antitumor activity by stabilizing the tumor suppressor p53. NADH quinone oxidoreductase 1 may be a major factor in conferring on 17AAG as well as its parent compound the advantage that they specifically accumulate in tumor cells (Chiosis *et al*., 2003; Workman, 2003), which also contributes to the explanation why Hsp90 inhibitors are not so generally

Table 1 Hsp modulator compounds (a few examples)

Compound	*Modulation*	*Targets*	*Potential indications*	*References*
Carbenoxonole	Inducer	Hsp70	Ulcer	Nagayama *et al*. (2001)
2-Cyclopenten-1-one	Inducer	HSF-1	Viral infections	Rossi *et al*. (1996)
Dexamethasone	Inducer	HSF-1	Ischemic heart disease, inflammation	Sun *et al*. (2000)
Geranyl-geranyl acetone	Inducer	Hsp70	Cerebral and cardiac ischemia	Ooie *et al*. (2001), Yasuda *et al*. (2005)
Paeoniflorin	Inducer	Hsp27, Hsp40, Hsp70	Not identified yet	Yan *et al*. (2004)
Proteasome inhibitors	Inducer	Hsp70	Neuro-degeneration	Kim *et al*. (2004)
Stannous chloride	Inducer	Hsp70	Tissue transplantation	House *et al*. (2001)
Glycyrrhizin	Co-inducer	HSF-1	Not identified yet	Yan *et al*. (2004)
Bimoclomol, arimoclomol, BRX-220	Co-inducer	HSF-1, membranes	ALS, diabetic neurophaty, pancreatitis, ischemic heart disease	Vigh *et al*. (1997), Jednakovits *et al*. (2000), Kurthy *et al*. (2002), Rakonczay Jr. *et al*. (2002), Kieran *et al*. (2004)
Geldanamycin, 17AAG	Inhibitor	Hsp90	Cancer	Whitesell *et al*. (1994), Neckers & Neckers (2005)
Radicicol, cyclo-proparadicicol	Inhibitor	Hsp90	Cancer	Soga *et al*. (1998), Yang *et al*. (2004)
PU3, PU24F-Cl (purin-based molecules)	Inhibitor	Hsp90	Cancer	Chiosis *et al*. (2001)
Dihydroxy-phenylpyrazole	Inhibitor	Hsp90	Cancer	Kreusch *et al*. (2005)
Trichostatin-A (histone deacetylase inhibitors)	Inhibitor	Hsp90	Cancer	Yu *et al*. (2002)

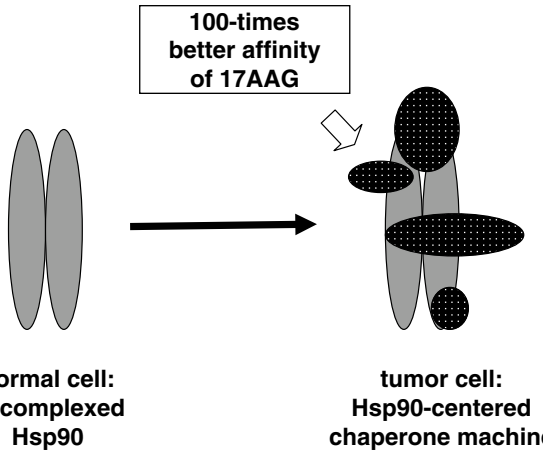

Figure 2 The tumor specificity of 17AAG, a geldanamycin analog inhibitor of Hsp90. 17AAG has a 100-times higher affinity towards the tumor-specific Hsp90 complexed by a large number of cochaperones than to the Hsp90 dimer, which is the predominant form of this chaperone in normal cells (Kamal *et al*., 2003; Lee *et al*., 2004).

toxic to patients as one would expect from the pleiotropic roles of Hsp90 inhibited by them besides their higher affinity to the tumor-specific, complexed form of Hsp90 mentioned before (Kamal *et al*., 2003; Vilenchik *et al*., 2004).

Combination therapies, applying low doses of Hsp90 inhibitors together with conventional chemotherapeutic agents, seem to be an effective way to target various cancers. For example, in the case of Bcr–Abl-expressing leukemias, a low dose of geldanamycin is sufficient to sensitize these cells to apoptosis in the presence of ineffective concentrations of doxorubicin (Blagosklonny *et al*., 2001). 17AAG in combination with taxol showed enhanced cytotoxic effects on taxol-resistant ErbB2-overexpressing breast cancer cells (Munster *et al*., 2001; Sausville, 2001). Another approach is to combine 17AAG with angiogenesis inhibitors, which was proven to be highly successful in breast tumors (de Candia *et al*., 2003). As an alternative strategy the synthesis of geldanamycin hybrids, such as the adduct with steroids (Kuduk *et al*., 1999; 2000) as well as with inhibitors of the PI-3-kinase related survival pathway (Chiosis *et al*., 2001), conferred further selectivity and efficiency for these drugs besides their tumor-specific accumulation. Besides the success of 17AAG, other Hsp90 inhibitors also entered phase I trials, which shows the high therapeutic potential of these drugs (Neckers & Neckers, 2005).

Hsp90 inhibitors as Hsp inducers?

Hsp synthesis is induced by the activation of HSF-1. Hsp90 dissociates during HSF-1 activation due to a competitive binding of misfolded proteins to this chaperone (Morimoto, 2002). Hsp90 inhibitors may also cause the transcriptional activation of HSF-1 by disrupting Hsp/HSF-1 complexes. Indeed, geldanamycin and 17AAG were shown to activate HSF-1 (Kim *et al*., 1999b; Bagatell *et al*., 2000) and the expression of Hsp40, Hsp70 and Hsp90 (Sittler *et al*., 2001). Thus, the inhibition of Hsp90, paradoxically, leads to an increase in their overall amount as well, which should be taken into account as a potential disadvantage, when clinical applications of these drugs are designed. Indeed, both geldanamycin and 17AAG were shown to antagonize the action of cisplatin in human colon adenocarcinoma cells (Vasilevskaya *et al*., 2003).

Inhibition or activation of Hsp90 is cardioprotective?

Griffin *et al*. (2004) has recently demonstrated that radicicol, which activates Hsp expression by binding to Hsp90, induces Hsp expression in neonatal rat cardiomyocytes, and this increase in Hsp's confers cardioprotection to these cardiomyocytes. However, they found that radicicol induction of the Hsp's and cardioprotection are dependent on the inhibition of Hsp90 in cardiomyocytes, and concluded that inhibitors of the function of Hsp90's in the cell may represent potential cardioprotective agents. In contrast, another recent study shows that *in vivo* gene transfer of Hsp90 in the myocardium leads to a protection of the ischemic myocardium in pigs *via* a direct stimulation of eNOS by Hsp90 (Kupatt *et al*., 2004). It is well known that NO is cardioprotective; however, increased NO synthesis with concomitant superoxide synthesis leads to the formation of the cytotoxic peroxynitrite in certain cardiovascular disorders (Ferdinandy & Schulz, 2003). Therefore, further studies are necessary to evaluate the possible role of Hsp90 inhibitors in the therapy of ischemic heart disease.

Therapeutic potential of upregulation of Hsp's

While the inhibitors of Hsp90 are of potential therapeutic interest primarily in cancer therapy, upregulation of other Hsp's, especially that of Hsp70, has been shown to be of great therapeutic potential in a variety of diseases.

Hsp-based immunotherapies of cancers and infections

The ability of Hsp's to interact with a wide range of proteins and peptides, a property that is shared by major histocompatibility complex molecules, has made the Hsp's to be used in new immunotherapies of cancers and infections. Increased Hsp60, Hsp70 and Hsp72 may lead to tumor cell sensitization for immune attacks by two mechanisms: tumor cells may express Hsp's on their surface, which leads to their enhanced recognition by the natural killer cells of the native immune system (Multhoff *et al*., 1997; Multhoff, 2002), as well as a specific antitumor immunity may be induced by Hsp-related antitumor vaccination (Chu *et al*., 2000; Baker-LePain *et al*., 2003; Shigapova *et al*., 2005). Hsp induction may help these processes and may be overcome by the limitations of aging-(Wick & Grubeck-Loebenstein, 1997; Pawelec *et al*., 2002) and chaperone overload-induced (Csermely, 2001) immunosuppression. As these topics have been extensively reviewed recently (Ranford & Henderson, 2002; Srivastava, 2002; Manjili *et al*., 2004; Oki & Younes, 2004), no further details are given in our present review.

Tissue protection by induction and 'coinduction' of Hsp70 and other Hsp's

From the now classical observations of Currie *et al*. (1988), we know that heat-shock response has a significant role in

cardioprotection. Similarly, Hsp72 induction helps the survival of neurons after stroke (Yenari *et al*., 1998), as well as improves the efficiency of tissue transplantation (Perdrizet *et al*., 1993). Hsp70 induction eases the deleterious consequences of chronic diseases, such as diabetes, as revealed by the application of the Hsp coinducer compounds bimoclomol and BRX-220 (Nanasi & Jednakovits, 2001; Kurthy *et al*., 2002). Conditions like Alzheimer's, Parkinson's, Huntington's or prion disease, where the accumulation of misfolded proteins is the major cause of neurodegeneration (Warrick *et al*., 1999; Carmichael *et al*., 2000; Sittler *et al*., 2001), as well as conditions such as trauma, where neuroregeneration becomes necessary (Kalmar *et al*., 2002; 2003), also have beneficial effects from overexpression of Hsp40 and Hsp70.

There are various approaches to induce Hsp's using pharmacological interventions without any traditional forms of stress, such as heat shock. Proteasome inhibitors upregulate Hsp synthesis by increasing the amount of misfolded proteins, which compete for Hsp's with HSF-1 (Kim *et al*., 1999a; 2004). Some of the protein kinase inhibitors were also shown to induce Hsp27 induction (Kawamura *et al*., 1999). Stannous chloride has been shown as a nontoxic, efficient inducer of Hsp70, improving the success rate of tissue transplantations (House *et al*., 2001). Geranyl-geranyl acetone, a nontoxic Hsp70 inducer, has been shown to protect neurons against cerebral ischemia (Yasuda *et al*., 2005). Similarly, the antiulcer drug carbenoxolone has also been shown to be an inducer of Hsp70 (Nagayama *et al*., 2001) (Table 1).

Most of the above methods introduce a certain level of stress to the cells and thus provoke Hsp synthesis. However, in most of the diseases it seems to be more efficient if the administered drug does not induce Hsp's, and just helps the natural Hsp induction provoked by the natural stimuli on the cell. This help in Hsp induction was termed as chaperone 'coinduction' (Vigh *et al*., 1997). Chaperone coinducers act like 'smart drugs' by a selective interaction with only those cells which are in danger, and may provide an important novel therapy in a number of acute and chronic diseases.

The best-known chaperone coinducer is aspirin, which enhances Hsp70 synthesis (Jurivich *et al*., 1992). Recently, certain herbal extracts were also shown to possess Hsp coinducer activity (Yan *et al*., 2004). Another family of drug candidates exemplified by Bimoclomol helps the induction of Hsp70 synthesis by both perturbing various membrane structures and helping the release of putative lipid-signaling molecules, as well as by the prolongation of the binding of HSF-1 to the heat shock elements on the DNA (Vigh *et al*., 1997; Hargitai *et al*., 2003; Torok *et al*., 2003; Kieran *et al*., 2004). Bimoclomol binds to HSF-1 with a low affinity (Figure 3), which may contribute to its effect to prolong HSF-1 binding to DNA. Chaperone coinducers also stabilize membranes, which may be of special importance to prevent apoptotic events related to the decomposition of cardiolipin and the consecutive instabilization of the mitochondrial membrane (Torok *et al*., 2003). Recently, a successful application of the chaperone coinducer arimoclomol has been shown to delay the onset of amyotrophic lateral sclerosis (Kieran *et al*., 2004). Another chaperone coinducer, BRX-220, has been shown to protect against CCK-induced acute pancreatitis (Fujiwara *et al*., 2004) (Table 1).

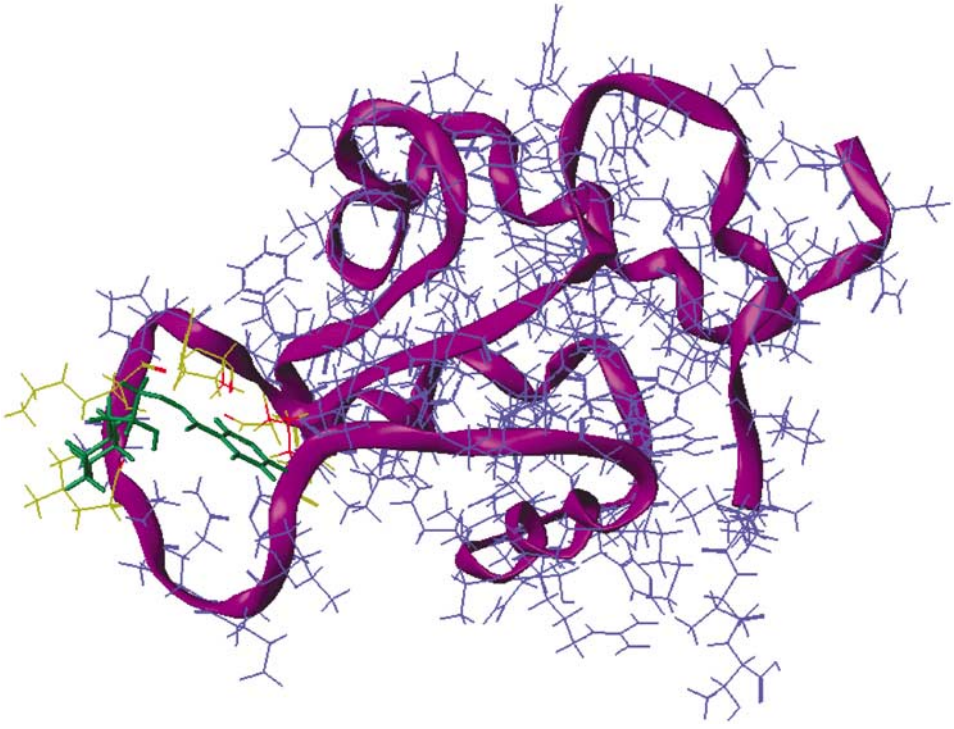

Figure 3 Bimoclomol binds to the HSF-1 with a low affinity (Hargitai *et al*., 2003). Fitting of /R/-bimoclomol to the 3D model of the DNA-binding domain of human HSF-1. The SiteID, FlexX and the FlexiDock modules of the Sybyl package were used sequentially to identify the loop domain as the active center (yellow–red amino acids) of the DNA-binding domain of human HSF-1 and dock bimoclomol (green) into this site. The calculated binding energy for the /R/-bimoclomol was $-26\,\text{kcal}\,\text{mol}^{-1}$. The backbone of the polypeptide is shown (purple).

Diminished heat stress response in hyperlipidemia and aging: potential use of Hsp inducers as anti-ischemic agents?

It is well known that aging leads to a diminished heat shock response in several tissues, thereby resulting in the deterioration of stress adaptation (Demirel *et al*., 2003) (for a review, see Soti & Csermely, 2003). We have recently reported that experimental hyperlipidemia also leads to diminished heat-stress response, as measured by Hsp70 expression in the ischemic heart of rats (Csont *et al*., 2002). It is of great interest that both aging and hyperlipidemia have been shown to decrease the cardioprotective effects of the ischemic preconditioning, that is, the endogenous adaptive response of the heart to ischemic stress (for reviews, see Ferdinandy *et al*., 1998; Baxter & Ferdinandy, 2001; Ferdinandy, 2003). The role of Hsp's in the mechanism of preconditioning has been extensively reviewed elsewhere (Latchman, 2001; Snoeckx *et al*., 2001). It has been shown that the cardioprotective effect of preconditioning is linked to the functions of Hsp70, Hsp27, and alphaB-crystalline. Heat stress or Hsp70 gene transfection into rat hearts has been shown to protect the ischemic myocardium (Arnaud *et al*., 2001; Jayakumar *et al*., 2001). Therefore, it is plausible to speculate that the loss of the protective effect of preconditioning in these disease states is related – at least in part – to diminished heat stress response. We have recently shown using DNA-microarray assay that hyperlipidemia alters the expression of several chaperones in the rat myocardium, including upregulation of Hsp86, metallothionein and glutathione-*S*-transferase, as well as downregulation of proteasome component C9, ubiquitin-like protein FUBI, Hsp105, calreticulin and chaperonin subunit 5 epsilon (Puskas *et al*., 2004).

The mechanisms by which hyperlipidemia and aging lead to diminished heat stress response are not known. It is suggested, however, that the altered membrane lipid composition and physical state (fluidity) of membranes are decisive factors in the processes of perception and transduction of stress into a signal that triggers the transcriptional activation of stress protein genes. Nevertheless, Hsp inducers and coinducers may have great therapeutic potential in aging and hyperlipidemia to regain the endogenous adaptation of the heart to ischemic stress.

Cellular membranes act as alternative thermosensors to regulate Hsp expression

Elucidation of primary sensors that perceive various stress stimuli and transducers that carry signals culminating in the expression of a particular Hsp is of great interest for the development of novel Hsp inducers and coinducers. According to the classical model, the common primary signal for induction of Hsp's is an increase in the amount of denatured proteins within the cell during stress and their competition with HSF (Morimoto, 1998) (see above for more refs). Hsp's are present in abnormal levels in a variety of human diseases and aging, but there is no evidence for concomitant modification of the kinetics of accumulation of denatured proteins that could justify the changes observed in the expression of Hsp's (Vigh *et al*., 1998; Vigh & Maresca, 2002). For example, curcumin, a well-known dietary pigment with potent anti-inflammatory, antioxidant and anticancer effects, causes heat shock response in transformed cell lines such as leukemia, breast, colon, etc.; however, it has no effect on nontransformed cell lines (Khar *et al*., 2001). Since no differences in the pattern of protein denaturation have been reported between normal and transformed cell lines, these data imply that the protein denaturation is not (or not only) the mechanism by which Hsp's are upregulated. There is also evidence for differential expression patterns of different Hsp's in the same cell type in response to the same stimulus (Heads *et al*., 1995). For example, herbimycin-A provokes Hsp70 synthesis and protects against a subsequent lethal heat stress; however, it does not activate the transcription of other Hsp genes such as hsp90, hsp60, hsp27 and grp78 in cultured neonatal cardiomyocytes (Morris *et al*., 1996). Monophosphoryl lipid A, a nontoxic derivative of endotoxin with potent anti-ischemic effect, induced a remarkable accumulation of Hsp70, but did not change the expression of Hsp27, Hsp32 and Hsp90 in adult rat cardiac myocytes (Nayeem *et al*., 1997). Selective stimulation of small Hsp's, Hsp27, and alfaB-crystalline but not Hsp70 was shown by anisomycin following heat stress in C6 glioma cells (Kato *et al*., 1999), by sphingosine1-phospate in osteoblasts (Kozawa *et al*., 1999), by hydroxyurea in B16 murine melanoma cells (Eskenazi *et al*., 1998), by short-chain fatty acids in the rat intestinal epithelial cells (Ren *et al*., 2001) or by thrombin in aortic smooth muscle A10 cells (Hirade *et al*., 2002), respectively. These observations indicate that Hsp expression in response to various stressors is regulated by differential control mechanisms rather than by uniform mechanisms.

On the basis of experimental evidence, it is proposed that, besides protein denaturation, specific membrane domains may act as alternative sensors or 'cellular thermometers'. Stress-induced membrane perturbations are converted into signal(s) leading to activation of heat-shock genes (Carratu *et al*., 1996; Horvath *et al*., 1998; Vigh *et al*., 1998; Vigh & Maresca, 2002; Shigapova *et al*., 2005). Changes in membrane composition and/or fluidity alter the 'set point' for Hsp expression, with expression initiated at lower temperatures in cells with more fluid membranes (Carratu *et al*., 1996; Horvath *et al*., 1998; Chatterjee *et al*., 2000; Shigapova *et al*., 2005) (Figure 4).

Cellular membranes as potential targets for Hsp inducers

Although the Hsp coinducing effects of bimoclomol were shown to be mediated *via* HSF-1 as described above, bimoclomol does not affect protein denaturation in the cells (Vigh *et al*., 1997; Hargitai *et al*., 2003). It has been shown that bimoclomol and its analogs specifically interact with and significantly increase the fluidity of negatively charged membrane lipids. Accordingly, the Hsp coinducing activity of bimoclomol is highly susceptible to the fatty acid composition and fluidity of membrane of target cells. In addition, bimoclomol is an efficient inhibitor of bilayer-to-nonbilayer lipid-phase transitions. Consequently, while sensitizing the cellular membranes at mild heat shock conditions, the drug protects against irreversible membrane damage at higher temperatures (Torok *et al*., 2003). Thus, even subtle alterations of the lipid phase of membranes caused by aging or pathophysiological conditions may influence membrane-initiated signaling processes either through fluidity changes or by specific interactions of membrane lipids with receptor proteins localized in the membrane. Taken together, it is highly conceivable that plasma membrane, which is the barrier to the

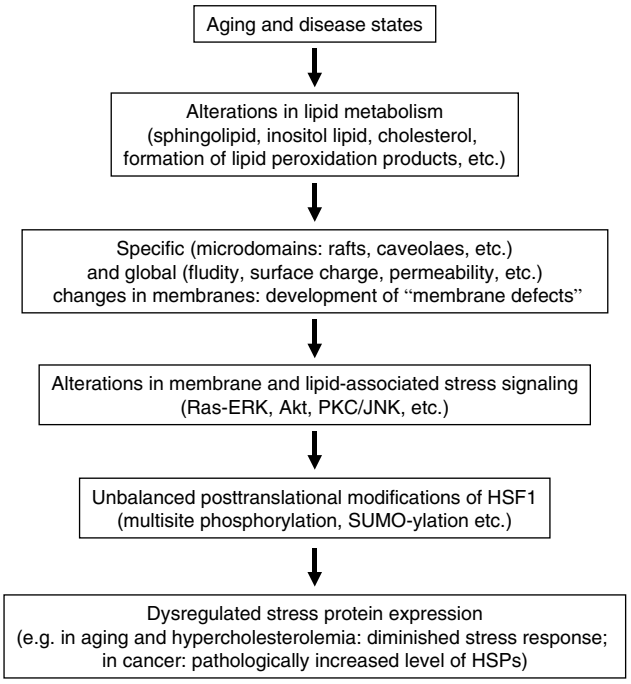

Figure 4 Cascade of events from membrane defects to dysregulated stress protein response.

external environment and well suited for sensing stress, acts also as an important regulatory interface. In line of this concept, recently it was speculated that the reduced HSF-1 and Hsp levels in diabetes are the result of reduced membrane fluidity (Hooper & Hooper, 2005). In fact, as a result of glycation, oxidative stress and insulin deficiency, diabetes is associated with less fluid membranes in human mononuclear leucocytes and platelets (Tong *et al*., 1995; Srivastava, 2002). Since heat treatment itself causes membrane hyperfluidization, it is not surprising that the daily hot water immersions in patents with type II diabetes improve glycemic control and reduce neuropathic symptoms (Hooper, 1999).

Perturbation of the organization and phase state of acidic glycerophospholipids, the major determinants of the activity of membrane proteins (van Klompenburg *et al*., 1997), by nontoxic drugs like bimoclomol may influence the structure/activity of membrane-bound proteins without direct drug–protein interaction. Protein function influenced by membrane fluidity and/or microheterogeneity has been suggested for phospholipase A_2, which is stimulated by heat shock, resulting in arachidonic acid release (Honger *et al*., 1996; Samples *et al*., 1999). Arachidonic acid stimulates HSF–DNA binding, increases phosphorylation of HSF-1 and upregulates transcription of the *Hsp70* gene in HeLa cells (Jurivich *et al*., 1994). Elevated activity of membrane-bound phospholipases and the resultant release of free fatty acids and diacylglycerols could also enhance the membrane association and activation of protein kinase C (PKC) isoforms responsible for the phosphorylation of HSFs. In agreement with these data, administration of a PKC activator phorbol ester compound in combination with heat shock markedly enhanced Hsp70 expression in K562 cells (Holmberg *et al*., 1997). Several other pharmacological modulators of kinase/phosphatase activities can alter the different regulatory steps of the heat shock response. Highlighting the complexity of this point, overexpression of inducible Hsp70 downregulated the basal activities of protein kinase A, various free and membrane-associated PKC isoforms and the MAP kinase pathways including c-Jun N-terminal kinase and p38 stress-activated protein kinase (p38 SAPK) (Kiang *et al*., 2002). Through the activation of the glycosylation of membrane sterols, cholesterol glucoside is rapidly accumulating in cells from mold to humans by exposure to environmental stress, and cholesterol glucoside production is followed by the activation of certain PKCs and induction of Hsp's (Kunimoto *et al*., 2002). Cholesterol glucoside accelerated the binding of HSF-1 to HSE and upregulated Hsp70 synthesis in human fibroblast (Kunimoto *et al*., 2002). Orally administered cholesterol glucoside apparently showed antiulcer activity in rats *via* HSF activation and Hsp70 induction (Kunimoto *et al*., 2003). A bimoclomol-related compound, BRX-235, was shown to induce phosphorylation of p38 SAPK, implying that the molecule acted upstream of p38 SAPK (Denes *et al*., 2002; Kabakov *et al*., 2005). HSF-1 appears to have several additional layers of regulation in the cell, including Ras/ERK1/GSK3/14-3-3 pathway (Hamaguchi *et al*., 2003; Wang *et al*., 2003), Akt-induced inhibition of GSK-3β (Bijur & Jope, 2000), small G-protein signalling such as Ras (Engelberg *et al*., 1994; Murakoshi *et al*., 2004) and oxidative stress-induced membrane translocation of Rac1 (Xu *et al*., 2000; Han *et al*., 2001), all potential targets for Hsp modulator development. Noteworthy that simvastatin, the known hydroxymethyl-glutaryl-CoA reductase inhibitor antihyperlipidemic drug, blocked the oxidative stress-induced membrane translocation of Rac1 (Negre-Aminou *et al*., 2002).

It is highly conceivable that the above findings are linked to those hypothetic signal transduction pathways which transmit the heat stress signal from membranes to DNA to induce expression of Hsp's. However, a lipid-selective association of a subpopulation of Hsp's with membranes, leading to increased molecular order, may in turn lead to downregulation of the heat shock gene expression (Torok *et al*., 1997; 2001; Tsvetkova *et al*., 2002). Such a 'crosstalk' between the primary stress sensor in the membranes and Hsp's suggests a feedback mechanism in the regulation of heat-shock genes, explaining the known temporality of induction of Hsp's. These findings show that knowledge on pathways of stress signaling will provide several molecular targets for further development of Hsp modulators.

Conclusions and future perspectives

Chaperones play a major role in the mechanism of endogenous stress adaptation of several tissues. However, altered chaperone function has been associated with the development of several pathologies; therefore, chaperone modulators became a new and emerging field of drug development. Inhibitors of Hsp90 recently emerged as a very promising tool to combat various forms of cancer. On the other hand, activation of chaperone induction proved to be an efficient tool for the recovery from a large number of diseases, such as, for example, ischemic heart disease, diabetes and neurodegeneration. Development of several Hsp modulators has already reached clinical phases. Due to the promising results, specific chaperone modulators could be one of the future blockbuster drugs on the market for several different therapeutic indications.

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Expressed as the sole Hsp90 of yeast, the α and β isoforms of human Hsp90 differ with regard to their capacities for activation of certain client proteins, whereas only Hsp90β generates sensitivity to the Hsp90 inhibitor radicicol

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Heat shock protein 90 (Hsp90) is a molecular chaperone required for the activity of many of the most important regulatory proteins of eukaryotic cells (the Hsp90 'clients'). Vertebrates have two isoforms of cytosolic Hsp90, Hsp90α and Hsp90β. Hsp90β is expressed constitutively to a high level in most tissues and is generally more abundant than Hsp90α, whereas Hsp90α is stress-inducible and overexpressed in many cancerous cells. Expressed as the sole Hsp90 of yeast, human Hsp90α and Hsp90β are both able to provide essential Hsp90 functions. Activations of certain Hsp90 clients (heat shock transcription factor, v-src) were more efficient with Hsp90α, rather than Hsp90β, present in the yeast. In contrast, activation of certain other clients (glucocorticoid receptor; extracellular signal-regulated kinase-5 mitogen-activated protein kinase) was less affected by the human Hsp90 isoform present in these cells. Remarkably, whereas expression of Hsp90β as the sole Hsp90 of yeast rendered cells highly sensitive to the Hsp90 inhibitor radicicol, comparable expression of Hsp90α did not. This raises the distinct possibility that, also for mammalian systems, alterations to the Hsp90α/Hsp90β ratio (as with heat shock) might be a significant factor affecting cellular susceptibility to Hsp90 inhibitors.

Heat shock protein 90 (Hsp90), an essential molecular chaperone, catalyzes the final activation step of many of the key regulatory proteins in eukaryotic cells (the Hsp90 'clients'). The list of proteins that are Hsp90 clients is impressive and ever-expanding (reviewed in http://www.picard.ch [1,2]). It includes several of the important determinants of multistep carcinogenesis, such as ERBB2, C-RAF, CDK4, AKT/PKB, steroid hormone receptors, mutant p53, HIF-1α, survivin and telomerase (hTERT). Genomic studies in yeast have recently addressed the breadth of the Hsp90 clientele, revealing that up to 10% of the proteome may be subject to Hsp90 regulation [3,4].

Most simple eukaryotes have only a single form of cytosolic Hsp90 (e.g. *Drosophila* [5] and *Caenorhabditis elegans* [6]). Although budding yeast (*Saccharomyces cerevisiae*) has two isoforms of cytosolic Hsp90, one constitutively expressed at high level (Hsc82) and the other strongly heat-inducible (Hsp82) [7], these most probably arose as the result of the ancestral

Abbreviations
AD, activator domain; BD, DNA-binding domain; CT, C-terminal activator/modulator; DO, complete dropout glucose medium; ERK, extracellular signal-regulated kinase; GR, glucocorticoid receptor; HSF, heat shock transcription factor; Hsp90, heat shock protein 90; MAP kinase, mitogen-activated protein kinase; v-src, v-src tyrosine kinase; Y2H, yeast two-hybrid.

duplication of the *S. cerevisiae* genome [8], as most yeast species have just a single Hsp90.

Vertebrates also have two major forms of cytosolic Hsp90 (Hsp90α; Hsp90β), isoforms that are generally around 85% identical in amino acid sequence [9,10]. Hsp90β is expressed constitutively to a higher level than Hsp90α in most tissues and is important for long-term cellular adaptation, differentiation, and evolution. The other isoform, Hsp90α (in humans, a form with 86% identity and 93% similarity in sequence to Hsp90β), is generally stress-inducible and may therefore be a more cytoprotective form of Hsp90 [11]. Hsp90α is also expressed to high level in many cancers [9], as well as extracellularly, where its effects on the activity of metalloproteinase 2 may be important in cancer cell metastasis [12].

Although the differential patterns of expression of Hsp90α and Hsp90β suggest that these isoforms may not be completely equivalent in function, there is as yet no firm genetic evidence for a functional difference between Hsp90α and Hsp90β [11]. During the heat shock response, many mammalian cell types display strong heat shock transcription factor (HSF)-directed induction of Hsp90α. This induction of Hsp90α will increase the α/β isoform ratio, an increase that might be a significant factor in Hsp90-dependent actions [13]. In the yeast heat shock response, HSF-directed elevation of Hsp90 level is required in order to facilitate the activation of an Hsp90 client protein kinase needed for high-temperature growth [14]. This kinase, in turn, activates a transcription factor responsible for a significant fraction of the non-HSF-dependent events of gene induction in yeast subjected to heat shock stress [14].

The human genome appears to have just two functional genes for Hsp90α and one for Hsp90β [10]. So essential is the Hsp90 function conferred by these genes that it is possible that neither Hsp90α nor Hsp90β can be inactivated completely in vertebrate systems, creating a situation where the remaining isoform would have to provide all the essential functions for cytosolic Hsp90. Hsp90β loss is known to cause embryonic lethality in the mouse [15]. Whereas it might be feasible to generate Hsp90α or Hsp90β gene knockouts in particular animal tissues, it is not clear whether this is a realistic strategy for revealing any functional differences between the isoforms. We have therefore investigated yeast strains that express, to similar levels, either human Hsp90α or human Hsp90β as their sole Hsp90. Here we report a study of the activation of various Hsp90 clients and Hsp90 inhibitor sensitivity in such strains; analysis that showed that many mammalian clients are able to be activated by both Hsp90α and Hsp90β. Whether Hsp90α or Hsp90β is expressed in the yeast, however, has a dramatic effect on Hsp90 inhibitor sensitivity. This raises the intriguing possibility that the α/β isoform ratio may be an important determinant of such inhibitor sensitivity in mammalian cells. In an independent study, yeasts expressing either Hsp90α or Hsp90β were recently used to study the effects of some naturally occurring sequence polymorphisms in the human genes for Hsp90 [16].

Results

PP30[hHsp90α] and PP30[hHsp90β] – yeast strains that express human Hsp90α or Hsp90β as their sole Hsp90

S. cerevisiae strains PP30[pHSC82b], PP30[pHSP82] and PP30[hHsp90β] are *hsc82Δ hsp82Δ* double mutant cells that express, from a plasmid-borne Hsp90 gene, either the native yeast Hsc82, the native yeast Hsp82 or human Hsp90β as their sole, essential Hsp90 [17]. For the current study, we constructed in this genetic background an additional strain in which the Hsp90 present is human Hsp90α (PP30[hHsp90α]; see Experimental procedures). Western blotting using an antiserum that recognizes, with comparable efficiencies, both of the yeast and both of the human isoforms of Hsp90 indicated that the levels of Hsp90 expression in strains PP30[pHSC82b], PP30[pHSP82], PP30[hHsp90α] and PP30[hHsp90β] were comparable, although the Hsp90β expression of PP30[hHsp90β] appeared to be slightly lower than that of the other three strains (Fig. 1A). These yeasts, isogenic but for their *Hsp90* gene, might therefore be expected to exhibit similar phenotypes. Nevertheless, as the studies below reveal, the strains expressing human Hsp90α or Hsp90β do exhibit some differences. These isoforms are therefore not completely identical in their *in vivo* actions, at least when expressed in yeast.

Phenotypic differences between strains PP30[hHsp90α] and PP30[hHsp90β]

Investigating the properties of the strains expressing human Hsp90α or human Hsp90β as their sole Hsp90, we found no defect in respiratory growth, cell wall integrity or the ability to withstand osmostress (properties defective in certain Hsp90 mutants of yeast [3,14,18,19]; unpublished data). Both strains were growth-arrested when exposed to the mating pheromone α-factor (data not shown), and so were not defective in this Hsp90-dependent response [20]. Also, when rendered histidine prototrophic through the introduction of an *HIS3* vector, both PP30[hHsp90α] and PP30[hHsp90β] grew well at 30 °C in the presence of

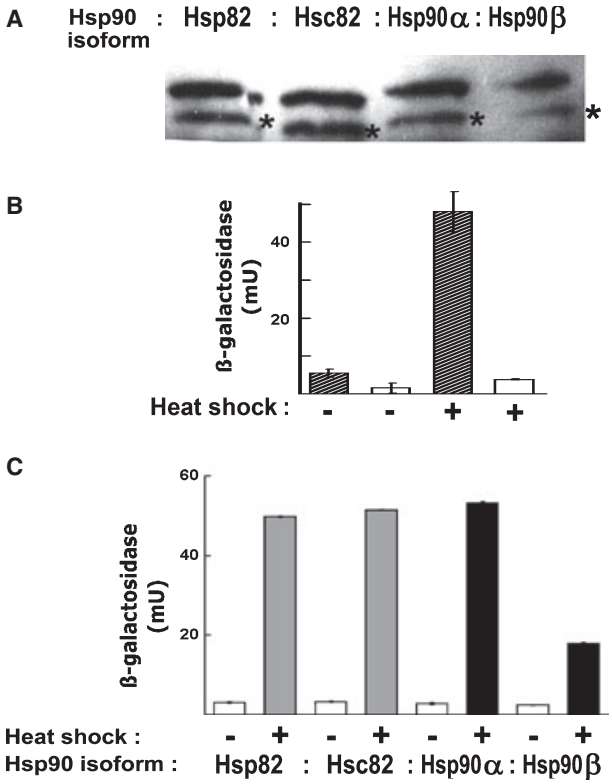

Fig. 1. (A) Measurement of the relative levels of Hsp90 expression in strains PP30[pHSC82b], PP30[pHSP82], PP30[hHsp90α], and PP30[hHsp90β]. Ten micrograms of total soluble protein was gel fractionated, and then western blotted; the blot was then probed with anti-(*Achlya* Hsp90) monoclonal serum. The bands indicated by an asterisk correspond to a slightly degraded, N-terminally truncated form of Hsp90 that is often present in total cell extracts of yeast [45]. (B) Levels of HSE2-*LacZ* reporter gene activity in strain PSY145* with wild-type Hsf1p [22] (hatched bars) or strain PSY145*HSF(1–583) with a CT domain-deficient Hsf1p [22] (open bars), showing that heat induction of HSE2-*LacZ* is dependent on the Hsf1p CT domain. (C) Measurements of HSE2-*LacZ* expression in strains PP30[pHSP82], PP30[pHSC82b], PP30[hHsp90α], and PP30[hHsp90β]; cultures either in growth at 25 °C (–) or heat shocked from 25 °C to 37 °C for 1 h (+). Measurements in (B) and (C) are the mean and SD of eight separate assays on each culture.

30 mM 3-aminotriazole. They are therefore not defective in the Hsp90-dependent activation of Gcn2p kinase [21].

In contrast, the strain expressing Hsp90β was slightly temperature-sensitive [PP30[hHsp90α], and exhibited growth on YPD to 39–40 °C, whereas PP30[hHsp90β] grew only to 36–37 °C (not shown)]. Growth of *S. cerevisiae* at high temperature requires the activity of the C-terminal activator/modulator (CT) domain of yeast HSF (Hsf1p). Cells expressing a CT domain-deficient Hsf1p exhibit no growth above 35 °C. This high-temperature growth defect is rescued by Hsp90 overexpression, revealing that this growth defect is primarily due to the low level of (Hsf1p-directed) Hsp90 expression in these cells [14,22].

To find whether heat activation of the Hsf1p CT domain is defective in our strains expressing a single Hsp90 isoform (all strains with a wild-type Hsf1p), we monitored a reporter gene (HSE2-*lacZ* [23]) that measures activity of the Hsf1p CT domain (HSE2-*lacZ* heat activation is completely lost in a yeast mutant that expresses normal Hsp90 but a CT domain-deficient HSF; see Fig. 1B). We found efficient HSE2-*lacZ* activation by heat shock in cells expressing either the native yeast Hsp82 or Hsc82, or the human Hsp90α (PP30[pHSC82b], PP30[pHSP82], and PP30[hHsp90α], respectively), but only moderate HSE2-*lacZ* activation in the identically stressed PP30[hHsp90β] (Fig. 1C). The induction of CT domain activity by heat stress is therefore less efficient with Hsp90β as compared to Hsp90α present in the yeast. As temperature sensitivity is normally associated with compromised activity of the Hsf1p CT domain [14,22], the compromised heat activation of this domain with Hsp90β present in the yeast (Fig. 1C) is a plausible explanation for the moderate degree of temperature sensitivity exhibited by strain PP30[hHsp90β].

Activation of mammalian Hsp90 clients by either Hsp90α or Hsp90β expressed in yeast

We were interested in whether mammalian Hsp90 clients would display any differences in activation when expressed in the PP30[hHsp90α] and PP30[hHsp90β] yeast strains, differences that might indicate a functional nonequivalence of human Hsp90α and Hsp90β. We therefore expressed in these strains three vertebrate Hsp90 clients whose activities are known to be Hsp90-dependent when expressed in yeast (client proteins, therefore, that have already been demonstrated to be activated by the native Hsp90s of yeast): glucocorticoid receptor (GR) [24], v-src tyrosine kinase (v-src) [25], and extracellular signal-regulated kinase-5 (ERK5) mitogen-activated protein (MAP) kinase [18].

GR assays indicated that human Hsp90α and Hsp90β, as well as the native yeast Hsp90s, were all capable of activating GR in these strains (Fig. 2).

Active v-src expression is normally lethal for yeast, an organism with very low intrinsic levels of tyrosine kinase activity [25]. With use of a galactose-inducible system for v-src expression, high levels of tyrosine phosphorylation were generated in response to v-src induction in PP30[hHsp90α] (Fig. 3B); an induction associated with strong growth inhibition (Fig. 3A). In contrast, the identically treated culture of strain PP30[hHsp90β] exhibited much lower levels of tyrosine

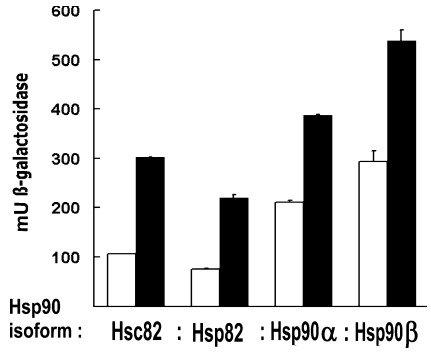

Fig. 2. Measurements of GR activity in 30 °C cultures of strains PP30[pHSP82], PP30[pHSC82b], PP30[hHsp90α], and PP30[hHsp90β], 3 h following addition of either 20 μM (open bars) or 50 μM (solid bars) dexamethosone. The data shown are the mean and SD of four separate assays on each culture. In the absence of inducer, activity levels were consistently less than 10 mU (units are defined as in [43,45]).

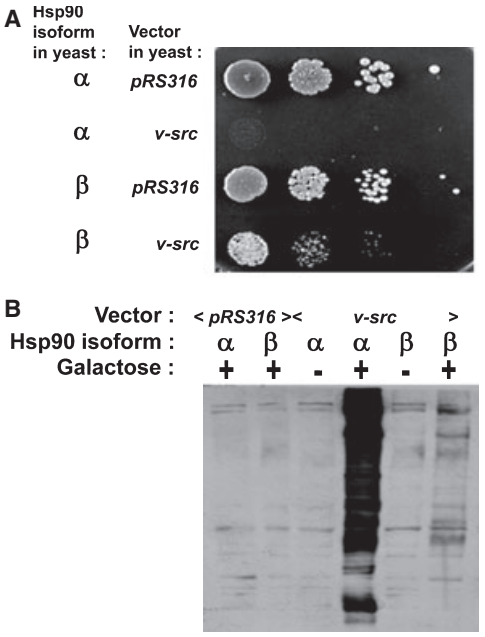

Fig. 3. (A) v-src exerts a much stronger dominant-negative effect in Hsp90α-expressing tha in Hsp90β-expressing yeast. Serial dilution of either PP30[hHsp90α] or PP30[hHsp90β], transformed either with empty pRS316 vector or the vector for galactose-inducible v-src expression, grown for 3 days at 29 °C on DO minus uracil and galactose plates. (B) Analysis of the levels of protein tyrosine phosphorylation before (–) or 1 h after (+) transfer of these PP30[hHsp90α] and PP30[hHsp90β] transformants from glucose to galactose medium. Detection was with antibody to phosphotyrosine.

phosphorylation (Fig. 3B), and the cells were also relatively much less sensitive to the lethal effects of the v-src expression (Fig. 3A). Hsp90α, but not Hsp90β, therefore facilitated the efficient production of active v-src in these strains.

Although MAP kinases are generally considered to have non-Hsp90-dependent activities [26], we recently found that human ERK5 MAP kinase is an Hsp90 client, at least when expressed in active form in yeast [18]. ERK5 is the human ortholog of the yeast Slt2p cell integrity MAP kinase (also an Hsp90 client); heterologous expression of ERK5 in yeast completely rescuing the effects of loss of this native Slt2p [3,18]. ERK5 activity in yeast is therefore readily monitored as the suppression of *slt2Δ* mutant phenotypes [18]. To determine whether ERK5 could still provide the cell integrity MAP kinase function when, in yeast cells, either human Hsp90α or Hsp90β replaced the native Hsp90, we constructed *slt2Δ* mutant versions of strains PP30[hHsp90α] and PP30[hHsp90β] (see Experimental procedures). These strains (PP30[hHsp90α]*slt2Δ* and PP30[hHsp90β]*slt2Δ*) were then transformed with either a control empty vector or a vector for constitutive ERK5 expression (pG1 and pG1-ERK5, respectively [18]), as well as a plasmid bearing the *YIL117w-LacZ* reporter gene [27], which monitors the activity of Rlm1p, a transcription factor activated by cell integrity MAP kinase.

Loss of cell integrity MAP kinase generates a number of characteristic phenotypes in yeast, including temperature and caffeine sensitivity [28–30] and loss of mating projection formation upon treatment with mating pheromones [31]. Plasmid pG1-ERK5 rescues these phenotypes of *slt2Δ* yeast [18]. It was also able to rescue these phenotypes in both PP30[hHsp90α]*slt2Δ* and PP30[hHsp90β]*slt2Δ*, the restoration of high-temperature (37 °C) growth being shown in Fig. 4A. Both isoforms of human cytosolic Hsp90 can therefore activate human ERK5 MAP kinase in yeast.

Rlm1p, the major *trans*-activator of cell wall genes in yeast, is activated through Slt2p-catalyzed phosphorylation [27,32,33]. *slt2Δ* mutant cells therefore display a pronounced Rlm1p activity defect. Hsp90 is required for the rescue of their Rlm1p activity defect by ERK5 expression, as such rescue is abolished by the T22I Hsp90 mutation or by Hsp90 inhibitor treatment [18]. As shown in Fig. 4B, ERK5 expression provided an appreciable rescue of the Rlm1p activity of PP30[hHsp90α]*slt2Δ* and PP30[hHsp90β]*slt2Δ*, activity that was increased by two stress inducers of cell integrity pathway signaling, heat shock and caffeine. This is yet further evidence that both Hsp90α and Hsp90β are able to activate human ERK5 expressed in yeast.

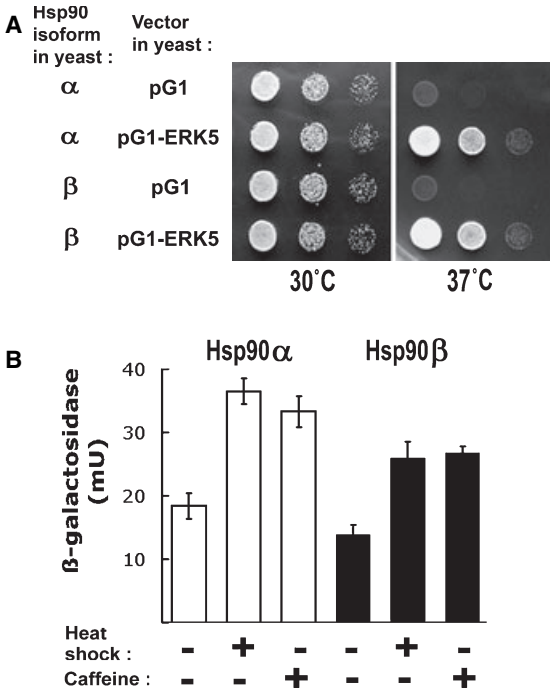

Fig. 4. (A) Growth (3 days in YPD) at either 30 °C or 37 °C of PP30[hHsp90α]*slt2Δ* and PP30[hHsp90β]*slt2Δ* cells transformed with pG1 or pG1-ERK5. (B) Measurements of *YIL117c-LacZ* expression in PP30[hHsp90α]*slt2Δ* (open bars) and PP30[hHsp90β]*slt2Δ* (black bars) transformed with pG1-ERK5, either in growth at 25 °C (unstressed), heat shocked from 25 °C to 37 °C for 1 h, or exposed for 1 h to 8 mM caffeine at 25 °C. In the absence of ERK5 expression, expression levels were less than 2 mU.

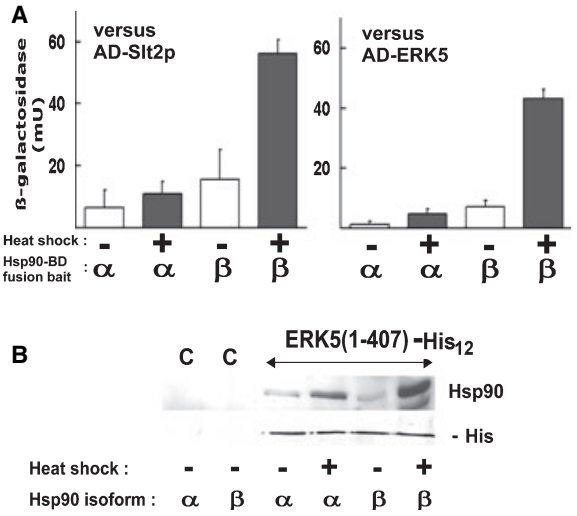

Fig. 5. (A) The relative strengths of Hsp90α-BD–AD-Slt2p, Hsp90β-BD–AD-Slt2p, Hsp90α-BD–AD-ERK5 and Hsp90β-BD–AD–ERK5 Y2H interactions, both at 30 °C and 1 h following a 30 °C to 39 °C heat shock. These measurements of interaction-responsive *LacZ* expression in strain PJ694 reveal that AD-Slt2p and AD-ERK5 bind more strongly to Hsp90β-BD than to Hsp90α-BD in this system. The control cells (all exhibiting less than 0.1 mU β-galactosidase activity, not shown) were those expressing AD-Slt2p or AD-ERK5 but with empty pBDC vector, as the basal levels of *LacZ* expression in this system are generally due to the AD fusion [49]. (B) Determination of Hsp90 associated with nickel resin-retained, wild-type ERK5(1–407)-His_{12} in extracts from PP30[hHsp90α]*slt2Δ* and PP30[hHsp90β]*slt2Δ* cultures, either in growth at 25 °C, or heat shocked from 25 °C to 39 °C for 1 h. The blots were probed with anti-(*Achlya* Hsp90) and anti-tetra-His sera. Control lanes (C) are the extracts from unstressed, non-ERK5-expressing cultures of the same strains.

Two MAP kinase clients show a stronger interaction with Hsp90β as compared to Hsp90α

Slt2p and ERK5 are Hsp90 client MAP kinases that both acquire stronger capacity for Hsp90 binding *in vivo* when phosphorylated by the upstream MAP kinase kinase, Mkk1/2p. Their interactions with the native Hsp90 of yeast are therefore strengthened by conditions of stress, such as heat shock, that activate cell integrity pathway signaling to Mkk1/2p [3,14,18]. We used the yeast two-hybrid (Y2H) system to determine the relative strengths of *in vivo* interaction of these two MAP kinases with the two isoforms of human Hsp90. In the yeast Hsp90s, a C-terminal Gal4p DNA-binding domain (BD) extension preserves the essential Hsp90 functions *in vivo*, whereas positioning this BD at the N-terminus of Hsp90 inactivates the chaperone [34]. We therefore constructed strains that express Y2H 'bait' fusions comprising Hsp90α and Hsp90β with C-terminal BD extensions (Hsp90α-BD, Hsp90β-BD; see Experimental procedures). These were then mated to cells expressing the previously described Gal4p activator domain (AD)-Slt2p and AD-ERK5 'prey' fusions [3,18]. Expression of the *GAL7* promoter-regulated *LacZ* gene in the resulting diploid strains, a gene reporter of protein–protein interaction, was then analyzed. As shown in Fig. 5A, both the Slt2p and ERK5 MAP kinases displayed stronger Y2H interactions with Hsp90β than with Hsp90α.

Consistent with Slt2 and ERK5 acquiring an enhanced capacity for Hsp90 binding *in vivo* in response to Mkk1/2-directed phosphorylation of the MAP kinase activation loop [3,14,18], Y2H interaction of these MAP kinases with the two isoforms of human Hsp90 was strengthened by heat shock (Fig. 5A). The stronger interaction of ERK5 with Hsp90β, relative to Hsp90α, was then confirmed through an analysis of extracts of PP30[hHsp90α]*slt2Δ* and PP30[hHsp90β]*slt2Δ* cells expressing a functional [18] ERK5(1–407)-His_{12} fusion. More Hsp90β, relative to Hsp90α, was associated with the nickel resin-retained ERK5(1–407)-

His_{12} (Fig. 5B). As this, and the Y2H interactions in Fig. 5A, essentially reflect the formation of a late-stage complex of the Hsp90 chaperone cycle [3,14,18], it is possible that MAP kinase complexes with Hsp90β in yeast progress more slowly through this chaperone cycle than do the equivalent complexes with Hsp90α (see Discussion).

Expression of Hsp90α or Hsp90β markedly affects cellular sensitivity to the Hsp90 inhibitor radicicol

We recently reported that strain PP30[hHsp90β] is extremely sensitive to Hsp90 inhibitors [35]. This, however, is not a general effect of human Hsp90 expression in yeast, as the cells expressing Hsp90α were not sensitized to the Hsp90-targeting antibiotic radicicol. Instead, strain PP30[hHsp90α] was relatively radicicol-resistant, displaying levels of sensitivity comparable to that of isogenic strains expressing either of the two isoforms of the native yeast Hsp90 (PP30[pHSC82b], PP30[pHSP82]); Fig. 6A,C,D. Remarkably, low radicicol levels (to 4 μM) were found to increase the final biomass yields of PP30[pHSP82], relative to the other strains tested (Fig. 6). In addition, at high temperature (37 °C as compared to 30 °C), the presence of the Hsp82 isoform of yeast Hsp90 in these cells rendered cells much less susceptible to radicicol inhibition as compared to comparable expression (Fig. 1A) of the 97% identical Hsc82 (compare Fig. 6B,C). In normal yeast (although not these engineered strains), Hsp82 is the strongly heat-inducible isoform of Hsp90, whereas Hsc82 is constitutively expressed [7]. As far as we are aware, the data in Fig. 6A–C represent the first evidence of a phenotypic difference generated by comparable expression (Fig. 1A) of the different isoforms of native Hsp90 in yeast.

With 30 °C 4 μM radicicol treatment of proliferating PP30[hHsp90β] cells, the cells continued to enlarge, but their growth totally lacked organization (rhodamine–phalloidin staining revealed almost instant loss of any actin organization following Hsp90 inhibitor treatment; data not shown). After 6 h, many of these cells displayed an apparent arrest of DNA and vacuolar segregation between the mother and daughter (Fig. 6D; middle image). By 24 h, over half had adopted the terminal phenotype of enlarged, misshapen cells, their elongated shape being consistent with a general failure of the acto-myosin contractile ring formation that normally leads to cytokinesis (Fig. 6D; left-hand cell cluster in right-hand image). With such 4 μM radicicol treatment, all of these phenotypes were displayed by PP30[hHsp90β], but not the more resistant PP30[hHsp90α] (Fig. 6D). At this

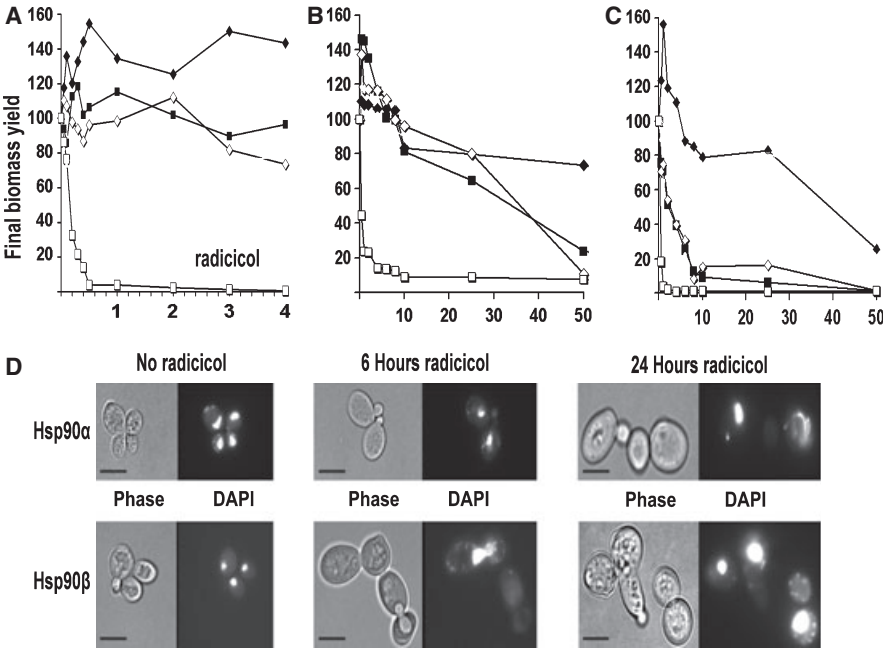

Fig. 6. (A–C) Only Hsp90β, not Hsp90α, sensitizes yeast to radicicol. Final biomass yields, expressed as a percentage of that of cells with no inhibitor, for cells expressing just a single isoform of either yeast Hsp90 (◆, Hsp82; ■, Hsc82) or human Hsp90 (◇, Hsp90α; □, Hsp90β), cultured for 42 h in the presence of (A) 0–4 μM radicicol, 30 °C, (B) 0–50 μM radicicol, 30 °C, or (C) 0–50 μM radicicol, 37 °C. (D) Morphologic differences between PP30[hHsp90α] and PP30[hHsp90β] cultured for 6 or 24 h at 30 °C in the presence of 4 μM radicicol.

radicicol concentration, the latter strain was not arrested in growth (Fig. 6A,B).

Discussion

In this work, we have investigated how the presence of Hsp90α or Hsp90β – as the sole Hsp90 in yeast cells – influences both the activation of certain clients in these cells and cellular sensitivity to the Hsp90 inhibitor radicicol. The most striking finding was that it is only expression of Hsp90β, not comparable expression of Hsp90α, which renders yeast highly sensitive to radicicol (Fig. 6). This raises the distinct possibility that, in mammalian systems as well, alterations to the Hsp90α/Hsp90β ratio (as with heat shock) may be a significant factor affecting sensitivity of cells to Hsp90 inhibitors. Up to now, the Hsp90α/Hsp90β isoform ratio has never been considered as a possible influence on Hsp90 drug resistance. Instead, the total level of the drug target (Hsp90) in cells, and the amount of this Hsp90 that becomes locked into complexes with client proteins [36] have generally been considered to be important factors in such resistance. Nevertheless, the true picture as regards the determinants of Hsp90 drug resistance is considerably more complicated than this, as studies of yeast mutants have revealed that altered resistance can arise with mutation to Hsp90, with altered cochaperone function and with the loss of plasma membrane drug efflux pumps [35].

The results of this study point to the two isoforms of human cytosolic Hsp90 differing in the relative efficiencies with which they activate certain Hsp90 clients, at least in yeast. Cells that express Hsp90β as their sole Hsp90 are moderately heat-sensitive, which may be due in part to lowered Hsf1p activity (Fig. 1C). Activations of GR and ERK5 were seemingly efficient with either Hsp90α or Hsp90β in the yeast (Figs 2 and 4). In contrast, activation of v-src was clearly compromised with Hsp90β rather than Hsp90α present in the cells (Fig. 3). Evidently, therefore, Hsp90α engages in a much more productive chaperone cycle leading to v-src activation in yeast, as compared to Hsp90β. Among src tyrosine kinases, v-src exhibits a much higher dependence on Hsp90 relative to c-src [1,25]. The former is just one of many mutant oncogenic proteins that tend to accumulate as Hsp90-containing multiprotein complexes in cancer cells; cells that are often found to be overexpressing Hsp90α at a high level [36]. Future studies should therefore address whether diverse oncogenic proteins – with activities that often exhibit a high dependence on Hsp90 function – are, in general, more efficiently activated by Hsp90α than by Hsp90β.

Hsp90 tends to transiently bind its client proteins, in a chaperone cycle thought to take place over a time scale of minutes [37,38]. In yeast, Hsp90β undergoes stronger Y2H interaction with MAP kinase clients than Hsp90α (Fig. 5). As detection of *in vivo* protein–protein interaction by the Y2H approach requires a fairly long association of 'bait' and 'prey' fusions in the nucleus of the living cell, these stronger MAP kinase Y2H interactions with Hsp90β as compared to Hsp90α (Fig. 5A) are consistent with a longer residence time of these clients in the form of multiprotein complexes *in vivo* when associated with Hsp90β as compared to Hsp90α – an indication that Hsp90β may progress rather more slowly through the chaperone cycle than Hsp90α. Y2H interactions with Hsp90 are generally only detected when the chaperone cycle is slowed [3].

In mammalian cells, the fraction of the cellular Hsp90 existing in the form of multiprotein complexes with client proteins appears to be a major determinant of Hsp90 drug sensitivity, the high sensitivity of certain cancer cells to these drugs apparently being associated with the large pool of mutant client proteins sequestering much of the Hsp90 into Hsp90–client complexes [36]. Thus, the high radicicol sensitivity of PP30[hHsp90β] relative to the other strains tested (Fig. 6) may, in part, be due to a higher Hsp90 fraction in this strain existing as multichaperone complexes with high affinity for client proteins, rather that as the latent uncomplexed chaperone.

The ATPase reaction of Hsp90 is thought to constitute the rate-limiting step of the Hsp90 chaperone cycle *in vivo*, ATP turnover rate therefore being an important determinant of the length of time for which a client remains Hsp90-bound [39–41]. The question therefore arises of whether more inefficient Hsp90β operation in yeast relates to the extremely low intrinsic ATPase of this Hsp90β [41]. Nevertheless, intrinsic ATPase activity measurements on purified vertebrate Hsp90s indicate that this activity is not appreciably different for Hsp90α as compared to Hsp90β [ATP turnover rates for recombinant chick Hsp90α and human Hsp90β are 0.025 and 0.015 min^{-1} (30 °C), respectively [42]; for recombinant human Hsp90α and 90% pure rat Hsp90β, they are 0.046 and 0.035 min^{-1} (37 °C), respectively (C. Söti, unpublished data)]. *In vivo*, however, a number of other factors may come into play to affect this activity. A still unexplored factor is whether Hsp90α differs significantly from Hsp90β in its regulation by cochaperones. For example, heat shock increases the levels of Aha1p, a cochaperone that activates the ATPase activity of Hsp90. Aha1p levels will therefore increase in cells under the same heat stress conditions that generate an increased Hsp90α/Hsp90β

ratio [43]. This, in turn, may affect the operation of the Hsp90 chaperone machine.

Experimental procedures

Yeast strains and yeast culture

Cultures were grown at 30 °C or 33 °C, either on complete dropout glucose medium (DO) [44] or on YPD medium [2% (w/v) glucose, 2% Bacto peptone, 1% yeast extract, 20 mg·L^{-1} adenine). Radicicol was purchased from Sigma (Poole, UK).

Derivatives of strain PP30 that express, as their sole Hsp90, the native Hsc82 or Hsp82 of *S. cerevisiae* (PP30[pHSC82b], PP30[pHSP82]), as well as human Hsp90β (PP30[hHsp90β]), have been described previously [35]. A plasmid (pH90α) for human Hsp90α expression in *S. cerevisiae* was constructed by PCR amplification of the Hsp90α ORF using the forward primer AAATAAGTCG ACATGCCTGAGGAAACCCAG (*Sal*I site underlined; Hsp90α start codon in bold) and the reverse primer CTTC ATCTGCAGTTAGTCTACTTCTTCCAT (*Pst*I site underlined; stop codon position in bold). This PCR product was cleaved with *Sal*I and *Pst*I, and then inserted into *Sal* I–*Pst*I-cleaved pHSCprom (an expression vector that comprises the *LEU2* vector YCplac111 with *S. cerevisiae HSC82* promoter and *ADHI* terminator inserts [45]), thereby creating pH90α. Fusion of the *HSC82* promoter to the human Hsp90α sequence was confirmed by sequence analysis. Transformation of pH90α into *S. cerevisiae* PP30[pHSC82] (MAT**a** *trp1-289*, *leu2-3,112*, *his3-200*, *ura3-52*, *ade2-101*^{oc}, *lys2-801*^{am}, *hsc82::kanMX4*, *hsp82::kanMX4* [pHSC82]), and then curing of the pHSC82 *URA3* vector by restreaking onto plates containing 5-fluoroorotic acid (Melford Laboratories, Ipswich, UK), were as done as previously described [46], leading to a strain (PP30[hHsp90α]) that expresses human Hsp90α as its sole Hsp90.

Determination of client activations

Measurements of HSE2-*LacZ* expression, GR expression and v-src expression were all done as previously described [17,25,43,45]. Viability of v-src-expressing yeast strains was determined on SGC-URA plates in dot spot experiments. Plates were incubated for 3 days at 29 °C.

To express human ERK5 MAP kinase in place of the native Slt2p cell integrity MAP kinase in cells with either Hsp90α or Hsp90β, *slt2Δ* mutant versions of PP30[hHsp90α] and PP30[hHsp90β] were generated. First, strain PP30*slt2Δ* was constructed by *hphMX4* cassette [47] deletion of the *SLT2* gene in PP30[pHSC82]. The *LEU2* vectors pH90α (this study) and pH90β [35] were then inserted into this PP30*slt2Δ*, and this was followed by 5-fluoroorotic acid curing of the pHSC82 *URA3* vector, as previously described [46]. The resultant strains (PP30[hHsp90α]*slt2Δ*; PP30[hHsp90β]*slt2Δ*) were then transformed with the *TRP1* plasmids pG1 and pG1-ERK5 (control empty vector and vector for *TDH1* promoter-driven ERK5 expression, respectively [18]) or pHis-ERK5(1–407) (a vector for *MET25* promoter-regulated expression of a functional truncated ERK5 with a C-terminal 12xHis tag) [18].

Western blot analysis

Total protein extracts were prepared and western blots prepared as described previously [46]. Antisera used at 1 : 2500 dilution were mouse monoclonal antibodies to *Achlya ambisexualis* Hsp90 (Stressgen, Victoria, Canada) or tetra-His (Qiagen, Crawley, UK).

Two-hybrid studies

Two-hybrid baits that consist of human Hsp90α and Hsp90β fusions with a C-terminal BD extension (Hsp90α-BD; Hsp90β-BD) were generated by homologous recombination within yeast, essentially as previously described [34,48]. ORFs of these human Hsp90s were initially amplified by two sequential PCR amplifications. The first PCR used primers that possess 3′ sequence homologies to these Hsp90s but 5′ homologies to plasmid pBDC [34] (Hsp90α, forward primer GCTTGAAGCAAGCCTCGATGCCT GAGGAAACCCAGACCCAA, reverse primer CAGT AGCTTCATCTTTTCGGTCTACTTCTTCCATGCGTGA; Hsp90β, forward primer GCTTGAAGCAAGCCTCGAT GCCTGAGGAAGTGCACCATGGA, reverse primer CA GTAGCTTCATCTTTCGATCGACTTCTTCCATGCGA GA). The second PCR used a universal pair of primers [34,48]. PJ69-4α [48] was then transformed with the product of this second PCR and *Nru*I-digested pBDC, so as to generate, through homologous recombination within PJ694α yeast, genes for Hsp90α-BD or Hsp90β-BD fusions. PJ694a cells expressing the AD-Slt2p and AD-ERK5 fusions (described previously [3,18]) were then mated to PJ694α expressing Hsc82-BD, Hsp82-BD [34], Hsp90α-BD, or Hsp90β-BD. The resultant PJ69-4 diploids (now expressing both AD- and BD-fusions) were selected on DO lacking histidine and tryptophan. Automated measurement of the β-galactosidase activity due to basal and stress-induced expression of the interaction-responsive, *GAL7* promoter-regulated *LacZ* gene of PJ69-4 was as previously described [3,18,49]. The data shown (mean and SD of eight individual assays) are expressed relative to the control diploid PJ69-4 cells containing pBDC lacking a gene insert and the plasmid for AD-fusion expression [as the low basal *LacZ* expression levels in this system are generally due to the AD-protein fusion, the even lower *LacZ* expression level in cells containing an Hsp82-BD 'bait' and empty AD vector (pOAD) are essentially unaffected by stress [49]].

Drug sensitivity assays

Cells were inoculated into liquid DO containing the indicated level of inhibitor, to a final density of 6×10^5 cells·mL^{-1}. Final cell density was monitored after growth at either 30 °C or 37 °C, as indicated in the legend to Fig. 6.

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Zinc supplementation boosts the stress response in the elderly: Hsp70 status is linked to zinc availability in peripheral lymphocytes

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Abstract

Chaperones and zinc are indispensable for proper immune function. All the zinc status, the immune function and the stress response decline during aging. Here we studied the effect of nutritional zinc and zinc homeostasis on the stress response in healthy old subjects recruited during the ZincAge European Union project that either underwent or not a 48-day zinc supplementation. Inducible Hsp70 levels were determined at basal conditions as well as after heat shock in the CD3+ and CD3− subset of lymphocytes by a two-color FACS analysis. Short term zinc supplementation resulted in a marked increase in both basal as well as stress-induced Hsp70 levels in lymphocytes from healthy elderly donors with a higher impact on CD3+ cells. Heat inducibility showed a strong correlation with basal Hsp70 level, and both basal as well as stress-induced Hsp70 highly correlated with intracellular zinc availability. In conclusion, short term oral supplementation with zinc safely and efficiently induces the stress response in lymphocytes of old donors. The stress response may be a candidate pathway connecting zinc deficiency with aging and immunosenescence. Thus, proper dietary zinc intake may emerge as a chaperone inducer and an anti-aging mechanism in the immune system.

Keywords: Stress response; Hsp70; Zinc; Zinc supplementation; Lymphocytes

1. Introduction

Zinc is one of the most important trace elements in the body (Stefanidou et al., 2006). It has a catalytic/regulatory role in many enzymes, maintains the structural integrity of various proteins (such as superoxide dismutase and zinc-finger transcription factors) and modulates protein–protein interactions. At the cellular level zinc is essential for cell proliferation and survival, contributes to genomic stability and antioxidant defense, which highlights its crucial role in aging and age-dependent degenerative diseases. Zinc is indispensable for proper immune function. Zinc deficiency increases susceptibility to infections, compromises both innate immunity and the T-cell compartment (Shankar and Prasad, 1998; Rink and Haase, 2007). It is well documented that zinc status and immune function declines with aging, and zinc supplementation is beneficial to immune responses in the elderly (Haase et al., 2006b; Larbi et al., 2006).

Molecular chaperones are conserved and abundant proteins that guard the conformational homeostasis of proteins (Hartl, 1996). They maintain signaling, regulate proliferation, differentiation and apoptotic pathways (Sőti et al., 2005b; Sreedhar and Csermely, 2004). Chaperones (or stress proteins) confer cytoprotection and assure survival upon various stresses. The stress response is regulated by the heat shock transcription factor 1 (HSF-1), named after the archetype of proteotoxic stress. HSF-1 induces

Abbreviations: HSF-1, heat shock transcription factor 1; Hsp70, 70 kDa heat shock or stress protein; MFI, mean fluorescence intensity; PBMC, peripheral blood mononuclear cells.

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the transcription of various stress proteins. Inducibility of the major chaperone, the 70 kDa heat shock protein (Hsp70) reflects the robustness of the stress response, which is indispensable for adaptation (Voellmy, 2004). Both a single heat shock as well as transgenic Hsp70 induces longevity (Tatar et al., 1997), HSF-1 overexpression induces a twofold life-span extension, while HSF-1 knock-out markedly shortens life-span (Hsu et al., 2003; Garigan et al, 2002), demonstrating the central importance of a robust stress response in aging in invertebrate models. HSF-1 knock-out mice neither display heat shock response, nor show premature aging in 'sterile' laboratory conditions. However, they are hypersensitive to endotoxemia, which reinforces the role of the stress response in immune function and inflammation (Xiao et al., 1999). Moreover, both chaperone inducibility and chaperone function decrease during aging and in chronic inflammation characteristic to the elderly (Nardai et al., 2002; Sőti and Csermely, 2003; Arslan et al., 2006), therefore preserving their function is an attractive target in anti-aging therapies (Sőti et al., 2005a; Sőti and Csermely, 2005).

Zinc and chaperones are connected in many ways (Larbi et al., 2006; Arslan et al., 2006; Sőti and Csermely, 2005). Zinc is a potent inducer of Hsp70 in cell culture (Hatayama et al., 1993) including lymphoblasts form old, but not from young donors (Ambra et al., 2004). Both zinc deficiency and overdose inhibits the stress response in rodent thymus (Moore et al., 2003). Thus, proper dietary zinc is critical for mounting a robust stress response. The ZincAge project funded by the 6th Framework Program of the European Union aimed to study the role of nutritional zinc in healthy aging and in immunosenescence. We were interested to study the relationship of zinc homeostasis and aging with the stress response, an essential adaptive and survival mechanism in the immune system. Here we report on the effect of zinc status on the stress response of lymphocytes obtained form healthy elderly donors.

2. Materials and methods

2.1. Subjects and zinc supplementation

Twenty healthy old subjects (between 64 and 85 years of age) were enrolled according to the inclusion criteria required by the ZincAge European specific targeted research project (www.zincage.org) approved by the respective National Ethical Committees. Briefly, all subjects were originated from Italy, lived an independent life, were in a good general health conditions devoid of functional impairment and serious acute or chronic disease, without taking medical drugs or nutritional supplements. All subjects gave informed consent and underwent medical examination and clinical laboratory tests. Seven persons from the twenty healthy old subjects underwent an oral zinc supplementation which was performed with 10 mg pure zinc/day (in the form of 50 mg zinc-aspartate Unizink 50, KÖHLER PHARMA Corp., Alsbach-Hähnlein, Germany) for 48 ± 2 days. All subjects admitted to zinc supplementation had values of plasma zinc below 11 μM at least at one test out of two made within one year.

2.2. Blood withdrawal and PBMC preparation

Heparinized whole blood samples after an overnight fast were withdrawn from each subject before and after zinc supplementation. The whole blood was centrifuged at 450*g* for 10 min at 4 °C to separate the plasma. Peripheral blood mononuclear cells (PBMC) were prepared by Ficoll-Hypaque ($d = 1.077$ g/ml) gradient centrifugation (450*g* for 30 min at 20 °C), washed twice in PBS, and aliquots were made in fetal bovine serum containing 10% DMSO and were frozen in liquid nitrogen until further use.

2.3. Plasma zinc and intracellular zinc ion availability determination

Plasma zinc levels were analyzed by induction plasma coupled mass spectrometry (ICP-MS). All plasma samples and standard were diluted 1:10 with a solution containing the following reagents: 0.1% Triton X-100, to maintain a stable emulsion with the diluted sample and 0.15% HNO_3, to ensure that trace elements are maintained in solution and to aid washout of these elements between samples. External calibration solutions containing zinc (blank to 2000 ppb), were prepared by serial dilution of parent 1000 ppm stock (VHG Labs, Manchester, NH, USA), using the same solution used to dilute the samples. Measurement of plasma zinc was performed with a Thermo XII Series ICP-MS (Thermo Electron Corporation, Waltham, MA, USA), using an external calibration curve containing zinc (blank to 2000 ppb). The instrument was operated with a Peltier-cooled impact bead spray chamber, single piece quartz torch (1.5 mm i.d. injector) together with Xi interface cones and a Cetac-ASX 100 autosampler (CETAC Technologies, Omaha, Nebraska, USA). A Burgener Trace nebuliser was used as this device does not block during aspiration of clinical samples. The instrument was operated in standard mode (non-CCT), using 1400 W RF power, 1.10 L/min nebuliser gas flow, 0.70 L/min auxiliary gas flow, 13.0 L/min cool gas flow, 70 ms dwell time, 30 s sample uptake and 35 s wash time (two repeats per sample). Zinc ion availability of PBMC was measured with zinpyr-1 (ZP1) (Neurobiotex, Galveston, TX, USA) by flow cytometry, as described (Malavolta et al., 2006)). Briefly, PBMCs (1×10^6) were stained with 20 μM Zinpyr-1 for 30 min at 37° C and 5% CO_2 in HEPES-buffered "zinc-free" RPMI Medium supplemented with 1 mM EDTA. Following incubation, cells were analyzed with a flow cytometer (Coulter Epics XL, Coulter, Hialeah, FL, USA). The population of lymphocytes was selected using FSC and SSC and the ZP1 derived fluorescence was detected at 525 nm using 488 nm excitation wavelength. Data for zinc ion availability are reported as the mean fluorescence intensity normalized to the mini-

mum fluorescence obtained after the addition of 50 μM *N,N,N′,N′*-tetrakis (2-pyridylmethyl)ethylenediamine (TPEN) (Sigma–Aldrich, MI, Italy).

2.4. Cell culture and heat shock treatment

PBMC were gently thawed in RPMI 1640 medium, supplemented with 10 mM Hepes, 10% fetal bovine serum, 2 mM L-glutamine, 100 μg/ml streptomycin and 100 IU/ml penicillin (all from Gibco–Invitrogen, Carlsbad, CA, USA) and incubated for 16 h at 37 °C in a 5% CO_2 incubator at isobaric oxygen. Each PBMC sample was divided into control and heat shock aliquot. Heat shock was performed at 43 °C for 1 h in an Eppendorf thermomixer at a cell density of 10^6/ml. The induction of the heat shock response was allowed to develop for 4 h at 37 °C in a 5% CO_2 incubator. Control PBMCs of the same person were treated identically except for exposing the cells to heat shock. Then cells were harvested, washed with PBS supplemented with 2% fetal bovine serum, counted and the viability was assessed using a CASY TT cell counter (Scharfe Systems, Bielefeld, Germany). Viability was over 90%.

2.5. Intracellular staining and flow cytometry

Cells were fixed and permeabilized in 250 μl Cytofix/Cytoperm solution (BD Biosciences Pharmingen, San Diego, CA, USA) at 4 °C for 20 min. Cells were then washed two times with Perm/Wash solution (BD Biosciences Pharmingen, San Diego, CA, USA) and incubated with flourochrome-conjugated mouse monoclonal antibodies [anti-CD3 allophycocyanin (APC) conjugated antibody (345767, BD Biosciences, San Diego, CA, USA) and anti-Hsp70 fluorescein isothiocyanate (FITC) conjugated antibody (SPA-810FI, Stressgen, Alberta, Canada)] at 4 °C for 30 min. (CD3 immunostaining either in intact non-permeabilized or in permeabilized cells gave identical results.) For compensation cells were stained either with anti-CD3-APC conjugated antibody or with anti-CD3-FITC conjugated antibody (345763, BD Biosciences, San Diego, CA, USA) as described above. After washing once with Perm/Wash solution and once with PBS with 2% FBS cells were fixed with 2% paraformaldehyde in PBS and analyzed with a FACSCalibur flow cytometer using the CellQuest software (BD Biosciences, San Diego, CA, USA). Approximately 5000 live lymphocytes from each sample were electronically gated according to granularity and size in the forward versus side scatter (Fig. 1A). Differentiation between CD3+ and CD3− subpopulations was achieved in the CD3-APC vs. forward scattergram (Fig. 1B). Hsp70-related fluorescence was obtained in a 530/30 (FITC) filter, and the relative Hsp70 protein level was expressed as the mean fluorescence intensity (MFI) of FITC from logarithmic histograms. As a standard, two parallel vials of lymphocytes isolated from the buffy coat of a healthy subject (purchased from the Hungarian National Blood Transfusion Service, approved by the National Ethical Committee, and in accordance with the Helsinki regulations) were used in each experiment including heat shock treatment, staining and FACS analysis.

2.6. Statistical analysis

Data were statistically analyzed using Statistical Package for the Social Sciences software version 15.0 for Windows (SPSS Inc., Chicago, IL, USA). Variables are expressed as mean ± standard error of the mean (SEM). Means were either compared by using the Student's paired samples or independent samples *t*-test. Correlation of continuous variables was analyzed by the Spearman algorithm. A p value (two-tailed) <0.05 was considered statistically significant.

3. Results

3.1. Characteristics of the stress response in lymphocyte populations

In order to gain insight into the stress response in specific subpopulations of lymphocytes, we set up a two-channel FACS analysis, using CD3 (Fig. 1A and B), a T-lymphocyte marker, and Hsp70, the major cytosolic stress protein. Initial experiments were performed on pooled PBMCs from healthy donors. While basal Hsp70 level reflects steady-state chaperone capacity, stress-induced Hsp70 level determines the robustness of adaptation to a noxious insult. Heat shock, a model of fever was selected not only as the archetype of stress but also as an important physiopathological determinant of lymphocyte function. The optimal conditions of induction were found to be 43 °C for 1 h and 4 h of recovery time to allow Hsp70 synthesis (data not shown) and used in subsequent comparative studies.

Looking at the Hsp70 distributions, a high and a low Hsp70-expressing population could be observed, and the high population could respond more dynamically to heat shock (Fig. 1C all, 37 *vs.* 43 °C). Further examining the relationship between baseline and heat-induced Hsp70 levels, we investigated the stress response in PBMC obtained from old donors. We found a significant correlation between these variables in the CD3+ lymphocyte populations, while no such strong association existed in CD3− lymphocytes (Fig. 2). Moreover, the strong correlation persisted with respect to inducibility, indicating that it is indeed a relationship between the basal level and the mounting of the stress response. We did not observe any gender-specific effect.

3.2. Zinc supplementation boosts the stress response

Next, we tested the effect of clinical zinc supplementation on the stress response. Seven healthy elderly subjects received 10 mg zinc/day for 48 ± 2 days, and blood was withdrawn before and after the supplementation. Zinc sup-

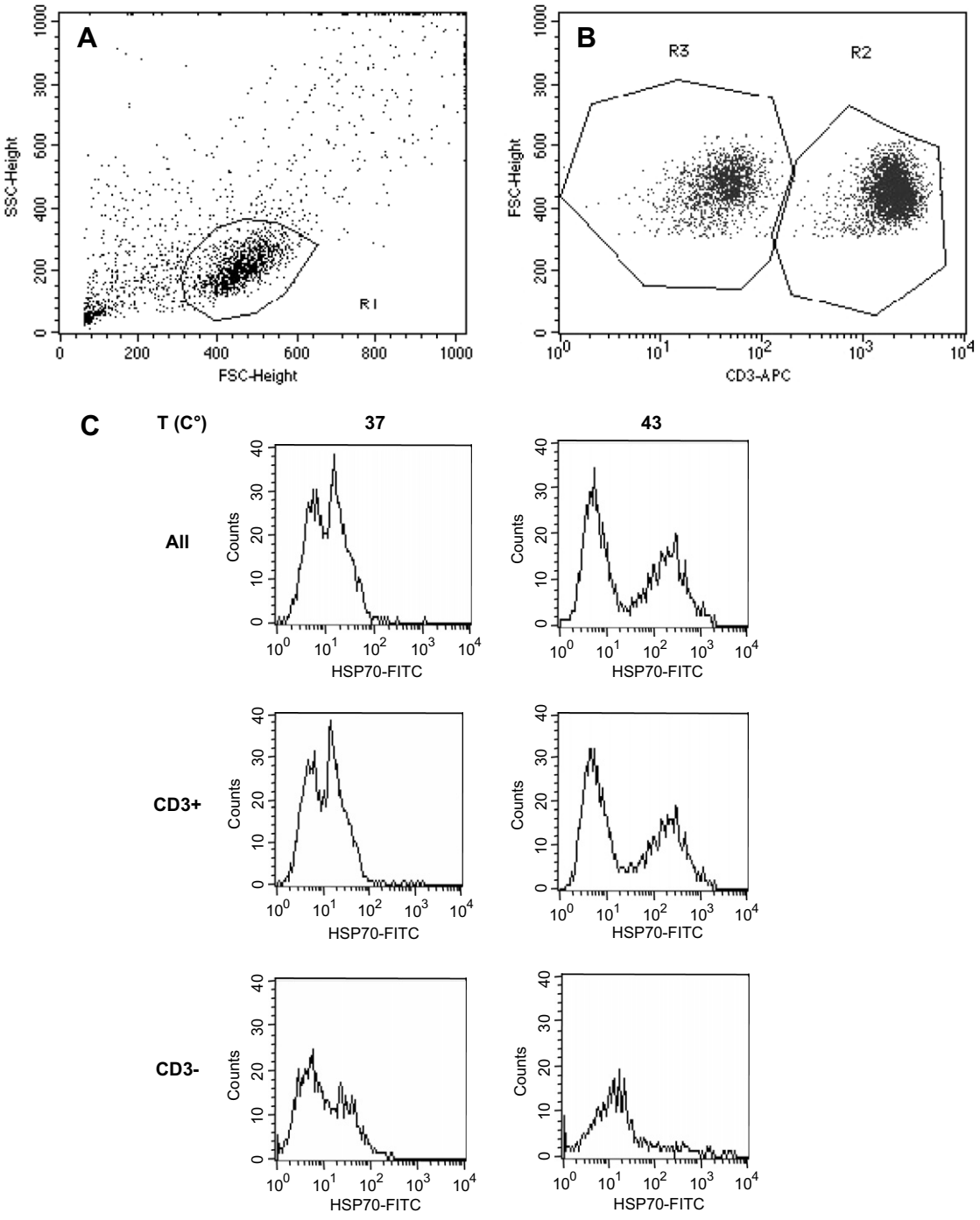

Fig. 1. Differential stress response in different lymphocyte populations. (A) Scatterplot distribution of lymphocytes in the forward vs. side scattergram. (B) Differentiation of CD3− (R3) and CD3+ (R2) lymphocyte populations using an anti-CD3-APC fluorescence. (C) Histograms of basal (37 °C) and heat-induced (43 °C) Hsp70 levels in total, CD3+ and CD3− populations by depicting the anti-Hsp70-FITC fluorescence. The figure is a representative of three independent experiments.

plementation did not significantly increase plasma zinc concentration, however, it promoted a significant improvement of zinc availability, an intracellular measure of free zinc ion available for cellular reactions, therefore a better marker of zinc homeostasis (Table 1). Fig. 3 shows the individual characteristics of the stress response in the total lymphocyte population before and after zinc supplementation. All the basal (panel A) and heat-induced Hsp70 levels (B) as well as the inducibility (C) were boosted by oral zinc supplementation, as can be seen from the upward trend of the individual subjects. However, heat induction of Hsp70 showed a more uniform and stronger increase.

Statistical analysis showed a significant increase of the heat-induced Hsp70 as well as the stress-inducibility upon zinc supplementation, while the basal Hsp70 level displayed a tendency to increase, probably due to the limited

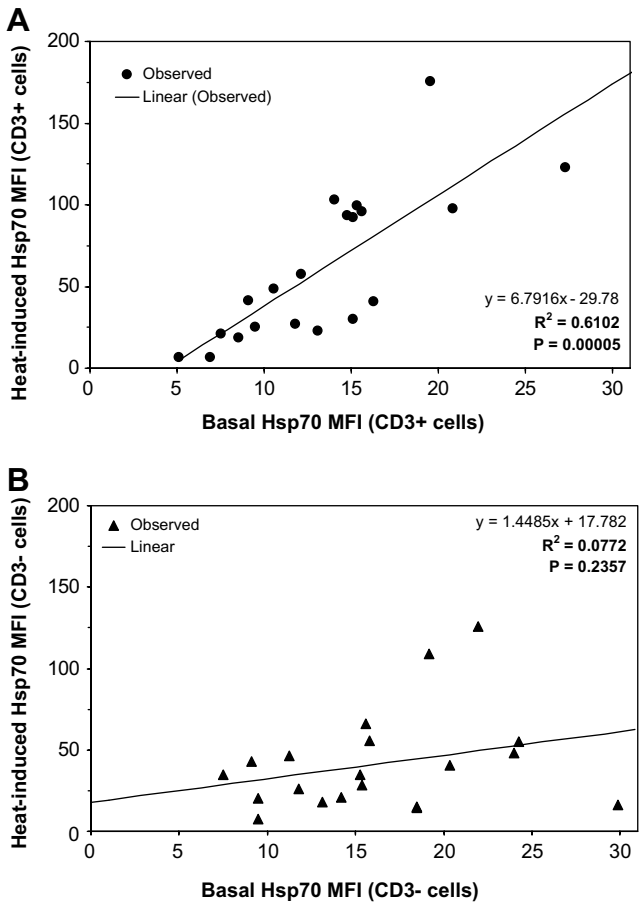

Fig. 2. Relationship between basal and heat-induced Hsp70 levels in CD3+ and CD3− lymphocyte populations from old donors. PBMC were obtained from healthy elderly patients ($n = 20$). Hsp70 levels were expressed as the mean fluorescence intensity (MFI). Observed heat-induced (43 °C) vs. basal (37 °C) Hsp70 MFIs were plotted from CD3+ (A) and CD3− (B) lymphocyte populations of the same individual. Bivariate linear correlations were calculated using the Pearson's algorithm and the parameters are indicated on the charts.

Table 1
Age of the subjects and zinc status before and after zinc supplementation

	Age (years)	Plasma Zn (μM)	Zn availability
Before Zn suppl.	74.7 ± 2.0	12.1 ± 0.7	1.26 ± 0.03
After Zn suppl.	—	13.5 ± 1.5	1.39 ± 0.02
P	—	0.367	0.013

Data ($n = 7$) were obtained as described in Section 2 and shown as mean ± SEM. p values were calculated using the Student's paired samples t-test.

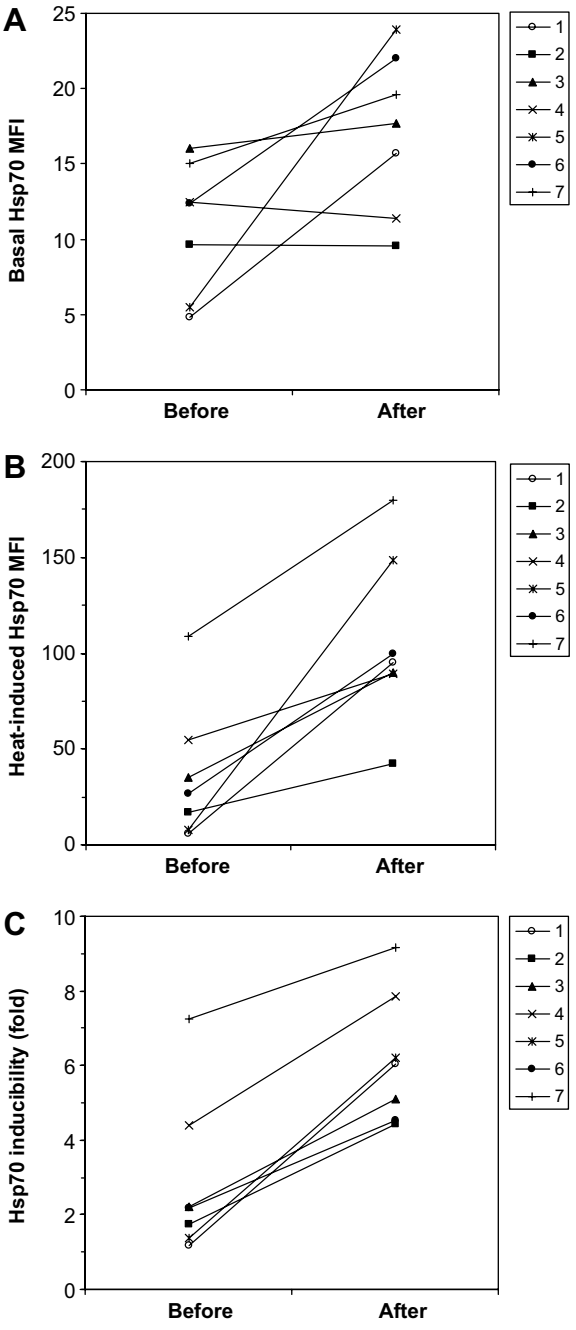

Fig. 3. Hsp70 status of lymphocytes from individual healthy old subjects before and after clinical zinc supplementation. PBMC were obtained from the same patients ($n = 7$) before and after a 48-day oral zinc supplementation (10 mg pure zinc/day) and the experiments were performed as described in Section 2. Individual basal (37 °C (A)) and heat-induced (43 °C (B)) Hsp70 levels are indicated as the mean fluorescence intensity (MFI), Hsp70 inducibility (C) is given as the 43 °C/37 °C ratio.

number of subjects (Fig. 4). Zinc affected the CD3− population to a smaller extent. It should be also noted that CD3− lymphocytes displayed a higher basal level but a less intense heat induction of Hsp70 than CD3+ cells (Fig. 4). Furthermore, the lymphocyte fraction that highly express Hsp70 (gated at Hsp 70 MFI > 18) showed a sharp increase both under basal as well as heat-induced conditions upon zinc supplementation (basal: 9.6 ± 2.9% before zinc vs. 22.1 ± 6.3% after zinc, $p = 0.026$; heat-induced: 22.0 ± 16.7% before zinc vs. 57.8 ± 5.5% after zinc, $p = 0.004$, respectively).

To further investigate the effect of orally taken zinc on the stress response, we compared the Hsp70 status of subjects (Age: 76.6 ± 1.7, $n = 10$) after zinc supplementation with that of subjects (Age: 72.2 ± 1.8, $n = 10$. $p = 0.095$ for age) not having taken zinc tablets. Fig. 5 demonstrates

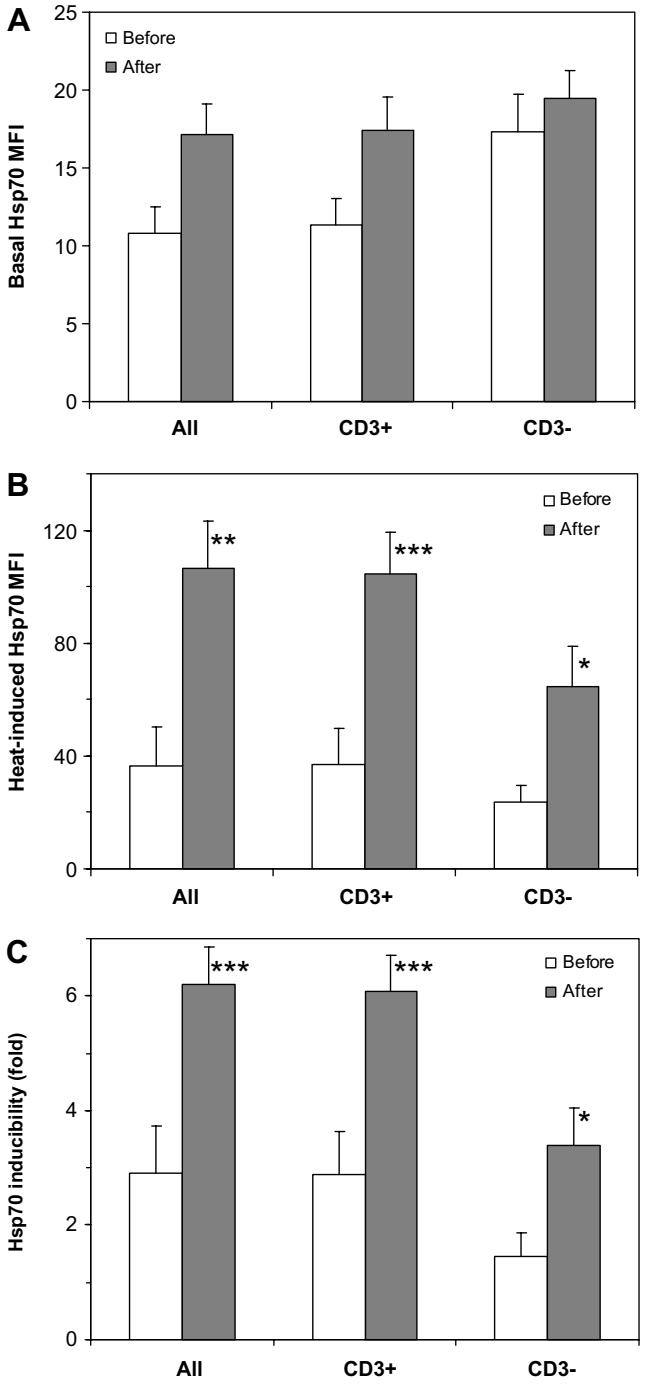

Fig. 4. Hsp70 status of different lymphocyte populations from healthy old subjects before and after clinical zinc supplementation. PBMC were obtained from the same patients ($n = 7$) before and after a 48-day oral zinc supplementation (10 mg pure zinc/day) and the experiments were performed as described in Section 2. Basal (37 °C, (A)) and heat-induced (43 °C, (B)) Hsp70 levels were expressed as the mean fluorescence intensity (MFI), Hsp70 inducibility (C) is given as the 43 °C/37 °C ratio. Bars represent the mean + SEM of the total (All), the CD3+ and the CD3− lymphocyte populations, respectively. p values were calculated using the Student's paired samples t-test and levels of significance are as follows: * denotes $p < 0.05$, ** denotes $p < 0.005$, *** denotes $p < 0.001$.

that zinc-supplemented subjects had higher Hsp70 levels and inducibility in all lymphocyte populations.

3.3. Robustness of the stress response correlates with intracellular zinc availability

After having seen the effect of clinical zinc supplementation, we were interested to analyze the relationship between zinc status and Hsp70 levels/inducibility. We clustered the subjects according to either plasma zinc or intracellular zinc availability in low or high groups. Subjects with high zinc availability displayed a higher steady state Hsp70 level and a more robust stress response. Plasma zinc levels did not significantly differ between groups (Table 2). Using the same subjects, further calculation of bivariate linear correlations of basal Hsp70 vs. zinc availability and heat-induced Hsp70 vs. zinc availability showed that both the basal as well as the heat-induced Hsp70 level correlated significantly with intracellular zinc availability in a concentration-dependent manner (basal Hsp70 vs. intracellular zinc availability: $R^2 = 0.3260$, $p = 0.005$; heat-induced Hsp70 vs. intracellular zinc ion availability: $R^2 = 0.4137$, $p = 0.002$). In contrast, plasma zinc level seemed to have no significant correlation with heat-induced Hsp70 levels ($R^2 = 0.0048$, $p = 0.772$). We observed no gender-specific effect.

4. Discussion

Loss of adaptation to stress is a hallmark of aging. A major molecular mechanism behind is a decrease in heat shock response, a fundamental cytoprotective and survival mechanism in several models (Sőti and Csermely, 2003; Arslan et al., 2006), including human blood cells and extracellular chaperones of the elderly (Njemini et al., 2002; Rea et al., 2001; Singh et al., 2006). Since molecular chaperones are intimately involved in signaling and proliferation, a less robust stress response may not only cause limited survival, but may also contribute to the compromised reactivity of old lymphocytes to various inflammatory stimuli, a phenomenon called immune cell anergy (Pawelec, 2006). Besides, looking at two lymphocyte subpopulations it turned out that CD3− cells had a significantly higher basal and a lower heat-induced Hsp70 level then their CD3+ counterparts. Whether the CD3− B-cells (and NK-cells) have almost no stress tolerance (in terms of heat shock response) only in elderly subjects and if it relates to immune function is an intriguing open question.

Stress tolerance, the adaptation to an environmental noxa depends on the robustness of the stress response, i.e. the inducibility of stress proteins by the heat shock transcription factor (Voellmy, 2004). Preconditioning (exposure to a mild stress, like hormesis [Verbeke et al., 2001; Rattan, 2004]) results in elevations in Hsp70 and other stress proteins, a more robust stress response and a better adaptation with implications in longevity. However, large elevation in Hsp70 is only transient and the remaining protein level is insufficient to meet the increased demand during stress. To our knowledge, no molecular mechanism has been associated with a better mounting of the stress

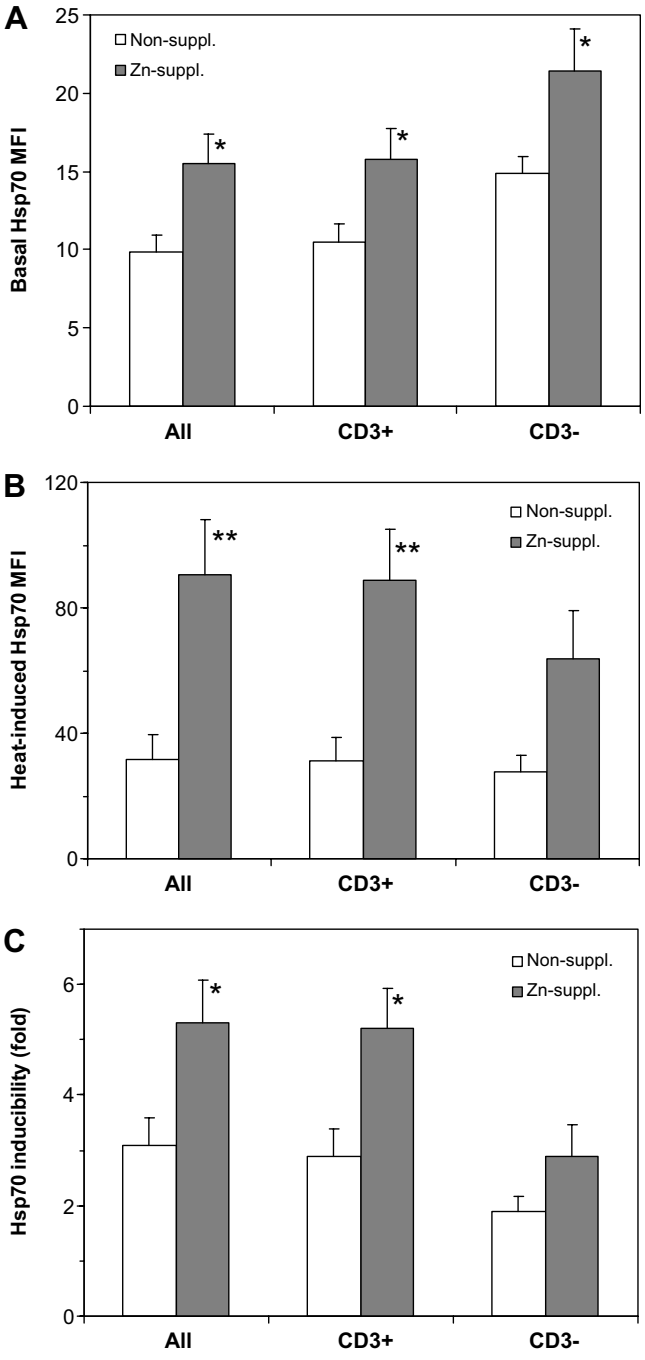

Fig. 5. Hsp70 status of different lymphocyte populations from healthy old subjects undergoing or not clinical zinc supplementation. PBMCs were obtained from patients not supplemented (Non-suppl., $n = 10$) or supplemented (Zn-suppl., $n = 10$) with zinc (10 mg pure zinc/day for 48 ± 2 days) and the experiments were performed as described in Section 2 . Basal (37 °C, (A)) and heat-induced (43 °C, (B)) Hsp70 levels were expressed as the mean fluorescence intensity (MFI), Hsp70 inducibility (C) is given as the 43 °C/37 °C ratio. Bars represent the mean + SEM of the total (All), the CD3+ and the CD3− lymphocyte populations, respectively. p values were calculated using the Student's independent samples t-test and levels of significance are as follows: * denotes $p < 0.05$, ** denotes $p < 0.01$.

response after a previous/chronic mild stress. We observed that basal Hsp70 level in the CD3+ lymphocytes strongly correlates ($R^2 = 0.6102$) with its own inducibility (Fig. 2A). Our results imply that Hsp70 may indeed operate a positive feedback loop chaperoning its own synthesis upon a subsequent stress, providing a framework for stress tolerance. Furthermore, one can predict that cells possessing higher basal Hsp70 levels might have been primed by hormetic means and have better chances to adapt to stress. On the contrary, continuously very high level, such as strong overexpression of Hsp70 would compromise cell physiology (Seo et al., 1996), but this threshold either may not be reached in physiological setting or in the old cells analysed in the current study. Further studies are needed to explore the molecular details and its applicability as a biomarker of stress tolerance.

Zinc is indispensable for proper immune response. Zinc deficiency is detrimental, while zinc supplementation is beneficial to T-lymphocyte function (Shankar and Prasad, 1998; Rink and Haase, 2007). Both zinc status and immune function declines with aging (Haase et al., 2006b; Larbi et al., 2006). Here we obtained evidence that nutritional zinc intake modulates the stress response. Short term zinc supplementation markedly improved all aspects of lymphocyte Hsp70 homeostasis involving also CD3− cells, in spite of their less reactivity to heat shock (Fig. 4). This effect was highly significant even in a relatively small study population. The observations were extended and confirmed towards subjects that were or were not supplemented with zinc (Fig. 5). Intriguingly, plasma zinc was mostly in the normal range for the subjects studied (Tables 1 and 2) and there was no significant difference of Hsp70 response between subjects having lower and higher than 11 μM plasma zinc concentration, respectively (data not shown). In contrast, intracellular zinc availability highly correlated with basal and heat-induced Hsp70 levels, strongly suggesting that low zinc availability exerts a negative effect on the stress response, and even without subclinical zinc deficiency (plasma Zn < 11 μM) zinc supplemented subjects show a more robust Hsp70 induction. Notably, plasma zinc concentration did not seem to affect Hsp70 inducibility. This may be in part attributed to the limited number of subjects and in part to the fact that plasma is often discussed not to be reliable to monitor zinc status in humans, because zinc occurs in the body primarily intracellularly and there is only a small portion of zinc in the plasma mainly bound to plasma proteins. Furthermore, plasma zinc is highly dynamic and is profoundly affected by several factors such as diurnal rhythm, stress, infection, starvation and plasma protein levels (Wood, 2000). Alternatively, zinc status is measured by erythrocyte membrane zinc (Bettger and Taylor, 1986) or metallothioneins (Sullivan et al., 1998), proteins which are responsible for the regulation of intracellular free zinc ions. Similarly to our results, Thomas et al. (1992) showed that metallothionein concentrations in erythrocytes elevated significantly while no changes in plasma zinc could be observed after zinc supplementation. Recent *in vitro* assessments (Malavolta et al., 2006; Haase et al., 2006a) and preliminary "*in vivo*" findings (Mocchegiani, 2007; Cipriano et al., 2006) suggest that intracellular

Table 2
Relationship between the zinc status and the stress response

Group (*n*)	Plasma Zn (μM)	Zn availability	Hsp70 status		
			MFI at 37 °C	MFI at 43 °C	Ind. (fold)
Low Zn (10)	11.8 ± 0.5	1.21 ± 0.02	9.4 ± 1.1	26.5 ± 4.8	2.7 ± 0.4
High Zn (10)	13.1 ± 1.1	1.39 ± 0.02	16.7 ± 1.5	97.4 ± 14.3	5.9 ± 0.7
P	0.068	<0.0001	0.001	0.001	0.001

Subjects were grouped according to their zinc availability (high Zn > 1.30 ⩾ low Zn). Data were obtained as described in Section 2 and shown as mean ± SEM. Basal (37 °C) and heat-induced Hsp70 (43 °C) levels are indicated as the mean fluorescence intensity (MFI), Hsp70 inducibility (Ind.) is given as the 43 °C/37 °C ratio. *p* values were calculated using the Student's independent samples *t*-test.

zinc availability (or labile zinc) of leukocytes may be a parameter that allows a good determination of zinc status. The intracellular zinc that is available for zinc-dependent enzymes is often called as the labile pool of zinc which is modulated by the release of zinc from intracellular vesicular stores or from metallothioneins what is essential to regulate many cellular processes such as apoptosis, stress response or cell cycle (Jacob et al., 1998; Malavolta et al., 2006). Moreover, since stress signals mobilize zinc by oxidation of protein sulfur ligands (Maret, 2006), the assessment of labile zinc represents an optimal target for establishing a link between stress response and zinc status. In addition to these, this method allowed also the determination of zinc in the cells analyzed while plasma zinc is not a cell specific indicator of zinc status. Our findings corroborates *in vitro* experiments that reported a stress response inducing property of zinc in various cell cultures and peripheral cells of different origin (Hatayama et al., 1993; Ambra et al., 2004; Bauman et al., 1993; Odashima et al., 2002; Qing et al., 2004 and reviewed in Arslan et al., 2006). Similarly, zinc in our study also increased the basal steady-state Hsp70 level in human lymphocytes, suggesting that zinc per se exerts a mild stress on a variety of cell types. This property of zinc might resembles an hormetic action (Rattan, 2004). In fact, the zinc-induced increase in Hsp70 elicited a more intense stress response (2- to 4-fold increase of heat-induced Hsp70) with no apparent damage. Moreover, PBMC from zinc-supplemented people had much better morphology (data not shown), arguing for a generally improved ability to cope with stress. Since blood samples were obtained and frozen under controlled conditions at different times by the same personnel, the effect observed was not due to differential sample preparation.

Chaperones and zinc may co-operate in other ways, as well. Besides, zinc is also an important regulatory metal in protein function and signaling. Both zinc and chaperones promote lymphocyte proliferation (Csermely et al., 1988; Huse et al., 1998; Schnaider et al., 2000). Lack of zinc results in decreased cell proliferation, skin and mucous membrane manifestations and decreased wound healing (Prasad, 2003), while chaperone upregulation facilitates wound healing and regeneration (Vígh et al., 1997). Moreover, zinc deprivation compromises the stress response in ketratinocytes, and in the Jurkat T-cell line (Larbi et al., 2006; Parat et al., 1998). An upregulation of HSF-1 was observed in T-cell clones both upon zinc deprivation and during *in vitro* aging, suggesting a compensatory mechanism. As HSF-1 is a zinc finger protein, it may be directly affected by fluctuations in intracellular free zinc ion availability. This is supported by the fact that free intracellular zinc is in the femtomolar range, around the KD of various zinc-binding proteins (Outten and O'Halloran, 2001) and p53 and the apurinic/apyrimidinic endonuclease are all inactivated by zinc deficiency (Ho and Ames, 2002; Ho et al., 2003). According to a recent intriguing hypothesis of Bruce Ames (Ames, 2006), shortage of micronutrients may promote the re-allocation of scarce micronutrients towards processes favoring short term survival at the expense of longevity. According to this, the stress response (a major adaptation mechanism) would either enjoy an intermediate priority (after basic cellular processes like oxidative metabolism, and before reproduction) and as such, would be downregulated during modest nutrient deprivation, as seen in the present study. It is very much in agreement with the fundamental role of the stress response in longevity (Sőti and Csermely, 2003). Therefore, beyond genomic stability, the stress response is another candidate mechanism to connect nutrient scarcity with aging and degenerative diseases, at least in the cases of zinc. Effect of other micronutrients on the stress response remains to be elucidated.

As a conclusion, we provided evidence that dietary zinc is a chaperone inducer and a major determinant of the stress response in human lymphocytes in the elderly. Since the stress response is a conserved and universal phenomenon, and the effect of zinc *in vitro* is fairly general, these findings may have wider implications in other bodily tissues as a possible anti-aging mechanism. However, whether this is a proof of principle or a special exception will be a subject of future studies.

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Forum Original Research Communication

Resveratrol Induces the Heat-Shock Response and Protects Human Cells from Severe Heat Stress

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ABSTRACT

Molecular chaperones play key roles in protein quality control, signal transduction, proliferation, and cell death, and confer cytoprotection and assure survival after environmental stress. The heat-shock response is implicated in a variety of conditions including ischemic diseases, infection and immunity, neurodegeneration, and aging. Physiologic and pharmacologic chaperone inducers were shown to be an efficient therapeutic approach in different acute and chronic diseases. Here we characterize resveratrol, a polyphenol from red wine, as an inducer of the heat-shock response. Resveratrol activated the heat-shock promoter and the expression of the major chaperone Hsp70 in cell lines and in human peripheral lymphocytes, comparable to moderate heat stress. This effect was not due to its antioxidant property, because 5 m*M* *N*-acetylcysteine was unable to activate the heat-shock response. Moreover, resveratrol failed to upregulate Grp78, and tunicamycin was unable to induce Hsp70, suggesting that the resveratrol-induced heat-shock response was not mediated by canonic endoplasmic reticulum stress. Resveratrol synergized with mild to moderate heat shock and conferred cytoprotection against severe heat stress. Our results reveal resveratrol as a chaperone inducer that may contribute to its pleiotropic effects in ameliorating stress and promoting longevity. *Antioxid. Redox Signal.* 10, 65–75.

INTRODUCTION

MOLECULAR CHAPERONES are conserved, abundant, and essential proteins that guard the conformational homeostasis of proteins (15, 58). Chaperones in the everyday setting maintain signal transduction and other molecular networks, regulate proliferation and differentiation, and they are important modulators of the immune response and apoptotic pathways (9, 30, 44, 45). Besides, chaperone (or stress) protein levels are subject to a sudden and transient increase with stress, a so-called stress or heat-shock response, a basic cellular adaptation mechanism (27). Chaperone induction is mediated at the transcriptional level by an autoregulatory feedback loop: an increase in misfolded proteins results in the release of heat-shock transcription factor 1 (HSF-1)[1] from the repressing Hsp90/Hsp70/Hsp40 complex, and a consequent transcriptional activation of various heat-shock genes. Beyond chaperones, a variety of regulatory mechanisms are involved in the regulation of HSF-1 activity, including phosphorylation, trimerization, nuclear import, and termination of transcription (52).

The heat-shock response confers stress tolerance, cytoprotection, and assures short- and long-term survival during severe environmental conditions. Both a single heat shock and transgenic Hsp70 induce longevity (47). HSF-1 overexpression induces a twofold life-span extension, whereas HSF-1 knockout markedly shortens the life span (13, 18) and compromises immune responses in invertebrate and vertebrate model organisms, respectively (13, 57). Moreover, proteotoxicity and cellular degeneration increase, whereas chaperone inducibility and chaperone function decrease during aging (3, 28, 29). Modulation of the chaperone levels has been shown to provide protection in a variety of pathophysiologic states, such as ischemia/reperfusion, inflammation/sepsis, and conformational diseases (16, 41, 46, 49). Thus, preservation or enhancement of the heat-

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shock response is a subject of intense research. Chaperone induction has been proven in preclinical studies to be an efficient therapeutic approach in cardiovascular and age-related degenerative diseases involving cancer, diabetes, and neurodegeneration (11, 42, 43). Besides physiologic stressors (exercise, sauna, calorie restriction), both synthetic and plant-derived small molecules are among promising lead compounds (4, 19, 21, 23, 40, 50).

Resveratrol, a plant-derived polyphenolic compound, was identified in 1992 as an ingredient of grape skin and red wine, reducing the risk of coronary heart disease (40). Since then, it has become clear that resveratrol displays an impressive therapeutic potential against cancer, ischemic injuries, cardiovascular and inflammatory diseases, as well as neurodegeneration (5, 20, 33). A potent antioxidant and antiinflammatory property is an important determinant of its mechanism of action. Besides, recent studies have shown that it induces stress resistance and longevity in a variety of organisms, such as yeast, invertebrates, and vertebrates (17, 48), and it may reprogram the bodily processes similar to calorie restriction, probably by acting on the sirtuin (SIR, silent information regulator) family of deacetylases (56). The puzzling similarity between the therapeutic benefits of chaperone induction and resveratrol has led us to explore the effect of resveratrol on the heat-shock response of mammalian cells. Here we provide evidence that resveratrol activates the stress response, exerts a synergistic effect with mild to moderate heat treatment, and confers cytoprotection against a lethal heat shock. Our results reveal resveratrol as a chaperone inducer that may contribute to its pleiotropic effects in ameliorating stress and inducing longevity.

MATERIALS AND METHODS

Materials

Reagents for cell culture and Lipofectamine transfection reagent were from Gibco-Invitrogen (Carlsbad, CA); solutions and antibodies for flow cytometry were from BD Biosciences (San Diego, CA); and Ficoll-Paque PLUS was from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). The hsp70.1pr/luciferase plasmid was a kind gift of Richard Morimoto (Evanston, IL), the CMV/β-galactosidase was from BD Biosciences. The Bright-Glow luciferase and the β-galactosidase assay kits were from Promega (Madison, WI). Materials for protein determination, gel electrophoresis, and Western blotting were from Bio-Rad (Hercules, CA). *Trans*-resveratrol (RSV), *N*-acetyl-L-cysteine (NAC), hydrogen peroxide (H_2O_2), tunicamycin, the anti–β-actin antibody and propidium iodide were from Sigma (St. Louis, MO). The anti-Hsp70 fluorescein isothiocyanate (FITC) conjugated antibody (recognizing inducible Hsp70) and the anti-Grp78 mouse monoclonal antibodies were from StressGen (Victoria, BC, Canada). Peroxidase-conjugated secondary antibodies were obtained from DAKO Cytomation (Glostrup, Denmark), and the ECL reagent was from New England Nuclear, Perkin Elmer Life Sciences (Boston, MA). Complete tablets were from Roche (Basel, Switzerland). All other reagents were from either Sigma or Fluka (Buchs, Switzerland).

Preparation of lymphocytes

Human peripheral lymphocytes were isolated from a mixture of buffy coats (purchased from the Hungarian National Blood Transfusion Service, approved by the National Ethical Committee, and in accordance with the Helsinki regulations), which were usually prepared from the venous blood of five young healthy donors. To obtain leukocyte-rich plasma, the buffy coats were first layered over 6% dextran in a ratio of 9:1 (vol/vol) and sedimented for 1 h at room temperature. Lymphocytes were then isolated by Ficoll-Paque PLUS (d = 1.077 g/ml) gradient centrifugation, according to the manufacturer's instructions, and washed twice in phosphate-buffered saline (PBS). Aliquots were made in RPMI-1640 medium containing 40% fetal bovine serum and 5% DMSO and were frozen in liquid nitrogen until further use.

Cell culture and treatments

Peripheral lymphocytes were gently thawed and cultured in RPMI 1640 medium, supplemented with 10 m*M* Hepes, 10% fetal bovine serum, 2 m*M* L-glutamine, 100 μg/ml streptomycin, and 100 IU/ml penicillin at 37°C in a 5% CO_2 incubator at isobaric oxygen. The African green monkey kidney fibroblast-like cell line COS-7 was cultured in DMEM 4500, whereas the human cervical carcinoma cell line HeLa was cultured in minimal essential medium supplemented with 10% fetal bovine serum, 2 m*M* L-glutamine, 100 μg/ml streptomycin, and 100 IU/ml penicillin. For each experiment, cells were treated first with resveratrol (RSV) or with *N*-acetyl-L-cysteine (NAC) at 37°C in a 5% CO_2 incubator for 10 h. Subsequent treatments were performed with hydrogen peroxide (H_2O_2) for 2 h or with tunicamycin for 16 h at 37°C in a 5% CO_2 incubator. Heat shock was carried out at 41–45°C for 20–60 min either in an Eppendorf thermomixer (lymphocytes), or in a circulating water bath (cultured adherent cells).

Transfection and HSF-1 reporter gene assay

COS-7 cells were plated in six-well plates at a density of 20% and transfected the next day with 1 and 0.5 μg of hsp70.1pr/luciferase and CMV/β-galactosidase plasmids, respectively, by Lipofectamin, according to the manufacturer's protocol. Treatments were made 24 h after transfection in complete medium. Eighteen-hour posttreatment cells were lysed in β-galactosidase lysis buffer (Promega), and activities were determined by Bright-Glow luciferase and β-galactosidase commercial assay kits and were expressed as a ratio.

Determination of Hsp70 levels by flow cytometry

The 10^6 lymphocytes or COS-7 cells were treated either with resveratrol or NAC for 10 h and were or were not heat shocked, as specified in the figure legends. The induction of the heat-shock response was allowed to develop for 4 h at 37°C. Then cells were fixed and permeabilized in 250 μl Cytofix/Cytoperm solution at 4°C for 20 min. Cells were then washed twice with Perm/Wash solution and incubated with an anti-Hsp70 fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal antibody and anti-CD3 Peridinin-chlorophyll-protein complex

(PerCP) conjugated antibody at 4°C for 30 min in Perm/Wash solution at a concentration recommended by the manufacturer. For compensation, cells were stained either with anti-CD3-PerCP–conjugated antibody or with anti-CD3 FITC–conjugated antibody. After washing once with Perm/Wash solution and once with PBS with 2% FBS, cells were fixed with 2% paraformaldehyde in PBS and analyzed with a FACSCalibur flow cytometer by using the CellQuest software (BD Biosciences). Approximately 10,000 live lymphocytes from each sample were electronically gated according to granularity and size in the forward *versus* side scatter. Differentiation between $CD3^+$ and $CD3^-$ subpopulations was achieved in the CD3-PerCP versus side scattergram. Hsp70-related fluorescence was obtained in a 530/30 (FITC) filter, and the relative Hsp70 protein level was expressed as the mean fluorescence intensity of FITC from logarithmic histograms.

Cell lysis and Western blotting

Cells were harvested, washed twice in PBS, then lysed in lysis buffer (20 m*M* Hepes, 100 m*M* NaCl, 1 m*M* EDTA, 1% NP40, pH 7.5, and 2× Complete protease inhibitor cocktail for 20 min at 4°C, vigorously vortexed, and centrifuged at 13,000 rpm for 10 min in a microcentrifuge. Protein content of the supernatants was determined by the Bio-Rad Bradford-assay with bovine serum albumin used as standard. Then 50 μg protein from detergent cell lysates was subjected to a 9% SDS-PAGE and electroblotted by using a semidry transfer apparatus on nitrocellulose membranes. Blots were blocked in 5% milk powder at room temperature, probed with the appropriate primary antibodies (Abs) overnight at 4°C, washed and incubated with peroxidase-conjugated secondary antibodies for an hour at room temperature, and developed by using an ECL reagent.

Cell-survival assay

The 10^6 cells per sample were incubated in the presence or absence of resveratrol for 10 h at 37°C, then treated with H_2O_2 or heat shocked, as indicated. Twenty-four hours later, cells were harvested, washed twice with PBS with 2% FCS, and stained with propidium iodide at a final concentration of 10 μg/ml in PBS with 2% fetal bovine serum at room temperature for 15 min. Flow-cytometric analysis was performed with a FACSCalibur flow cytometer and the CellQuest software by using 488 nm excitation and 630/22 emission filters, respectively. Cell debris was eliminated by gating according to side-scatter and forward-scatter detection, and ~10,000 cells were analyzed. The percentage of PI-negative (surviving) cells was calculated by single-parameter analysis of the PI-related fluorescence by using logarithmic histograms.

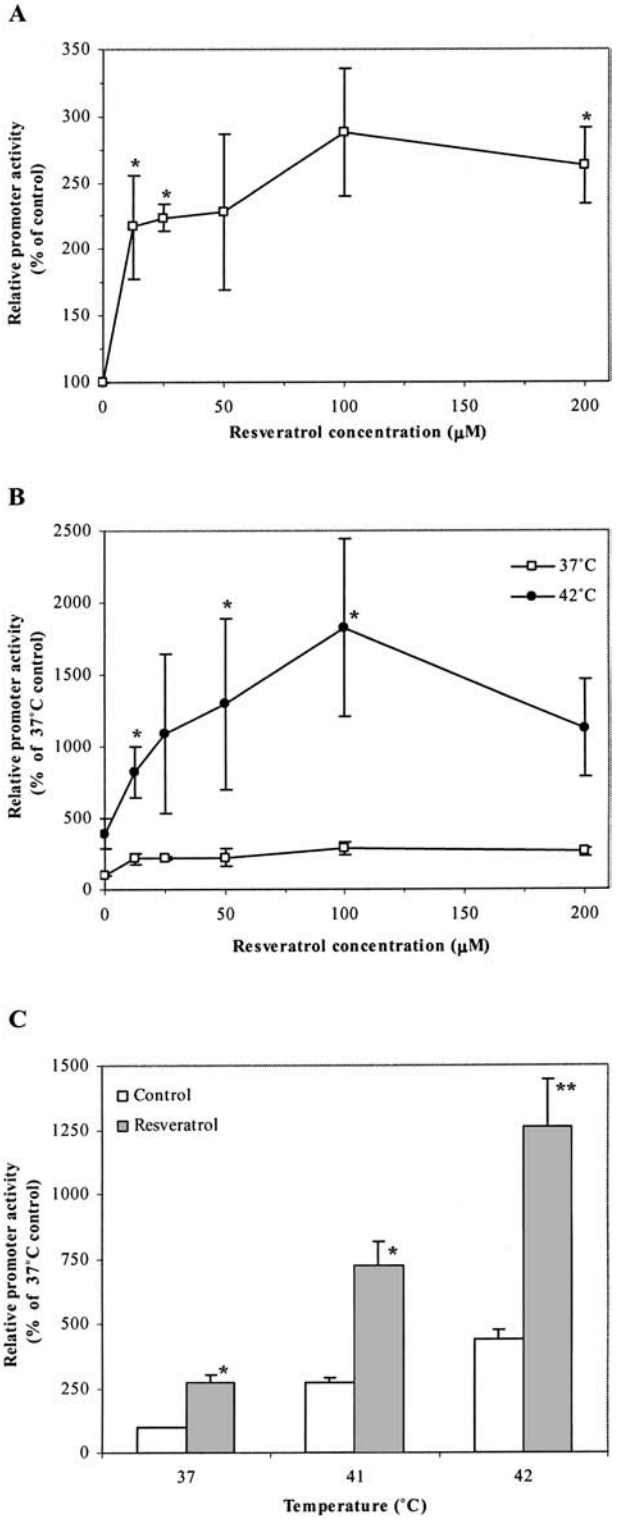

FIG. 1. Resveratrol induces heat-shock promoter activation and acts as a chaperone co-inducer. (**A**) Activation of the hsp70.1 promoter reporter by resveratrol at 37°C. (**B**) Resveratrol potentiates the heat-shock response in a concentration-dependent manner (the 37°C curve is displayed for comparison). (**C**) Resveratrol lowers the threshold of the heat-shock response. COS-7 cells were co-transfected with hsp70.1pr/luciferase and CMV/β-galactosidase and were incubated with the indicated concentrations of (**A, B**) or 50 μM (**C**) resveratrol for 3 h, and then were heat stressed for 20 min at the indicated temperatures (**B, C**) as described in Materials and Methods. After 18 h, enzyme activities were determined, and their ratio was expressed. Data represent mean ± SD of three experiments. * or **, significant differences at $p < 0.05$ or $p < 0.01$, respectively.

Statistical analysis

Data were statistically analyzed by using Statistical Package for the Social Sciences software version 15.0 for Windows (SPSS Inc., Chicago, IL). Variables are expressed as mean ± standard deviation (SD). Means were compared by using the Student's *t* test. A *p* value (two-tailed) < 0.05 was considered statistically significant.

RESULTS

Resveratrol activates heat-shock promoter–driven transcription

To investigate the effect of resveratrol on the stress response, we first asked whether it induced the activation of the heat-shock promoter containing consensus binding motifs of HSF-1. We transiently transfected COS-7 cells with a luciferase construct fused to the promoter of the major stress protein, the inducible isoform of Hsp70 (53) and observed a dose-dependent induction of the reporter activity at both 37°C and at 42°C (Fig. 1A and B). The EC_{50} values calculated from double reciprocal plots were 8.6 and 15.2 μM, respectively. A cytotoxic effect was already observed at 200 μM and became predominant at 400 μM, where the stress-response induction was compromised, as well (data not shown). Resveratrol potently induced heat-shock promoter–driven transcription in HeLa cells, as well (data not shown).

Because resveratrol potentiated the heat-shock response at 42°C (Fig. 1B), we tested whether they cooperate in a synergistic manner. To this end, cells were pretreated with resveratrol and subjected to heat stress at different temperatures. Figure 1C shows that resveratrol exerted an effect comparable to

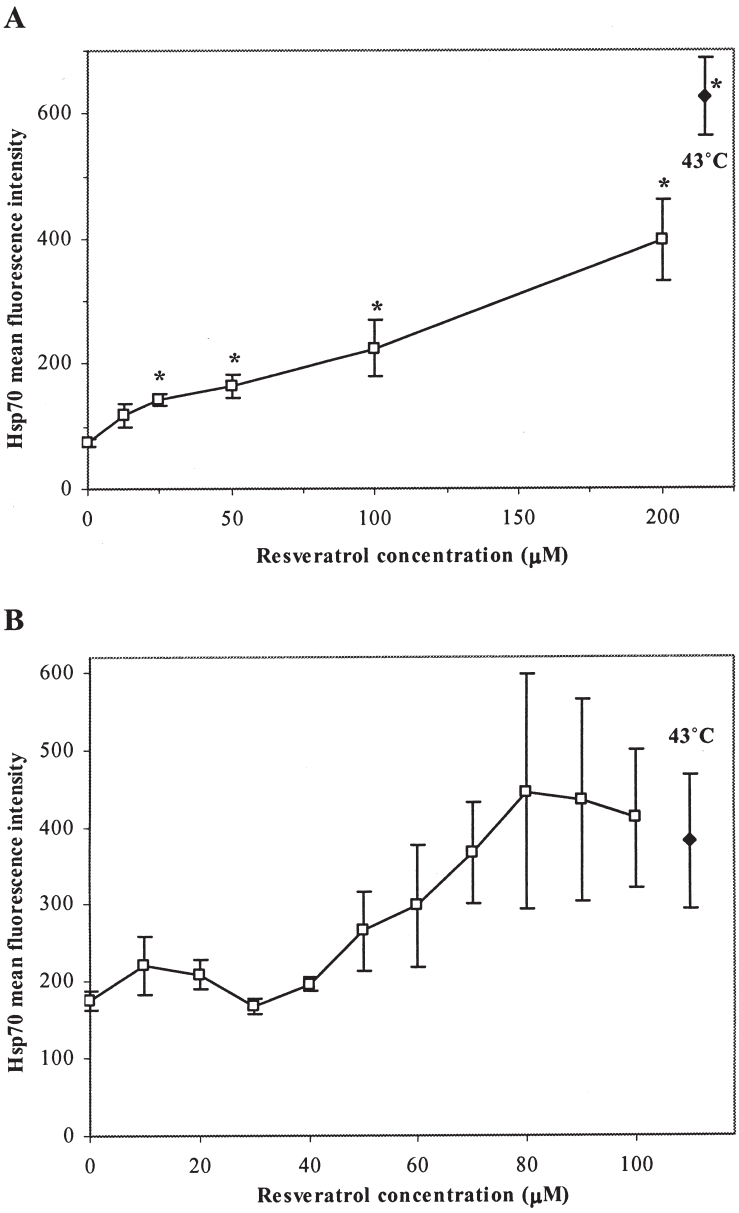

FIG. 2. Resveratrol induces Hsp70 protein expression in different cell types. COS-7 cells (**A**) or human peripheral lymphocytes (**B**) were incubated with the indicated concentrations of resveratrol for 15 h; then cells were harvested, stained with an anti-Hsp70 antibody FITC-conjugate, subjected to flow cytometry, and the mean fluorescence intensities were determined, as described in Materials and Methods. *Solid diamonds*, A 20-min (**A**) or a 60-min (**B**) heat stress at 43°C 4 h before analysis. Data represent mean ± SD of three (**A**) or two (**B**) experiments, respectively. *Significant difference at $p < 0.05$.

a 41°C heat shock, and a combination treatment at 41°C resulted in a higher heat-shock response than that at 42°C. Similarly, the combination of resveratrol with a 42°C treatment was highly synergistic, suggesting that resveratrol increases the robustness of adaptation to a harmful stimulus. HeLa cells similarly displayed a very pronounced synergy between resveratrol and heat shock (data not shown).

Resveratrol induces Hsp70 protein expression

To see whether the activating property of resveratrol would be translated into the expression of intrinsic chaperone proteins, COS-7 cells were treated with resveratrol, and the amount of the inducible Hsp70 protein was analyzed with flow cytometry (Fig. 2A). Resveratrol induced a fivefold accumulation of Hsp70 protein, close but not similar to the protein level after a 43°C heat stress. Maximal induction could not be properly determined because above 200 μM, resveratrol became cytotoxic (data not shown). After establishing resveratrol as a chaperone inducer in cell lines, we were seeking a more physiologic cellular model to characterize. Because the stress response is of central importance in the immune system, lymphocytes are governors of immune activity, show a weak stress response, and heat stress in the form of fever potentiates their function (30, 31, 38), we investigated human peripheral lymphocytes as a possible target of the action of resveratrol. Indeed, resveratrol treatment recapitulated the earlier findings on cell lines, although with different kinetics (Fig. 2B). Hsp70 induction seemed to start at the higher dose of 40 μM and reached a plateau at 80 μM, resembling to a sigmoid cooperative response curve. Even though none of the values, including the heat stress, reached statistical significance, the maximal Hsp70 induction of resveratrol tended to be higher than that of the 43°C treatment, suggesting a physiologically relevant stress response–inducing property.

Resveratrol's mechanism of action is mediated neither by its antioxidant property nor by a canonic ER stress response

A potent direct antioxidant property is a reminiscent feature of polyphenols and contributes to the beneficial effects of resveratrol. However, an increasing body of evidence argues for an indirect effect and implicates the upregulation of antioxidant enzymes and inhibition of redox-active signaling by resveratrol (35). It was also reported that the thiol antioxidant 1,2-dithiole-3-thione stimulated Hsp70 expression in dopaminergic neuronal cells (2). To mimic the presumed antioxidant property of resveratrol, we examined the possibility that a well-known thiol antioxidant, *N*-acetylcysteine (NAC) (14) would interfere with the stress response. But 5 m*M* NAC was unable to activate or coactivate the heat-shock promoter with heat stress in COS-7 and HeLa cells, respectively (Fig. 3A and data not shown). In contrast, it inhibited the heat-induced reporter activity, suggesting the role of reactive oxygen species in promoter activation. However, when H_2O_2 was tested at various concentrations, it did not induce the stress response at the HSF-1, the mRNA, or the protein level (E.M.V, Á.P., C.S., unpublished observations). Moreover, the inhibitory effect of NAC could not be observed in HeLa cells, arguing against a general mechanism (data not shown). Besides the results shown here, NAC had no influence on Hsp70 protein expression either in COS-7 cells or in peripheral lymphocytes (Fig. 3B and data not shown), which further argues against a general antioxidant property of resveratrol in its heat-shock–related effects.

It was recently reported that resveratrol induces longevity in *Caenorhabditis elegans via* the induction of a subset of ER-

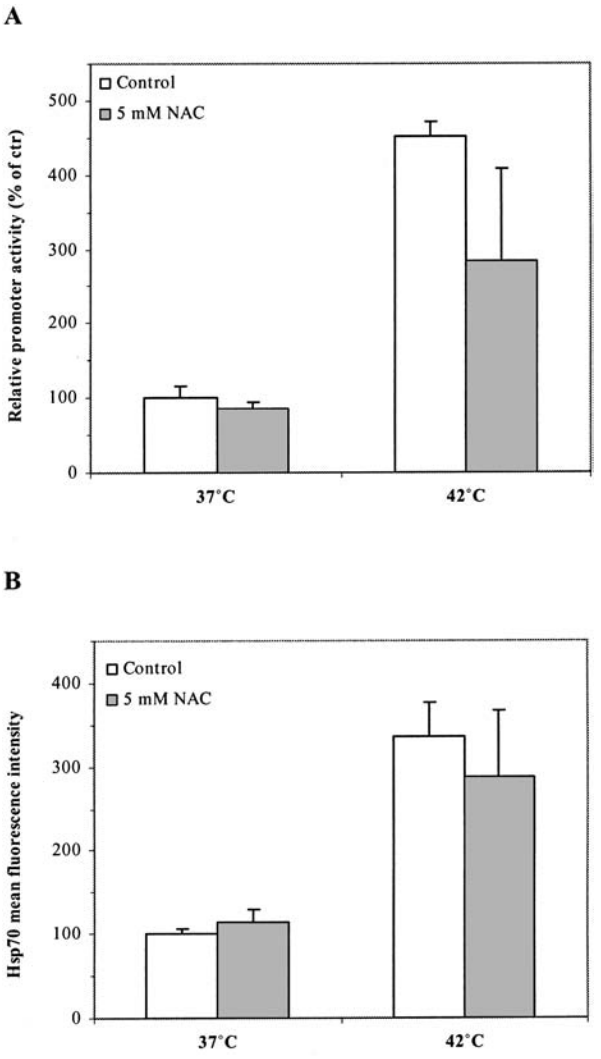

FIG. 3. The antioxidant *N*-acetylcysteine does not induce the heat shock response. (**A**) *N*-acetylcysteine (NAC) does not activate the hsp70.1-promoter reporter. COS-7 cells were co-transfected with hsp70.1pr/luciferase and CMV/β-galactosidase and were incubated with or without 5 m*M* NAC for 3 h, and then were heat stressed at 42°C or were kept at 37°C for 20 min, as described in Materials and Methods. After 18 h, enzyme activities were determined, and their ratio was expressed. (**B**) NAC does not induce Hsp70 protein expression. COS-7 cells were incubated with or without 5 m*M* NAC for 10 h, were heat stressed at 42°C, or were kept at 37°C for 20 min, as described in Materials and Methods. Four hours later, cells were harvested, stained with an anti-Hsp70 antibody FITC-conjugate, subjected to flow cytometry, and the mean fluorescence intensities were determined. Data represent mean ± SD of two experiments. *Significant difference at $p < 0.05$.

resident protein genes (51). Therefore, we analyzed possible crosstalk between the unfolded protein response/ER-stress pathway and the cytosolic heat-shock response. We found that tunicamycin, an inhibitor of core glycosylation, did not activate the heat-shock reporter. Furthermore, tunicamycin potently inhibited the heat-induced activation of the heat-shock promoter in both COS-7 and HeLa cells (Fig. 4A and data not shown). Notably, Hsp70 protein levels did not reflect the inhibition of the heat-shock promoter on tunicamycin treatment (Fig. 4B), raising the possibility that in these circumstances, transcriptional and posttranscriptional regulation of Hsp70 became uncoupled, which resulted in the preservation of adaptation to heat stress.

To acquire an insight into the effect of resveratrol on ER stress, Grp78, the major chaperone of the ER and a marker of the unfolded protein response, was analyzed with Western blotting. Figure 4C shows that neither resveratrol nor NAC induced Grp78 protein. Furthermore, neither agent interfered with Grp78 induction on tunicamycin treatment. Our results confirm earlier findings on *C. elegans*, in which resveratrol did not induce a canonic unfolded protein response, including the Grp78 orthologue (51). Neither they nor we found a general derangement in protein homeostasis in the models studied on resveratrol treatment (51, and data not shown). These observations suggest neither an overwhelming stress nor an imbalance in protein homeostasis, but a specific signaling event may mediate a selective induction of the cytosolic stress response by resveratrol.

Resveratrol protects cells from severe heat stress

Resveratrol has a well-documented chemopreventive and cytoprotective property because of its prominent action on free radicals and on the redox homeostasis (5, 35). Resveratrol pretreatment potently inhibited cell death under circumstances of oxidative stress in human peripheral lymphocytes (Fig. 5A). However, its ability to induce apoptosis may mask its beneficial effects under other stresses (12). We observed an augmented cell death in resveratrol-treated lymphocytes compared with control cells (data not shown).

Because molecular chaperones and the heat-shock response antagonize proteotoxic noxae, we tested whether resveratrol conferred cytoprotection against heat stress. Cells preconditioned with resveratrol displayed an increasing survival at increasing temperatures, even at the lethal 45°C heat shock (Fig.

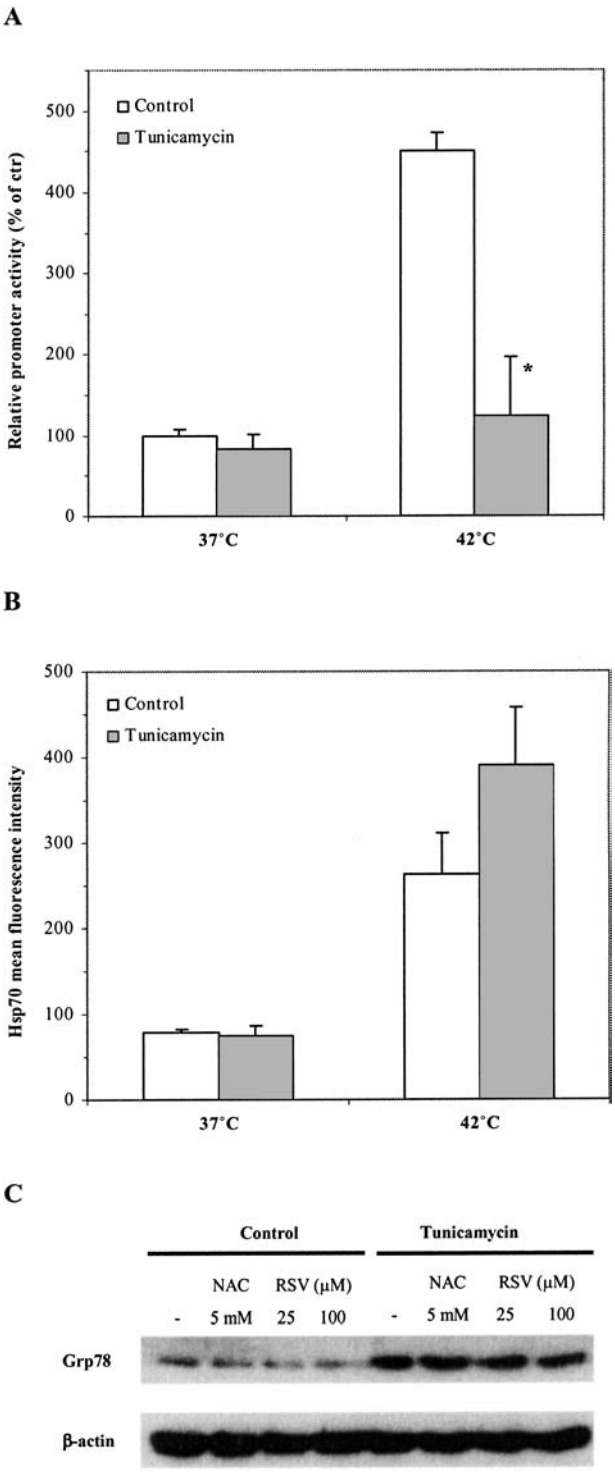

FIG. 4. Resveratrol does not use a canonic ER-stress pathway to induce the heat-shock response. (**A**) Tunicamycin does not activate the hsp70.1–promoter reporter and inhibits its heat-induced activation. COS-7 cells were co-transfected with hsp70.1pr/luciferase and CMV/β-galactosidase, and were incubated with or without 2 μg/ml tunicamycin for 3 h, and then were heat stressed at 42°C or were kept at 37°C for 20 min, as described in Materials and Methods. Eighteen hours later, enzyme activities were determined, and their ratio was expressed. (**B**) Tunicamycin does not induce Hsp70 protein expression. COS-7 cells were incubated with or without 2 μg/ml tunicamycin for 10 h, were heat stressed at 42°C, or were kept at 37°C for 20 min. Four hours later, cells were harvested, stained with an anti-Hsp70 antibody FITC-conjugate, subjected to flow cytometry, and the mean fluorescence intensities were determined. (**C**) Resveratrol does not induce Grp78 protein expression. COS-7 cells were incubated with or without NAC and resveratrol (RSV) at the indicated concentrations for 3 h, and then samples were supplemented with 1 μg/ml tunicamycin, and incubation was continued for 15 h. Cells were harvested, lysed, and subjected to immunoblotting for Grp78 and β-actin. Data represent mean $\pm$ SD, and the blot is a representative of two experiments. *Significant difference at $p < 0.05$.

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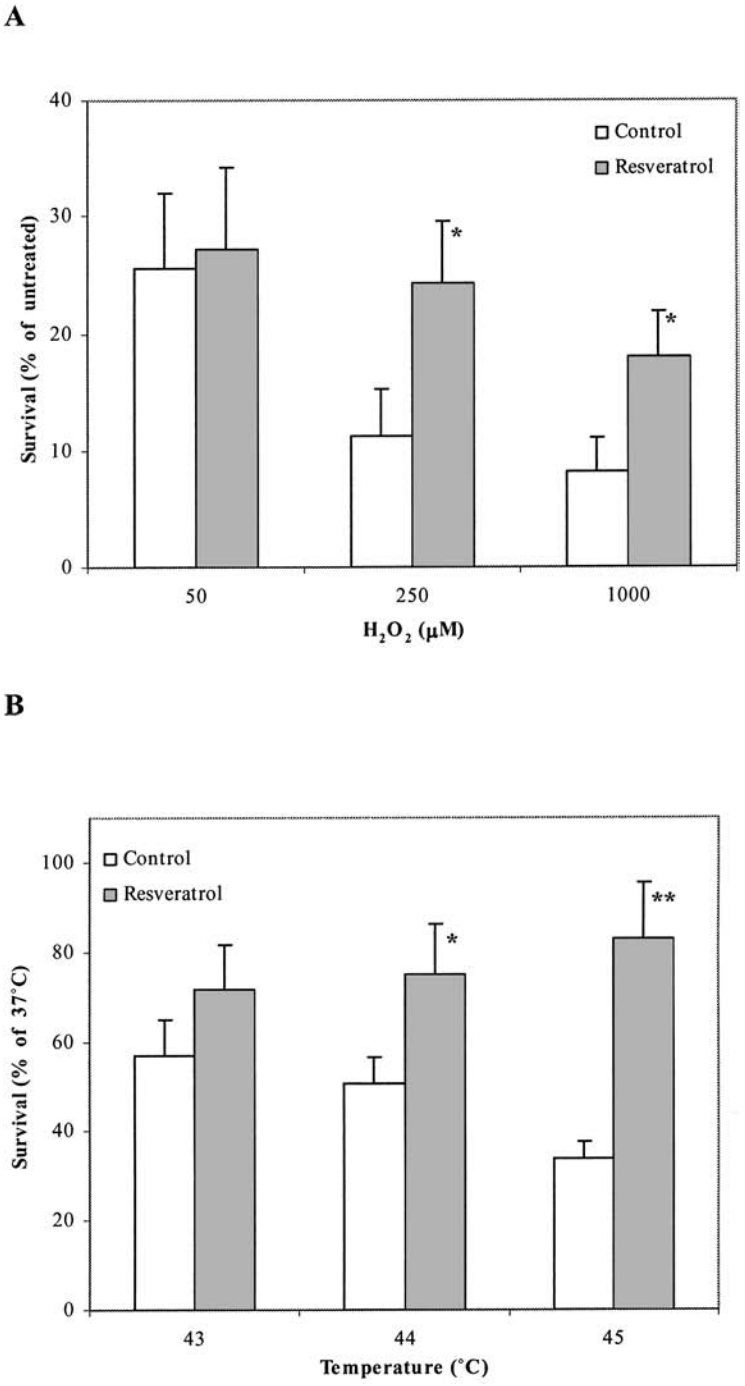

FIG. 5. Resveratrol confers cytoprotection against oxidative and heat stress. (**A**) Resveratrol protects the cells from hydrogen peroxide. (**B**) Resveratrol protects the cells from heat stress. Lymphocytes were incubated in the presence or absence of 100 μM resveratrol for 10 h at 37°C then treated with H_2O_2 for 2 h or heat shocked at the indicated temperatures for 30 min. After 24 h, cells were incubated with propidium-iodide and analyzed in a flow cytometer. The fraction of the propidium-iodide–negative (surviving) cells was expressed relative to the absolute and resveratrol-treated controls. Data represent mean ± SD of three experiments. *, **Significant differences at $p < 0.05$ or $p < 0.01$, respectively.

5B). These results suggest that resveratrol may ameliorate the consequences of proteotoxicity, probably *via* induction of the heat-shock response.

DISCUSSION

Resveratrol is a member of the polyphenol family of phytochemicals, plant-derived small molecules with potent and wide range of biological effects. Polyphenols, including resveratrol, possess antimicrobial, antitumor, antiinflammatory, antiischemic, and neuroprotective properties (12, 35, 36). Resveratrol is the first compound that has been recognized to promote longevity in several organisms (17, 48, 56), including mice kept on a high-calorie diet (6), predicting a tremendous benefit for human health. It has also been suggested that resveratrol acts *via* the sirtuin family of the NAD^+-dependent deacetylase family that connects metabolic stress and aging (17).

Our study identified resveratrol as an inducer of the heat-shock response. Resveratrol treatment of various mammalian, including human, peripheral cells activated HSF-1–dependent

transcription and accumulation of Hsp70 protein at a level comparable to the effect of a moderate heat stress. The EC_{50} value was in the low micromolar range (~10 μM), comparable to that of the activating effect on sirtuins (56). However, it is still much higher than the peak serum concentration after two glasses of red wine [2.4 n*M* unmodified and 180 n*M* derivatized resveratrol, respectively (5)]. These data recall a major obstacle of resveratrol research (*i.e.*, a gap between bioavailability and the effective concentration). In support of a physiologically relevant stress-inducing effect is the fact that use of a 100-mg/kg pharmacologic dose resulted in 9 μM unmodified and 680 μM derivatized resveratrol concentration, respectively (5). Furthermore, various organs can accumulate resveratrol 30-fold over the serum concentration; other polyphenols synergize with and inhibit the conjugation of resveratrol, and altogether they may put the red-wine consumption in the effective range.

The observed synergy of resveratrol with heat shock suggests that small or transient increases in resveratrol concentration may cooperate with other small stresses and promote the mounting of a more robust stress response. This is reminiscent of a chaperone co-inducer property (42, 50) and is highly beneficial, because reprogramming the cellular thermometer increases the stress tolerance and the fitness of the cell or organism.

By what mechanism does resveratrol induce the stress response? Studies with NAC strongly suggest that an antioxidant activity did not mediate this action. Moreover, our observations on protein aggregation and on the cytoprotective effect in case of simultaneous resveratrol and heat treatment argue against the possibility that resveratrol compromised global protein homeostasis (data not shown). In some circumstances, ER stress may induce cytosolic chaperones (32). In our model, the canonic unfolded protein response was unlikely to be involved in the resveratrol-induced stress response, because neither did tunicamycin activate the heat-shock response, nor did resveratrol induce the ER Hsp70 homologue UPR marker, Grp78. In other studies, resveratrol treatment seemed to upregulate the apoptosis-inducing transcription factor CHOP in colon carcinoma cells (54) and induced life-span extension through induction of a subset of nonclassic UPR genes (51), suggesting that resveratrol may interfere with and provide protection against stressful events in the ER. Thus, crosstalk between a noncanonic UPR and the cytosolic heat-shock response cannot be excluded.

Chaperone inducers may work along the heat-shock pathway: through direct binding and activation of HSF-1, inhibition of chaperones suppressing HSF-1, inhibition of the proteasome, or by other hitherto unknown mechanisms (4, 19, 21, 23, 42, 43, 50, 53). Alternatively, an interference with the phosphorylation of HSF-1 may also modulate the stress response, because protein kinase C, c-jun N-terminal kinase, and glycogen synthase kinase 3 all repress HSF-1 activation (52), and, in turn, a resveratrol-mediated inhibition of these kinases has been reported (34, 54). Interestingly, another phenolic acid derivate from cinnamon has recently been shown to activate the PI3-kinase Akt pathway (24) that exerts an inhibition on glycogen synthase kinase 3, suggesting a possible pathway for polyphenol-dependent HSF-1 activation.

Resveratrol belongs to the group of agents/stimuli that exhibit a reciprocal effect on HSF-1 activation (stress resistance) and NF-κB activation (inflammation). These processes have an opposite effect on longevity (5, 35, 53) and may be coordinately regulated branches of a survival program. This program is regulated by the level of environmental stress and operates with multiple sensors that transmit signals from the internal and external environment and use robust and redundant pathways that switch from costly reproduction to self-maintenance. One master regulator is the sirtuin family, which responds to food availability and may mediate the effects of calorie restriction (8, 22). It is tempting to speculate whether SIR2 is an upstream regulator of HSF-1. Evidence has suggested that SIR-2.1 may activate HSF-1 through the forkhead transcription factor Daf-16 in *C. elegans* (8). However, a direct inhibitory effect *via* chromatin structure was also described in yeast (39). In this context, resveratrol either may induce an SIR2-dependent activation or may inhibit a repression on the heat-shock response, or both (17, 51, 56). Whether resveratrol exclusively uses any of the pathways listed or promiscuously affects many of them simultaneously remains to be seen. Another exciting question is whether HSF-1 is needed for resveratrol to induce longevity, as was the case in daf-2 mutants (18).

Resveratrol exerted a cytotoxic effect both on lymphocytes and on cultured cell lines over 50 μM, accompanied by the downregulation of the stress response above 200 μM, in agreement of its proapoptotic activity (12, 54). Many chaperones exert a potent antiapoptotic effect, a possible escape and survival mechanism for tumor and normal cells, respectively (42, 43, 45). In this regard, the resveratrol-induced heat-shock response may be an aspecific compensatory mechanism in cytotoxicity. One study found a correlation between resveratrol treatment, apoptosis, and Hsp70 level in prostate cancer cells (7) that supports this hypothesis. In addition, the mechanistic insights presented in this study argue for a more specific mechanism of action.

Table 1. Effect of Resveratrol on the Stress Response and Thermotolerance in Various Mammalian Cells

	Lymphocyte	*COS-7*	*HeLa*
Heat-shock response			
Heat-shock promoter activation	N.D.	Yes	Yes
Synergy with heat shock	N.D.	Yes	Yes
Hsp70 protein induction	Yes	Yes	Yes
Thermotolerance	Yes	Yes	N.D.

N.D., not determined.

An important finding of the present work is that resveratrol protects cells from severe heat stress. It corroborates earlier results in which resveratrol rescued neuronal cells from mutant huntingtin proteotoxicity (33). In that study, polyglutamine toxicity was prevented by either SIR-2.1 overexpression or resveratrol treatment in *C. elegans*, suggesting that resveratrol may act *via* activation of SIR-2.1. Because HSF-1 is critical to combat aggregation (18, 28), we propose that resveratrol exerts its beneficial activity *via* the induction of the heat-shock response, a central protein quality-control mechanism. The conservation of this pathway in human cells is reasonable. Although HSF-1 maybe only one player in the signaling network that is regulated by the metabolic state, downregulation of the stress response may result in degenerative diseases (ischemic diseases, neurodegeneration, inflammation) as a consequence of overnutrition, all being major threats of Western civilization. Direct evidence indicates that hyperlipidemia blunts the stress response in the rat (10). Resveratrol, in contrast, lowers the temperature threshold of the heat-shock response, so cells become preconditioned to cope with a more severe, lethal stress, exactly what we have seen in the cytoprotection experiments. These effects were observed in various mammalian cell lines and peripheral cells, reflecting a conserved phenomenon (see Table 1).

Our data showed a biphasic effect of resveratrol on both heat shock–promoter activation and Hsp70 protein expression. This, as well as the cytotoxicity observed at higher doses, is a characteristic hormetic property (37). Hormesis is a dose–response phenomenon characterized by low-dose stimulation, high-dose inhibition. Similarly, a number of other phytochemicals, called hormetins, have been shown to evoke hormetic responses. The stress-response inducers curcumin and celastrol have both been demonstrated to induce the heat-shock response in a hormetic manner (1, 53).

The hormetic property of resveratrol also supports the "xenohormesis hypothesis," which postulates that small but harmful changes in the environment (famine, dryness, heat, cold, infection) induce the production of phytoalexins in plants (25). Most of these compounds are potent toxins, especially for insects (26), but their amount does not reach a critical threshold in bigger animals and promotes a hormetic response. Alternatively, phytoalexins seem to be danger signals and represent an altruistic courtesy of stressed plants toward ingesting animals, alerting them to prepare for and to survive during unfavorable environmental conditions. This principle was intuitively used for centuries in the form of herbal remedies by the ancient wisdom of folk therapy and of traditional oriental medicine. Indeed, xenohormetic compounds including resveratrol feature overlapping properties: they induce self-maintenance and protective mechanisms, such as improving energy metabolism (increasing insulin sensitivity), upregulation of phase II detoxification, antioxidant enzymes, and suppression of inflammatory pathways (26, 35), a key to a healthy life.

In conclusion, we propose that among other desired physiologic effects, resveratrol activates the stress response, a molecular pathway to combat stress, prevent disease, and induce longevity. Deeper understanding of the mechanism of action as well as the intricate interplay of resveratrol with various targets may lead to a better understanding of aging, stress, and longevity.

ACKNOWLEDGMENTS

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ABBREVIATIONS

ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; Grp78, 78-kDa glucose-regulated protein; HSF-1, heat-shock transcription factor 1; Hsp, heat-shock or stress protein, the number thereafter denotes molecular weight in kilodaltons; NAC, *N*-acetyl-L-cysteine; PBMC, peripheral blood mononuclear cells, RSV, resveratrol (3,4′,5-Trihydroxy-transstilbene); SIR, silent information regulator; UPR, unfolded protein response.

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The Heat Shock Connection of Metabolic Stress and Dietary Restriction

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Abstract: Molecular chaperones and the heat shock response guard and modulate protein conformation, protect proteins from misfolding and aggregation, and maintain signalling and organellar networks. Overnutrition and the metabolic syndrome represent a pro-aging condition, and dietary restriction is the most robust environmental intervention that induces longevity from yeast to mammals. In recent years a considerable effort has been made to elucidate the signaling pathways involved in metabolic signaling. Here we review the current understanding on the connection between metabolic stress, dietary restriction and the heat shock response and highlight results showing chaperone induction as a promising therapeutic strategy to promote healthy aging and to prevent metabolic disorders.

Keywords: Aging, caloric restriction, diabetes, heat shock response, resveratrol, sirtuin.

INTRODUCTION: STRESS, AGING AND THE HEAT SHOCK RESPONSE

Stress can be defined as an environmental change that induces damage at all the molecular, cellular and organismal level, respectively [1,2]. In the organisms several adaptive (stress) responses have evolved to promote survival via the acquisition of stress tolerance. If these responses can not eliminate damage, it results in a functional decline, a so-called distress, while hormesis is the induction of benefical effects by exposure to low doses of chemical or physical agents that are harmful at higher doses [3,4]. As aging can be considered as a chronic stress state [5], robust adaptive mechanisms are needed not only for instant survival but also to attain longevity.

An important target of stress at the cellular level is the proteome: during proteotoxic stress and in aging the dysbalance of protein homeostasis and the loss of both protein stability and function occur [6]; besides, protein conformational disorders such as Parkinson's, Alzheimer's, and Huntington's disease, respectively, may mimic the degenerative distress state of aging [7]. The maintenance of proteome integrity is regulated by a network of genes that link stress responses and lifespan. A key player in this regard is the chaperone network [7-10] which is responsibe to maintain and modulate protein conformation, to protect proteins from misfolding and aggregation, to promote translocation and assembly and disassembly of macromolecular complexes [11-14]. The induction of heat shock response is mediated by the heat shock transcription factor 1 (HSF1) [15]. Under normal conditions HSF1 is kept in an inactive, monomeric form by an inhibitory complex of Hsp-s. Upon proteotoxic insults, like heat shock, heavy metals and proteasome inhibitors, Hsp-s interact with denaturated and partially unfolded proteins, thus HSF1 is titrated out of the inhibitory complex. HSF1 trimerizes, becomes phosphorylated and is translocated into the nucleus, where it binds to consensus sequences of the promoter of heat shock genes (heat shock elements, HSEs). Recent evidence suggests that beyond this feedback loop translational elongation factor eEF1A and a thermometer non-coding RNA (HSR1) together participate in HSF1 activation [16,17]. According to this model, the heat shock derived translational shutdown and cytoskeletal collapse make eEF1A capable to bind the concomitantly formed HSR1. Thus the eEF1A-HSR1 complex is able to capture HSF1 released from the aforementioned chaperone complex and promotes trimerization and further activation steps, linking general translation and RNA metabolism to the heat shock response. After activation and nuclear translocation HSF1 is located within 30s in so-called stress granules [18-20]. These discrete subnuclear granules contain a plethora of proteins with unknown functions, including splicing factors suggesting a special micro-compartment for fine-tuning transcriptional responses during stress [18,21,22].

Mapping out the connection between the heat shock response and longevity has already begun with ambiguous results. The nematodal HSF1 ortholog HSF-1 overexpression induces longevity, while HSF-1 knock out shortens life-span in *C. elegans* [23,24], while mice lacking HSF1 do not display shorter lifespan [25]. Furthermore, studies in worms expressing GFP under the *hsp16.2* promoter demonstrated that the robustness of the heat shock response may predict lifespan [26]. However, a recent study using *hsp22* and *hsp70* reporter *Drosophila* strains reported a negative correlation between reporter expression and lifespan warranting further research in this direction [27].

OBESITY AND DIABETES AS A METABOLIC DISTRESS: THE PROTECTIVE ROLE OF MOLECULAR CHAPERONES

Overnutrition is one of the leading medical problems in the developed world. It is a result of an imbalanced diet,

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where the energy consumption is higher than the energy expenditure, and it leads to the development of the metabolic syndrome, characterized by obesity, type 2 diabetes, insulin resistance, dyslipidaemia and hypertension [28,29]. Metabolic syndrome is also a risk factor of atherosclerosis, stroke, cancer, arthritis and diabetes. Thus, the metabolic syndrome and diabetes may be perceived as a chronic metabolic distress which induces diseases and limits life expectancy.

Metabolic disturbances in diabetes induce a number of alterations in protein homeostasis. Glycation of the small chaperone α-crystallin of the lens compromises its chaperone activity and contributes to cataract formation [30]. High glucose-induced oxidative stress in the obese Zucker rat led to protein misfolding and aggregate formation could be cleared by autophagy, but not by the proteasome [31]. Elevated levels of HSF1, Hsp70 and Hsp90 that were found in the pancreas of diabetic monkeys may compensate for the altered protein homeostasis [32]. Intriguingly, decreased level of Hsp70 was found in the muscle of diabetic patients, as well as in the blood and in the liver of diabetic monkeys, where the level of HSF1 and Hsp70 and Hsp90 declined significantly [32,33] Indeed, impaired insulin signaling reduces HSF1 transactivation via the activation of glycogen synthase kinase-3 (GSK-3) and extra-cellular regulated kinase-1 (ERK-1) and c-jun N-terminal kinase (JNK) [34,35]. Decreased Hsp70 may not be able to inhibit JNK-induced inflammatory signaling, giving rise to a vicious cycle [35,36]. High glucose alters the interaction of the specific chaperone Hsp90 with two prominent clients endothelial nitric oxide synthase (eNOS) and inhibitor of kappaB kinase (IKK) providing a mechanistic basis for endothelial dysfunction [37,38]. How altered chaperone levels and function may contribute in other ways to peripheral insulin resistance, inflammation and to an altered extracellular stress signaling remains to be seen.

There are a number of experiments demonstrating the connection between diabetes and the heat shock response. Physical exercise is able to both raise chaperone levels and reverse diabetic alterations [34,35]. Besides, both the antidiabetic drug, metformin and the alkaloid, berberin promote the association of eNOS with Hsp90 and augment NO production and preserve endothelial function [39,40]. More importantly, a 41°C heat treatment once a week for 12 weeks improved glucose tolerance and reduced stress-activated signaling in rats fed by a high-fat diet suggesting a crosstalk between the development of HSF1 dependent thermotolerance and metabolic stress tolerance [41]. The chaperone co-inducer bimoclomol derivative, BRX-220 was also able to improve insulin sensitivity of both Zucker fatty diabetic rats, and streptozotocin-treated diabetic rats [42]. Finally, a recent study demonstrated that besides heat shock either overexpression or pharmacologic induction of Hsp70 by a novel chaperone co-inducer BGP-15 was sufficient to prevent the development of obesity-induced insulin resistance in fat-fed mice [43]. Strikingly, a 1-month treatment with BGP-15 significantly improved insulin sensitivity in insulin-resistant, nondiabetic human patients [44]. These protective effects may be related to an improved protein homeostasis, a more efficient modulation of signaling networks and to a better connectivity of subcellular organellar networks, such as the ER and the cytosol [8,45]. All these experiments highlight a vast therapeutic and preventive potential of chaperone induction in metabolic disorders.

DIETARY RESTRICTION IS AN INDUCER OF LONGEVITY AND OF THE HEAT SHOCK RESPONSE

Dietary (caloric) restriction, a moderate (30-40%) reduction of caloric intake with maintained nutrient supply is the most robust intervention that induces longevity from yeast to mammals. Dietary restriction also decreases the incidence of age-related diseases, such as cancer, diabetes, cardiovascular and neurodegenerative diseases by inducing changes in metabolism, protein biogenesis and turnover as well as by evoking resistance to a variety of stresses [46,47]. Thus, dietary restriction may emerge as a hormetic metabolic stress that activates various defense mechanisms to promote longevity.

Early studies showed that dietary restriction was able to restore the age-induced loss of Hsp70 transcription via the preservation of HSF1 activation in rat hepatocytes [48,49]. As a functional consequence, dietary restriction highly improved thermotolerance and was able to rescue 100% vs 50% of old rats subjected to hyperthermia [50]. These findings have been recapitulated in rat intestine, neural tissue, skeletal muscle and in heart myocardium, suggesting that a more efficient heat shock response induced by dietary restriction may improve muscle and neuronal function, protect from muscle cell loss and importantly may exert cytoprotection against ischemic episodes [51-55]. A small, but significant cytoprotection from heat stress could be transferred with blood serum to HepG2 cells by culturing them in serum obtained from human subjects undergoing caloric restriction, however, this study found no difference in basal Hsp70 levels [56]. Using transcriptional profiling, independent studies demonstrated a better maintenance of stress responses including chaperones both in liver and in muscle, respectively, of caloric restricted primates [57,58]. This effect was reflected in an efficient protection in both nematode and mouse models of misfolding models of neurodegenerative disorders [59,60]. Similarly to food restriction, the mimetic 2-deoxyglucose was shown to induce chaperones and protect neuronal cells from excitotoxic, oxidative and proteotoxic injury [61,62]. Intriguingly, 2-deoxyglucose blunted the Hsp70 induction and thermotolerance of prostate cancer cells suggesting that an otherwise hormetic intervention may induce a distress in the already stressed tumor cells and providing one plausible mechanism for the potent antitumor effect of dietary restriction and fasting [63]. Since an increased protein turnover is implicated in the 'cleansing' effects of dietary restriction, and chaperones are known to play an important role both in proteasomal degradation and in chaperone-mediated autophagy, it would be worth studying their crosstalk to gain a better understanding on the interplay between these processes [52,55,64-67].

THE HEAT-SHOCK RESPONSE IN THE DIETARY RESTRICTION-RELATED SIGNALING NETWORK

In recent years evidence has been accumulated that dietary restriction is not only a simple consequence of restriction of fuel and metabolism but initiates a highly regulated and orchestrated process that drives energy allocation from

reproduction to self maintenance during low food availability. Due to its versatility, short lifespan and ease of genetic manipulations much of the understanding has been obtained in the nematode *C. elegans*. Here we will mainly rely on data from worms and supplement it with findings from higher organisms.

A major endocrine sensor of food supply is insulin signaling. Reduction in insulin signaling extends life span in various organisms [68,69]. The essential role of HSF-1 and the chaperone-network in this pathway has been proven in the nematode *Caenorhabditis elegans*, acting in concert with the forkhead transcription factor FoxO/*daf-16* [24,70]. Consequently, insulin-signaling mutations delay the onset of polyglutamine toxicity in worms [71].

To address the mechanism of dietary restriction a number procedures have been developed in *C. elegans*. All of them induce a further life extension of already long-lived insulin signaling mutants suggesting that the effects of dietary restriction involve different mechanism apart form insulin-like signaling. A dilution of the food source bacteria from 10^{11} to 10^{8} *E. coli*/ml on solid agar extended lifespan of worms by 20-30%. Despite an increased stress resistance, the *C. elegans* HSF1 ortholog *hsf-1* was dispensable, however, it fully depended on the AMP-dependent protein kinase AMPK/*aak-2* and on FoxO/*daf-16* [72,73]. A similar longevity by the dilution of the bacterial food peptone was also mediated by *daf-16*, however, the contribution of *hsf-1* is, as yet, unknown [74]. The reduction of pharynx pumping rate by a genetic mutation (*eat-2*) depends on the mitochondrial gene *clk-1* while *daf-16*- and *hsf-1* are indispensable, moreover, the necessity of *clk-1* has been shown in the bacterial dilution protocol, too [24,73,75]. Yet, bacterial dilution combined with *eat-2* mutation produced a synergistic longevity [73]. Separate studies from two labs employed liquid medium, where diluting bacteria extended lifespan which was also largely *daf-16*-independent, however, depended on neuroendocrine signals by either the transcription factors FoxA/*pha-4* or Nrf2/*skn-1*, respectively [76,77]. Notably, *pha-4* also seemed to mediate the longevity of *eat-2* mutants [76]. The involvement of the heat shock response in these models has not been addressed. Dietary restriction in *Drosophila* appeared to be FoxO-independent [78]. The results highlighted above argue that a partial reduction of food source in invertebrates is heterogeneous intervention targeting various nodes of a highly connected signaling network and draws attention to the careful design and interpretation of research in mammalian models.

After reaching the adult stage, *C. elegans* displays a robust 50% increase in lifespan either in axenic medium almost devoid of calories or even in the total absence of the food source *E. coli* [79-81]. The life extending mechanism of these interventions is totally different from a partial food reduction, since *(i)* serial dilution of bacteria on the plates below a threshold (cca. 10^{7} *E. coli*/ml) killed worms within two days [73], *(ii)* the longevity by bacterial deprivation was independent on FoxO/*daf-16* and AMPK/*aak-2* [80], *(iii)* the presence of metabolically active live bacteria, or their product prevented the food deprivation-induced longevity [79,82]. Indeed, both the longevity induced as well as a potent protection in several proteotoxic models by bacterial deprivation required *hsf-1* [60]. It may be hypothesized that beyond the optimum food supply that finely tunes metabolism a signal produced by live microorganism exerts a constitutive inhibition on stress responses and HSF-1. Further research is necessary to reveal the nature and the pathway of the inhibition. For a summary of genes involved in dietary restriction-induced longevity please refer to Table **1**.

Another important nutrient sensor is the target of rapamycin (TOR) kinase which is activated by both nutrient supply and insulin. Inhibition of TOR in wild type and in *eat-2* mutant worms induces longevity by activating translation and inhibiting autophagy [66,83]. Reduction of translational output *per se* induces thermotolerance and longevity, the latter being additive to that of both insulin-like and *eat-2* mutants [83,84]. Similarly, both insulin-like and *eat-2* mutants require autophagy for their longevity, however, activation of autophagy is not sufficient to extend lifespan [66,67]. Though there is no direct evidence on the role of the protea-

Table 1. Requirement of Genes for Longevity in Various Dietary Restriction (Mimetic) Interventions *C. elegans*. See the text for details and for references. * B. Dancsó and C. Sőti, unpublished results, ** Tóth ML, Dancsó B, Csermely P and Sőti C, submitted manuscript

Intervention	**Genes (human ortholog)**						
	***daf-16* (FoxO)**	***aak-2* (AMPK)**	***hsf-1* (HSF1)**	***clk-1* (CLK1)**	***skn-1* (Nrf2)**	***pha-4* (FoxA)**	***sir-2.1* (SirT1)**
Bacterial dilution	yes	yes	no	Yes	no	no	no
Peptone dilution	yes	yes	?	?	?	?	?
Pharynx pumping (eat-2)	no	no	no	Yes	?	yes	yes/no
Liquid DR #1	no	?	?	?	yes	?	?
Liquid DR #2	partial	partial	?	?	?	yes	?
Bacterial deprivation	no	*no	yes	?	?	?	*no
Resveratrol	yes/no	yes	**yes	?	?	?	yes

somal system in dietary restriction, a close relationship can be suspected. A proper protein homeostasis seems to be a critical determinant in longevity and closely connected to the mechanism of dietary restriction. Namely, translation modulates protein output, molecular chaperones guard protein conformation and protein disposal clears the damaged and unnecessary proteins and provides fuel from intrinsic stores to build new proteins, elements of an adaptive response.

HSF1 and chaperones are involved in several physiological and housekeeping processes like signaling and immunity, however, the exact contribution of their significance related or unrelated to protein homeostasis has not been addressed. Taking use of the poikilothermic nature of *C. elegans* we investigated the effect of dietary deprivation on the lifespan of wild type and *hsf-1* loss-of function animals at temperatures below and above the ambient 20ºC. Fig. (**1**) shows that decreasing temperature progressively lengthens the lifespan of both the wild type and *hsf-1* worms to a similar extent (to 280% mean lifespan at 15ºC) suggesting that at the temperatures tested there is a uniform need of *hsf-1* for survival. Besides, dietary deprivation progressively extends the lifespan of wild type worms with increasing temperature. However, this effect is completely abolished in *hsf-1* mutants. These data indicate that either the effect of dietary deprivation is independent of the protein homeostatic burden or HSF-1 mediates the longevity effect via both protein-maintenance and independent means. Indeed, it has been shown that HSF1 regulates 3% of the genome in yeast and supports such core cellular functions as cell size, translation and ribosome biogenesis as well as glucose metabolism in mammalian tumor cell lines [85,86]. It may well be, that HSF1 orchestrates a network that optimizes fuel utilization and overall cellular and organismal functions in the postmitotic worm to attain efficient self maintenance and longevity. However, the mechanisms and the gene network involved remains to be identified.

RESVERATROL AND SIR2 ARE ACTIVATORS OF HSF1

The plant polyphenol resveratrol recapitulates the dietary restriction-induced longevity of yeast, invertebrates and mice on a high-fat diet (but not on a normal diet) [87-91]. Other studies, nevertheless, could not show an increased longevity upon resveratrol treatment in invertebrate and normal fed mouse trials [92,93]. Resveratrol has been shown to act via the Sir2 (silent information regulator) sirtuin family of NAD^+-dependent protein deacetylases in a FoxO/*daf-16*-independent manner [88,94]. Sir2 deacetylates a number of key regulatory proteins including FoxO, PPARγ, and its activator PGC-1α and, responsible for survival under stress and the metabolic change [95-97]. Genetic activation of Sir2/*sir-2.1* induces a lifespan extension in worms and mice overexpressing the major mammalian ortholog SirT1 show reduced energy expenditure, improved metabolism and protection from diabetes [98-100]. Sir2/*sir-2.1* seems to mediate some forms of dietary restriction including the *eat-2* mutation in *C. elegans* and SirT1 is required for dietary restriction-induced physical activity in mice [101-103]. However, in other studies dietary restriction by *eat-2* by bacterial dilution or by bacterial deprivation did not depend on Sir2 [73,80,83].

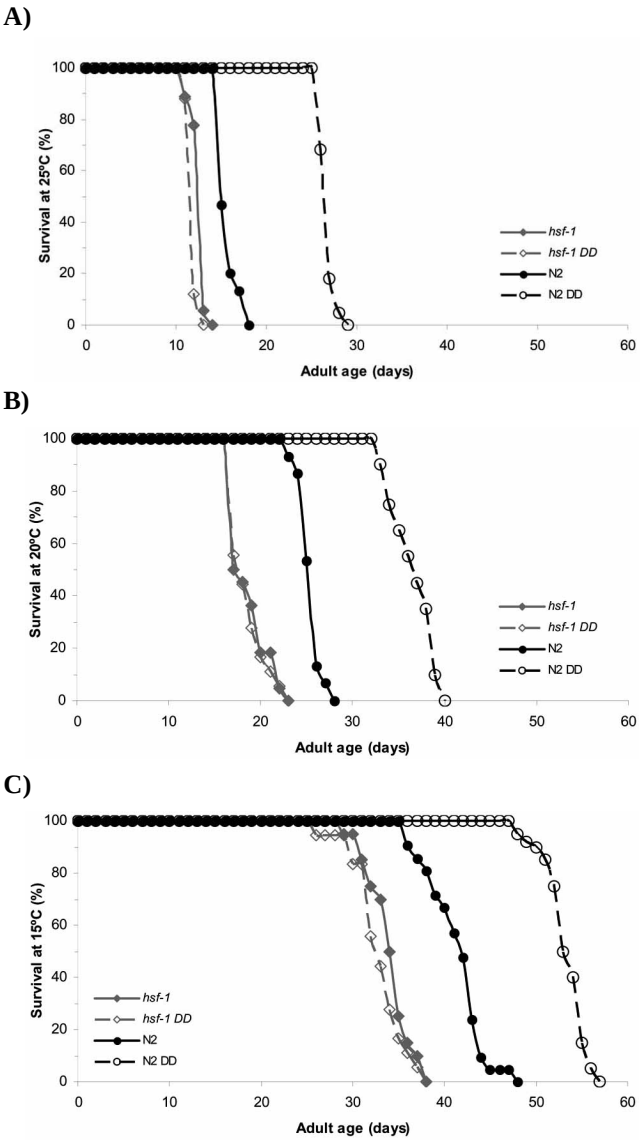

Fig. (1). HSF-1 mediates dietary deprivation-induced life-span extension at various temperatures. Survival curves of wild type (N2) and *hsf-1(sy441)* loss of function nematodes at 25ºC **(A)**, 20ºC **(B)** or 15ºC **(C)** in the presence (closed symbols) or absence (dietary deprivation, DD, open symbols) of the food source, *E. coli*. Synchronized strains were grown on NGM plates containing 50 μM FudR (5-fluoro-2'-deoxyuridine). DD was initiated at day 4 of adulthood. Animals that crawled off the plate, exploded, bagged, or became contaminated were removed from the evaluation process. The figure shows representative curves of 3 independent experiments.

Both resveratrol and Sir2/SirT1 were protective in misfolding neurodegenerative models suggesting an improved protein homeostasis [104,105]. As a potential mechanism, we have shown that resveratrol specifically induces the heat shock response and protects various mammalian cells against heat stress and proposed a Sir2-dependent modulation of HSF1 [106]. Recently this has been experimentally demonstrated by showing that SirT1 deacetylates HSF1 and prolongs its binding to the heat shock promoter [107]. However, the exact contribution of the heat-shock response to resvera-

trol-induced longevity remained unknown. Recent results from our lab show that HSF-1 mediates resveratrol-induced longevity in a manner dependent on Sir-2.1 in *C. elegans* (Tóth ML, Dancsó B, Csermely P and Sőti C, submitted manuscript, Table **1**). These findings link the metabolic and proteotoxic stress responses in longevity regulation in invertebrates. How this interaction modulates aging in mammals remains to be determined.

SUMMARY AND PERSPECTIVES

The observations presented in this review demonstrate an intimate connection between metabolic disturbances, dietary restriction and protein homeostasis with a special emphasis on the HSF1-orchestrated heat shock response. Most experiments elucidating the dietary restriction-induced longevity regulation have been performed in invertebrates and analyzing one or two components of the signaling pathways. Future studies employing systems biology and mammalian models will reveal the topology of the signaling network and the place of HSF1 in response of dietary restriction. This may lead to the development of novel strategies to attain the final goal of healthy aging and to prevent or heal civilization diseases.

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Absence of effects of Sir2 overexpression on lifespan in *C. elegans* and *Drosophila*

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Overexpression of sirtuins (NAD^+-dependent protein deacetylases) has been reported to increase lifespan in budding yeast (*Saccharomyces cerevisiae*), *Caenorhabditis elegans* and *Drosophila melanogaster*[1–3]. Studies of the effects of genes on ageing are vulnerable to confounding effects of genetic background[4]. Here we re-examined the reported effects of sirtuin overexpression on ageing and found that standardization of genetic background and the use of appropriate controls abolished the apparent effects in both *C. elegans* and *Drosophila*. In *C. elegans*, outcrossing of a line with high-level *sir-2.1* overexpression[1] abrogated the longevity increase, but did not abrogate *sir-2.1* overexpression. Instead, longevity co-segregated with a second-site mutation affecting sensory neurons. Outcrossing of a line with low-copy-number *sir-2.1* overexpression[2] also abrogated longevity. A *Drosophila* strain with ubiquitous overexpression of *dSir2* using the UAS-GAL4 system was long-lived relative to wild-type controls, as previously reported[3], but was not long-lived relative to the appropriate transgenic controls, and nor was a new line with stronger overexpression of *dSir2*. These findings underscore the importance of controlling for genetic background and for the mutagenic effects of transgene insertions in studies of genetic effects on lifespan. The life-extending effect of dietary restriction on ageing in *Drosophila* has also been reported to be *dSir2* dependent[3]. We found that dietary restriction increased fly lifespan independently of *dSir2*. Our findings do not rule out a role for sirtuins in determination of metazoan lifespan, but they do cast doubt on the robustness of the previously reported effects of sirtuins on lifespan in *C. elegans* and *Drosophila*.

The role of sirtuins in ageing was discovered in budding yeast, where overexpression of *SIR2* increases replicative lifespan[5]. It was then reported that elevated sirtuin levels increase lifespan in the nematode *C. elegans*[1,2,6] and the fruitfly *Drosophila*[3], indicating an evolutionarily ancient role of sirtuins in longevity assurance[7]. Dietary restriction (reduced food intake short of starvation) extends lifespan in organisms ranging from yeast to mammals[8], and initial studies indicated that dietary restriction increases lifespan by activating sirtuins in yeast[9], *C. elegans*[10] and *Drosophila*[3]. Pharmacological activation of sirtuins has therefore been widely promulgated as a potential means to mimic dietary restriction and slow ageing in humans[11]. However, several aspects of the role of sirtuins in ageing have proved controversial[12]. Subsequent studies have indicated that sirtuins do not mediate the effects of dietary restriction on ageing, at least in budding yeast and *C. elegans*[13,14]. The plant-derived polyphenol resveratrol and other compounds have been reported to activate sirtuins and extend lifespan[15,16], but more recent findings have challenged both effects[17–20]. We therefore re-examined the effects of sirtuin overexpression on lifespan in *C. elegans* and *Drosophila*. In particular, we wished to exclude the possibility that the increased longevity observed in strains with overexpression of sirtuin genes is caused by differences in genetic background, or by the mutagenic effects of transgene insertion, which frequently confound studies of the genetics of ageing[4].

We first examined a high-copy-number *sir-2.1* transgenic *C. elegans* strain (LG100) carrying the integrated transgene array *geIn3* [*sir-2.1 rol-6*(*su1006*)] (ref. 1). As expected, this strain was long-lived (Fig. 1a and Supplementary Table 1). However, outcrossing (×5) of *geIn3* to wild type (N2) abrogated the increase in longevity (Fig. 1a and Supplementary Table 1) without affecting SIR-2.1 protein levels (Fig. 1b). This loss of longevity upon outcrossing was verified by an independent research team (Supplementary Table 2).

LG100 showed a neuronal dye-filling (Dyf) defect[21] that did not segregate with the transgene upon outcrossing (Supplementary Fig. 2a). Dyf mutants often show extended lifespan[22]. To determine whether the longevity of LG100 might be attributable to a *dyf* mutation, we derived from this strain three Dyf, non-Rol lines (lacking *geIn3*) and three non-Dyf, Rol lines (carrying *geIn3*). Dyf, non-Rol lines were long-lived and showed wild-type SIR-2.1 protein levels (Fig. 1c, d and Supplementary Table 3). Non-Dyf, Rol lines showed elevated SIR-2.1 protein levels but had wild-type lifespans. Dyf mutant longevity seemed to be partially dependent on *daf-16* (Supplementary Fig. 2b), as seen previously for other Dyf mutants[22]. The co-segregation of longevity with this *dyf* mutation, but not with *geIn3*, was previously noted by another research team (S. S. Lee, personal communication). Furthermore, knockdown of *sir-2.1* expression in LG100 using RNA-mediated interference did not suppress longevity, despite lowering SIR-2.1 protein to wild-type levels (Fig. 1e, f and Supplementary Table 4). Taken together, these results indicate that the longevity of LG100 is attributable to an unidentified *dyf* mutation (or possibly another mutation closely linked to the *dyf* locus), and that high-level overexpression of *sir-2.1* is not sufficient to increase lifespan in these strains.

A low-copy-number transgenic strain (NL3909) overexpressing *sir-2.1* (ref. 7) is also long-lived[2]. We confirmed the increased lifespan of NL3909 (*pkIs1642 [sir-2.1 unc-119] unc-119*(*ed3*)) relative to the control strain NL3908 (*pkIs1641 [unc-119] unc-119*(*ed3*)) (Fig. 1g and Supplementary Table 5). We also observed an apparent increase in SIR-2.1 protein levels in NL3909 relative to NL3908 (Fig. 1h). Outcrossing (×6) of NL3909 once again abrogated longevity (Fig. 1g and Supplementary Table 5) without affecting SIR-2.1 protein levels (Fig. 1h and Supplementary Fig. 1c). This effect of outcrossing was independently verified (Supplementary Table 6). Thus, the longevity of NL3909 also seems to be attributable to effects of genetic background rather than to *pkIs1642*.

The duplication *mDp4* includes the *sir-2.1* locus, and the *mDp4*-containing strain DR1786 is long-lived[1]. We found that DR1786 is

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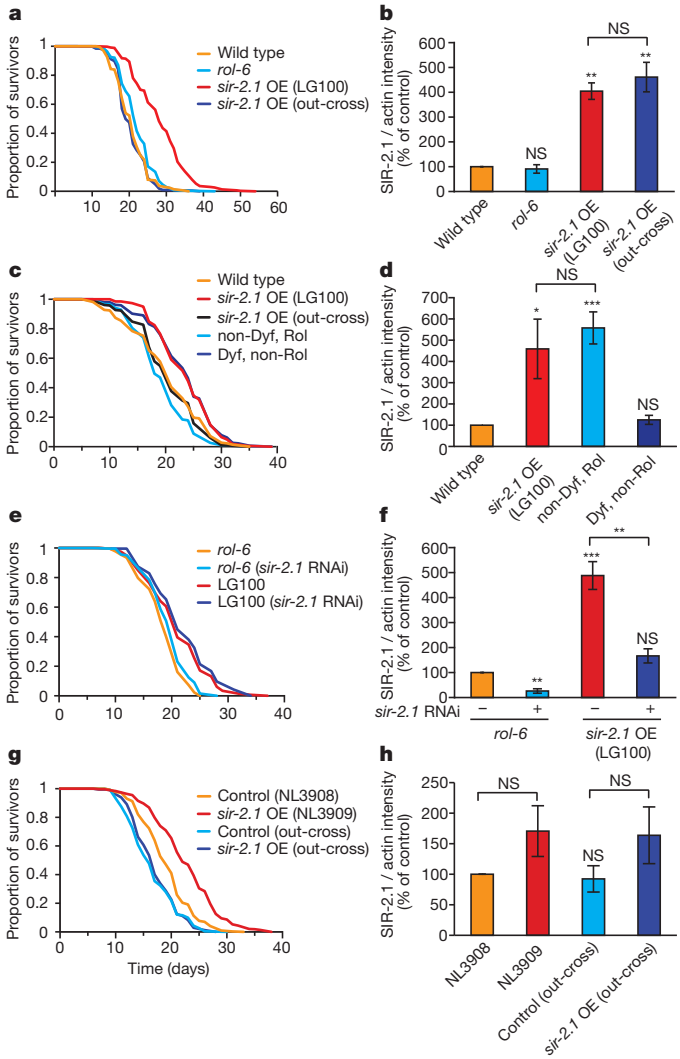

Figure 1 | Longevity of LG100 and NL3909 is not attributable to *sir-2.1* overexpression in *C. elegans*. **a**, **b**, Outcrossing of LG100 removes lifespan extension without affecting SIR-2.1 protein levels. Data in **b** are derived from western blots (mean of three trials, each using an independent protein preparation). A representative western blot is shown in Supplementary Fig. 1a. Quantitative reverse transcriptase PCR showed that *sir-2.1* mRNA is also elevated in both strains (data not shown). **c**, LG100-derived Dyf, non-Rol segregant lines are long-lived whereas non-Dyf, Rol lines are not. **d**, Non-Dyf Rol segregant lines have elevated SIR-2.1 levels, whereas Dyf, non-Rol lines do not. **e**, **f**, *sir-2.1* RNAi does not suppress LG100 longevity, but reduces SIR-2 protein levels. **g**, **h**, Outcrossing of NL3909 removes lifespan extension without affecting SIR-2.1 protein levels. See Supplementary Tables 1–5 for lifespan statistics for **a**, **c**, **e** and **g**, respectively. OE, overexpression. All error bars represent s.e.m. *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$; ***, $P < 0.001$; NS, not significant; Student's *t*-test (two-tailed). One remaining possibility is that the outcrossed *sir-2.1* strains both contain second-site mutations that suppress longevity effects. However, *daf-2* RNAi strongly induced longevity in both strains (data not shown), arguing against the presence of a general suppressor of longevity in each case.

indeed long-lived, and also shows elevated *sir-2.1* expression. However, longevity was not suppressed by *sir-2.1* RNA interference (RNAi) (Supplementary Fig. 3 and Supplementary Table 7) indicating causation by factors other than *sir-2.1*, either on *mDp4* or elsewhere in the genome.

In *Drosophila*, overexpression of *dSir2* reportedly increases lifespan relative to wild-type controls[3]. Overexpression was achieved using the GAL4-UAS binary system[23], with the largest increases in lifespan being produced by the combination of EP-UAS–*dSir2* ($dSir2^{EP2300}$) with a ubiquitously expressed tubulin–GAL4 driver. We outcrossed these

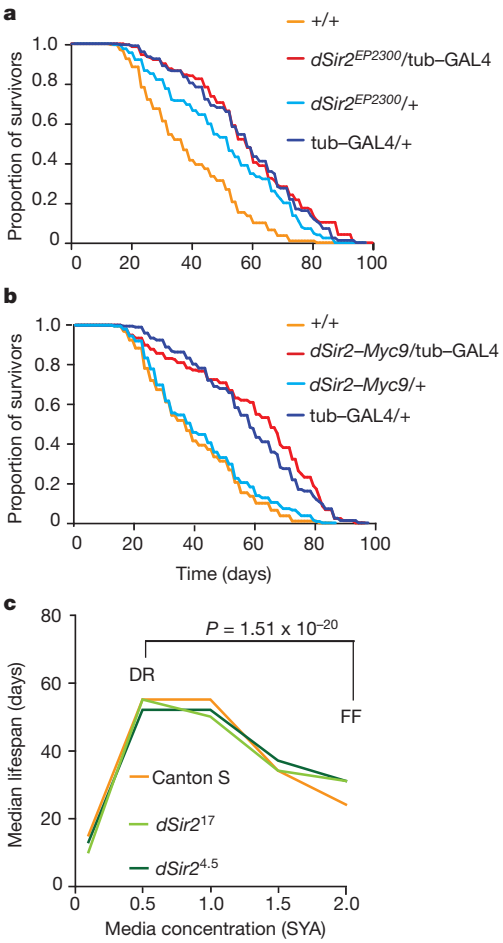

Figure 2 | Absence of effects of *dSir2* on lifespan in *Drosophila*. All lines were outcrossed into w^{Dah} (+/+). **a**, Lifespan in flies overexpressing $dSir2^{EP2300}$ driven via tubulin–GAL4 (tub–GAL4) is longer than in the wild type, but not longer than in the *tubulin–GAL4*/+ genetic control. Median lifespans: +/+, 39 days; $dSir2^{EP2300}$/*tubulin–GAL4*, 59 days; $dSir2^{EP2300}$/+, 53 days, *tubulin–GAL4*/+, 60 days. $P = 0.0006$ for comparison of $dSir2^{EP2300}$/*tubulin–GAL4* versus $dSir2^{EP2300}$/+; $P = 0.9295$ for $dSir2^{EP2300}$/*tubulin–GAL4* versus *tubulin–GAL4*/+; $P < 0.0001$ for $dSir2^{EP2300}$/*tubulin–GAL4* versus +/+. **b**, Lifespan in flies overexpressing *dSir2–Myc9* is longer than in wild type, but not longer than in the *tubulin–GAL4* control. Median lifespans: +/+, 39 days; *dSir2–Myc9*/*tubulin–GAL4*, 67 days; *dSir2–Myc9*/+, 41 days; *tubulin–GAL4*/+, 60 days. *dSir2–Myc9*/*tubulin–GAL4* versus *dSir2–Myc9*/+, $P = 0.0001$; *dSir2–Myc9*/*tubulin–GAL4* versus *tubulin–GAL4*/+, $P = 0.1354$; *dSir2–Myc9*/*tubulin–GAL4* versus +/+, $P < 0.0001$. All comparisons were made using log-rank tests, $n = 200$. **c**, The effect of dietary restriction on *Drosophila* lifespan is not *dSir2*-dependent. Flies were assayed over five concentrations of SYA media and data are presented as the median lifespan on each food concentration. All lines were outcrossed into Canton S (+/+). *P* values confirm that all flies respond normally to dietary restriction when median lifespans are compared for dietary restriction (DR) versus fully-fed (FF) conditions[30].

two transgenes (×6) into the control white Dahomey (w^{Dah}) background. When assayed on a medium similar to that used in the original study, EP-UAS–*dSir2*/*tubulin–GAL4* flies were longer-lived than wild-type controls, as previously reported[3] (Fig. 2a). However, they did not live longer than the *tubulin–GAL4*/+ control flies (Fig. 2a). This implies that lifespan extension is due to transgene-linked genetic effects other than the overexpression of *dSir2*. Lifespan was assayed on a range of food media (see Methods for details) to test for nutrient dependence of any effect. However, in no case were EP-UAS–*dSir2*/*tubulin–GAL4* flies longer-lived than one or both transgenic controls (Supplementary Fig. 4).

The lack of an observable effect on lifespan could reflect the relatively modest increase in *dSir2* expression in EP-UAS–*dSir2*/*tubulin–GAL4*

flies, both in terms of messenger RNA levels (Supplementary Fig. 5) and protein levels (increased by 35% relative to wild type; Supplementary Fig. 6). We therefore created lines with a higher level of overexpression of *dSir2* (UAS–*dSir2–Myc9*/*tubulin–GAL4*). Here, *dSir2* mRNA and protein levels were robustly increased relative to wild type (an increase of 318% relative to wild-type protein levels; Supplementary Figs 5 and 6). We examined recombinant protein raised in *Escherichia coli* to check that the presence of the Myc tag did not interfere with dSir2 histone deacetylase activity, as measured by deacetylation of the fluorophore-containing p53 substrate (Fluor de Lys) or of native acetylated histone H4 substrates, and it did not (Supplementary Fig. 7). We also found that dSir2 histone deacetylase activity was unaffected by addition of resveratrol in either assay (Supplementary Fig. 7). We saw no increase in lifespan in UAS–*dSir2–Myc*/*tubulin–GAL4* flies relative to *tubulin–GAL4*/+ controls, either on a food medium similar to that used in the original study (Fig. 2b), or relative to either control on a range of other media (Supplementary Fig. 4b, c, f). An independent research team also saw no increase in lifespan in UAS–*dSir2–Myc9*/*tubulin–GAL4* flies (Supplementary Fig. 8). These results indicate that the previously observed longevity of EP-UAS–*dSir2*/*tubulin–GAL4* flies was not attributable to elevated expression of *dSir2*, and that stronger, ubiquitous overexpression of *dSir2* also does not extend fly lifespan.

The role of sirtuins in the extension of lifespan by dietary restriction in yeast and *C. elegans* is controversial, with several groups reporting that sirtuins are not required for lifespan extension via dietary restriction in both organisms[8]. In *Drosophila*, it was reported that dietary restriction does not increase lifespan in *dSir2* deletion-mutant flies[3]. We tested this too, using the deletion alleles $dSir2^{4.5}$ (tested previously[3]) and $dSir2^{17}$. We first outcrossed these alleles (Supplementary Fig. 9a) into the Canton S wild type (see Methods), which was used in the previous dietary-restriction study[3]. We then checked the effect of each allele on *dSir2* gene expression. The allele $dSir2^{17}$ abrogated *dSir2* mRNA, indicating that this is a null allele. By contrast, $dSir2^{4.5}$, which contains a relatively small deletion at the 5′ end of the gene, did not reduce *dSir2* mRNA levels (Supplementary Fig. 9b, c).

To reassess the role of *dSir2* in dietary restriction in *Drosophila*, we compared lifespans of wild-type (Canton S), $dSir2^{4.5}$ and $dSir2^{17}$ homozygotes. All genotypes responded similarly and normally to dietary restriction in trials conducted by two independent research teams (Fig. 2c and Supplementary Fig. 10), hence the effect of dietary restriction on lifespan did not require *dSir2*.

In this study, we were unable to verify the effect of sirtuin overexpression on lifespan in either *C. elegans* or *Drosophila*. Increased lifespan was seen in two *C. elegans* lines with elevated *sir-2.1* expression, derived from independent studies, as previously reported, but in each case this was abrogated by outcrossing. Overexpression of *sir-2.1* does exert effects on traits other than lifespan. For example, *geIn3* is neuroprotective in a worm model of neuron dysfunction in Huntington's disease[24] and, notably, this effect is not attributable to the *dyf* mutation (Supplementary Fig. 11). Moreover, both NL3909 and its outcrossed derivative are thermotolerant (M. Somogyvári and C. Sőti, unpublished data). In *Drosophila*, lines overexpressing *dSir2* were longer-lived than wild-type controls, as previously reported, but they were not longer-lived than lines containing the appropriate transgenic controls. The fact that all transgenic lines were longer-lived than the Dahomey wild type into which they had been outcrossed could reflect heterosis in the vicinity of the transgene inserts, or a mutagenic effect of the GAL4 insert.

Lifespan was not increased either by overexpression of *sir-2.1* from its own promoter in *C. elegans,* or by ubiquitous overexpression of *dSir2* from a heterologous promoter in *Drosophila*. Our findings call into question the robustness of earlier reports of a role for sirtuins in longevity assurance on the basis of overexpression in *C. elegans* and *Drosophila*, and also the role of *dSir2* in the response to dietary restriction in *Drosophila*. However, sirtuins can affect lifespan in animals under certain conditions: *C. elegans daf-2(e1370)* mutants are hypersensitive to genetic effects on lifespan[25], and in these mutants, deletion of *sir-2.1* reproducibly increases lifespan[6] (Supplementary Fig. 12).

Our finding that resveratrol does not activate the histone deacetylase activity of dSir2 using a native histone H4 peptide is consistent with earlier findings using yeast SIR2 and mammalian SirT1 (refs 17, 18). Resveratrol increased *Drosophila* lifespan in one study[26] but not in another[27]. In principle, this could reflect sensitivity of resveratrol effects to subtle differences in culture conditions. If this were the case, our findings would indicate that such effects are not attributable to direct activation of dSir2 by resveratrol.

METHODS SUMMARY

Nematode strains and maintenance. Nematodes were maintained on nematode-growth-medium agar at 20 °C, with *E. coli* OP50 bacteria as a food source. Nematode strains used included: wild type (N2), GA707 *wuEx166 [rol-6(su1006)]* (*rol-6* control), LG100 *geIn3 [sir-2.1 rol-6(su1006)] dyf-?(wu250)*, NL3909 *pkIs1642 [sir-2.1 unc-119] unc-119(ed3)* and the control strain NL3908 *pkIs1641 [unc-119] unc-119(ed3)*.

Nematode lifespan measurements. These were performed as previously described[28], at 20 °C. To prevent progeny production, 5-fluoro-2′-deoxyuridine (FUdR) was added to seeded plates, to a final concentration of 10, 40 or 50 μM. Before testing the effects of RNAi on lifespan, worms were kept for two generations on the RNAi bacteria. The statistical significance of effects on lifespan was estimated using the log-rank test, performed using JMP, Version 7 (SAS Institute).

***Drosophila* stocks and maintenance.** *Tubulin–GAL4* and $dSir2^{EP2300}$ were obtained from the Bloomington Stock Center. The *dSir2–Myc2* and *dSir2–Myc9* lines were generated by germline transformation into strain w^{04}. The $dSir2^{4.5}$/SM6B, $dSir2^{17}$/Cyo and Canton S lines were gifts from S. Pletcher, J. Rine and S. Helfand. All lines were outcrossed at least six times into the relevant controls. Experiments were performed at 25 °C on a 12 h:12 h light:dark cycle at constant humidity.

***Drosophila* lifespan assays.** Flies were bred at standard density, allowed to mate for 48 h after emerging, then sorted into ten females per vial. Vials were changed every 48 h, and deaths per vial were scored until all flies were dead. In overexpression studies, $n = 200$. In *dSir2*-mutant studies, $n = 100$. For statistical methodology, see earlier.

dSir2 deacetylation assays. We used both the SirT1 Fluorimetric Drug Discovery Kit (Enzo Life Sciences) and an HPLC-based acetyl-histone-H4 deacetylation assay[29]. *dSir2* and *dSir2–Myc* were cloned into pET SUMO (Invitrogen) and proteins were purified on HisPur cobalt spin columns (Thermo Scientific).

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to D.G. (david.gems@ucl.ac.uk).

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METHODS

Nematode strains and maintenance. *Caenorhabditis elegans* were cultured under standard monoxenic conditions[31,32]. Strains used included N2 (wild type), GA707 *wuEx166 [rol-6(su1006)]*, HT1593 *unc-119(ed3)*, LG100 *geIn3* [*sir-2.1 rol-6(su1006)*] *dyf-?(wu250)*, NL3908 *pkIs1641* [*unc-119*] *unc-119(ed3)* and NL3909 *pkIs1642* [*sir-2.1 unc-119*] *unc-119(ed3)*.

Outcrossing of nematode strains. LG100 was outcrossed with N2 and the Rol trait was used to detect the presence of *gIn3*. NL3908 and NL3909 were outcrossed using HT1593 *unc-119(ed3)*. Rescue of Unc (uncoordinated movement) was used to detect the presence of the transgene array.

Isolation of Dyf, non-Rol and non-Dyf, Rol lines. LG100 was crossed with N2 and lines were established from individual F_2 animals with Dyf, non-Rol or non-Dyf, Rol phenotypes. The Dyf phenotype was identified by staining with the dye 1,1′-dioctadecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate (DiI) and looking for absence of dye uptake into the amphid and phasmid neurons. Non-Dyf, Rol F_2 animals that were heterozygous for the *geIn3* transgene array (the *rol-6* marker is dominant) were identified by the presence of non-Rol animals in the F_3, and were excluded.

RNAi in *C. elegans*. Animals were fed *E. coli* containing the HT115 vector, either with or without a portion of the *sir-2.1* gene cloned into it. The *sir-2.1* feeding strain was obtained from the Ahringer RNAi library[33]. Worms were maintained on RNAi feeding strains for two generations before lifespan measurements. One day before starting measurements, FUdR was applied to seeded plates at 10 μM to prevent progeny production.

Analysis of SIR-2.1 protein levels in *C. elegans*. Protein was prepared from synchronous nematode cultures (L4 larvae and young adults) raised on *E. coli* OP50 or RNAi bacteria for two generations. Western blots were performed with anti-actin monoclonal antibodies (Santa-Cruz Biotechnology) and an anti-SIR-2.1 polyclonal antibody (provided by A. Gartner[34]). For all assays, 3–5 replicate worm cultures were used.

Neuroprotection assays in *C. elegans*. To test for sirtuin-mediated protection from expanded polyglutamines (polyQs), we crossed GA919 (*geIn3* dissociated from *dyf-?(wu250)*) to strains carrying integrated polyQ arrays. These polyQ strains co-express the first 57 amino acids of human huntingtin with either 19 or 128 Gln residues fused to cyan fluorescent protein and expressed from the *mec-3* promoter, and YFP expressed from the *mec-7* promoter in touch-receptor neurons[24]. The response to touch at the tail was tested as previously described[24]. Three trials were performed and 150–200 animals were tested per genotype.

Lifespan analysis in *C. elegans*. Lifespans of synchronized population cohorts were measured as previously described[28]. FUdR was applied to the plates at 10, 40 or 50 μM (see Supplementary Tables). Lifespan experiments were performed at 20 °C. A small proportion of animals were censored, usually due to uterine rupture, which mainly occurred at mid-adulthood (~day 9–11).

Statistical analysis of *C. elegans* data. The statistical significance of effects on lifespan was estimated using the log-rank test, performed using JMP, Version 7 (SAS Institute).

***Drosophila* stocks and maintenance.** *Tubulin–GAL4* and $dSir2^{EP2300}$ lines were obtained from the Bloomington Stock Center. *dSir2–Myc2* and *dSir2–Myc9* lines were generated by germline transformation. These were outcrossed into white Dahomey (w^{Dah}). The strains $dSir2^{4.5}$/SM6B (ref. 35) and $dSir2^{17}$/Cyo (ref. 36), provided by S. Pletcher and J. Rine, were outcrossed into Canton S. All lines were outcrossed at least six times. The presence of the deletion was detected by PCR using the following primers: 149F (5′-AGATATGACATAAGGCAGTGGC-3′), 1427R (5′-TCCCGTTAGCACAATGATCTTC-3′) and 3909R (5′-GAAGGCGG TAGCAATGG TGACAA-3′). Flies were maintained at 25 °C on a 12 h:12 h light: dark cycle at constant humidity.

Myc-tagged *dSir2*. The Myc tag was added to RE27621 (Riken) using standard techniques and cloned into pUASP. The construct was microinjected into w^{04} and the transformant lines *dSir2–Myc2* and *dSir2–Myc9* were recovered. Primers were: Sir5′R2 (5′-CAAGAATTCCAACGAGAATTTTACACAGGTCGTGTG-3′), Sir3′Xba (5′-ATC GAGTCTAGACACTGCTGCTAACTGTCCTGGAGG-3′) and MYC3′Xba (5′-GAGCT ATCTAGAGGATCCGAGGAGCAGAAGCTGATC-3′).

Lifespan assays in *Drosophila*. Flies were bred at standard density (~300 flies per 200-ml bottle), allowed to mate for 48 h after emerging (once mated) and then sorted into ten females per vial (experiments performed at University College London) or 35 per vial on 15% SYA (experiments performed at University of Michigan). Vials were changed every 48 h and deaths per vial were scored until all flies were dead. The numbers of flies used in lifespan assays were: overexpression studies, $n \approx 200$ (UCL) or $n \approx 350$ (U. Michigan); dietary-restriction studies, $n = 100$. For the overexpression studies, the fly-food recipes were as follows: SYA (100 g yeast, 50 g sugar, 15 g agar, 30 ml nipagin and, in most trials, 3 ml propionic acid per litre of food); ASG (20 g yeast, 85 g sugar, 10 g agar and 60 g maize per litre of food); ASG^1 (31 g yeast, 124 g sugar, 9 g agar, 53 g cornmeal and 25 ml nipagin per litre of food); 15% SYA (150 g yeast, 150 g sugar, 21 g agar and 15 ml tegosept). For the dietary-restriction trials, the food dilutions used were as follows: 15 g agar, 30 ml nipagin, 3 ml propionic acid, with yeast and sugar both altered to final concentrations of 10 g, 50 g, 100 g, 150 g or 200 g per litre of food. All food was prepared as previously described[27].

Genetic crosses in *Drosophila*. *Tubulin–GAL4/TM3* males were crossed to $dSir2^{EP2300}$, *dSir2–Myc2* or *dSir2–Myc9* virgin females, and $dSir2^{EP2300}$/+; *tubulin–GAL4*/+, *dSir2–Myc2*/+; *tubulin–GAL4*/+ or *dSir2–Myc9*/+; and *tubulin–GAL4*/+ females were selected from the progeny. For the controls, *tubulin–GAL4/TM3* males or $dSir2^{EP2300}$, *dSir2–Myc2* or *dSir2–Myc9* virgin females were crossed to w^{Dah} and $dSir2^{EP2300}$/+, *tubulin–GAL4*/+, *dSir2–Myc2*/+ or *dSir2–Myc9*/+ females were selected from the progeny.

Quantitative reverse transcriptase PCR in *Drosophila*. RNA was extracted from ten females at 10 days of age using standard techniques and transcribed into cDNA. Four biological replicates were run per genotype, each in triplicate. Samples were normalized to either actin5C or ribosomal protein 49 (RP49). Primers used were: Sir2-4 5′-GCTCTCCACCGTTGTCTGAGGGCC-3′ (ref. 3), Sir2-5 5′-GGCGGCAGCTGTGCTGCGATGAG-3′ (ref. 3), Actin5CF 5′-CAC ACCAAATCTTACAAAATGTGTGA-3′, ActinCR 5′-AATCCGGCCTTGCAC ATG-3′, RP49F 5′-ATGACCATCCGCCCAGCATCAGG-3′ and RP49R 5′-ATCTCGCCGCAGTAAACG-3′.

Analysis of dSir2 protein levels. Protein was extracted from 30 females at 7 days of age. Western blots were performed using antibodies c-myc 9E10 (Santa Cruz Biotechnology), p2E2 (Developmental Studies Hybridoma Bank) and tubulin (Sigma).

dSir2 deacetylation assays. Sequences encoding *dSir2* (RE27621) and *dSir2–Myc* were cloned into pET SUMO (Invitrogen) and proteins were purified on HisPur cobalt spin columns (Thermo Scientific). For the Fluor de Lys assay, using the SirT1 Fluorimetric Drug Discovery Kit (Enzo Life Sciences), results presented are the mean ± s.e.m. of three biological replicates. In each biological replicate, samples were run in triplicate. Final concentrations were: resveratrol and suramin, 0.2 mM; NAD^+ 0.1 mM. Deacetylation of native acetyl-histone-H4 peptide was monitored by HPLC[29]. Deacetylation of histone H4 amino-terminal peptide (SGRGKGGKGLGKGGA(acetyl-K)RHRC) (Biomatik) was carried out using 500 μM NAD^+, 100 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5 mM dithiothreitol and 0.05% Triton X-100, and monitored by HPLC (Agilent 1100) with an ACE C8-300 150 × 3.0 mm column. The elution profiles were analysed using Chemstation for LC 3D software.

Statistical analysis of *Drosophila* data. Survivorships and the response to dietary restriction were compared using the log-rank test and analyses were performed using JMP, Version 7 (SAS Institute).

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Misfolded proteins inhibit proliferation and promote stress-induced death in SV40-transformed mammalian cells

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ABSTRACT **Protein misfolding is implicated in neurodegenerative diseases and occurs in aging. However, the contribution of the misfolded ensembles to toxicity remains largely unknown. Here we introduce 2 primate cell models of destabilized proteins devoid of specific cellular functions and interactors, as *bona fide* misfolded proteins, allowing us to isolate the gain-of-function of non-native structures. Both GFP-degron and a mutant chloramphenicol-acetyltransferase fused to GFP (GFP-Δ9CAT) form perinuclear aggregates, are degraded by the proteasome, and colocalize with and induce the chaperone Hsp70 (HSPA1A/B) in COS-7 cells. We find that misfolded proteins neither significantly compromise chaperone-mediated folding capacity nor induce cell death. However, they do induce growth arrest in cells that are unable to degrade them and promote stress-induced death upon proteasome inhibition by MG-132 and heat shock. Finally, we show that overexpression of all heat-shock factor-1 (HSF1) and Hsp70 proteins, as well as wild-type and deacetylase-deficient (H363Y) SIRT1, rescue survival upon stress, implying a noncatalytic action of SIRT1 in response to protein misfolding. Our study establishes a novel model and extends our knowledge on the mechanism of the function-independent proteotoxicity of misfolded proteins in dividing cells.—Arslan, M. A., Chikina, M., Csermely, P., Sőti, C. Misfolded proteins inhibit proliferation and promote stress-induced death in SV40-transformed mammalian cells. *FASEB J.* 26, 766–777 (2012). www.fasebj.org**

Key Words: HSF1 · Hsp70 · sirtuin

Protein misfolding has been implicated in the etiology of neurodegenerative diseases and aging, although the molecular mechanisms remain elusive (1). Proteins may misfold due to inherent mutations (1), mistranslation (2), in response to denaturing stress, or from post-translational modifications (3–4) resulting in diverse non-native conformations. However, misfolding-induced exposure of hydrophobic surfaces generally results in incorrect protein-protein interactions, by which misfolded oligomeric species gain a cytotoxic function (5–6). These mechanisms are supported by studies using both repeat-expansion mutants linked to neurodegeneration and observations on non-disease-associated proteins (7–9). In these models, however, the loss of physiological function and misfolding-induced alterations in specific protein-protein interactions could not be ruled out. Hence, the exact contribution of misfolded ensembles, *per se*, to cytotoxicity is largely unclear.

Protein maintenance is achieved by a complex proteostasis network including molecular chaperones (10). Many chaperones are heat-shock proteins induced by proteotoxic stress *via* heat-shock factor-1 (HSF1; ref. 11). The heat-shock response is considered the prime sensor of misfolded proteins in the cytosol (12), suggesting that a common structural determinant of non-native ensembles mediates both their recognition and cytotoxicity. Indeed, chaperones confer protection by preventing misfolding, as well as by promoting clearance and sequestration of misfolded proteins into aggregates (13). The heat-shock response declines during aging, with a concomitant accumulation of protein aggregates, especially in postmitotic tissues (14–15). Moreover, both aging and chronic expression of polyQ expansions disrupt proteostasis in *Caenorhabditis elegans* (16–17). However, it is unknown how short-term expression of a single misfolded protein affects proteostasis in replicating cells.

Green fluorescent protein (GFP) and chloramphenicol-acetyltransferase (CAT) are widely used reporters in mammalian cells. A C-terminal fusion of a degron peptide to GFP has been reported to aggregate and induce cytotoxicity in *C. elegans* and in mammalian neurons (18), and a C-terminally truncated form of CAT (Δ9CAT) has been shown to form inclusion bodies in *Escherichia coli* (19). Both variants are almost identical to their wild-type counterparts in sequence, meaning no major difference in costs of their expression and degradation. They lack disease-associated se-

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quences, high-affinity interactors, and cellular function, which eliminates mechanisms originating from sequence-specific interactions and from losing functional benefits upon misfolding. Hence, we introduce both mutants as *bona fide* misfolded proteins in COS-7 cells, allowing the isolation of the gains of function of misfolded ensembles. In this report, we analyze their turnover, effect on the heat-shock response, chaperone-mediated protein folding, cell proliferation, and survival under basal and stress conditions. Finally, we address how genetic up-regulation of major stress-inducible defense mechanisms combat the challenge induced by protein misfolding.

MATERIALS AND METHODS

Constructs

pEGFP-C1 was obtained from Clontech Laboratories (BD Biosciences, San Jose, CA, USA). GFP-degron was a kind gift of Christopher Link (University of Colorado, Boulder, CO, USA). GFP-wtCAT was generated by cloning the PCR-amplified full-length CAT (pCAT3-Control vector; Promega, Madison, WI, USA) region into the *Bgl*II/*Pst*I sites of pEGFP-C1, using forward primer 5′-AAAGATCTATGGAGAAAAAAATCAC-3′ and reverse primer 5′-AACTGCAGTTACGCCCCGC-3′. For GFP-Δ9CAT cloning, the reverse primer was designed to omit sequences corresponding to the last 9 C-terminal amino acids (5′-AACTGCAGTTACTGTTGTAATTC-3′). Expression plasmids were kind gifts of the following colleagues: His-cBSA, Richard Voellmy (University of Miami, Miami, FL, USA); GFP170*, Elizabeth Sztul (University of Alabama, Birmingham, AL, USA); Hsp70.1pr-luc, Rick Morimoto (Northwestern University, Evanston, IL, USA); HSF1-myc-His, Lea Sistonen (Åbo Akademi University, Turku, Finland); Hsp70-myc, Kerstin Bellmann (Heinrich-Heine University, Düsseldorf, Germany); SIRT6-Flag, Haim Cohen (Bar-Ilan University, Ramat-Gan, Israel). Wild-type and deacetylase-deficient (H363Y) SIRT1 were obtained from Addgene (Cambridge, MA, USA). CMV-β-galactosidase was purchased from Promega, and pTK-luc was provided by László Hunyady (Semmelweis University, Budapest, Hungary). All constructs were verified by DNA sequencing.

Cell culture and transfection

COS-7 cells (SV40-transformed African green monkey kidney fibroblast-like cell line; CRL-1651; American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle medium (with 4.5 mg/ml glucose; Life Technologies–Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin, and 2 mM L-glutamine in a humidified incubator at 37°C with 5% CO_2. Cells were plated at a density of 2.8–3 × 10^4 cells/cm^2, 1 d before transfection. Transient transfections were performed by Lipofectamine LTX reagent (Invitrogen) with the indicated plasmids according to the manufacturer's guidelines at a total DNA (μg) to Lipofectamine (μl) ratio of 1:3. Cells were processed for analyses at 24 or 48 h post-transfection.

Purification of aggregates and Western blotting

Cells were lysed in 1% Triton X-100 in PBS with protease inhibitors (Complete; Roche, Mannheim, Germany) for 30 min at 4°C. After sonication on ice for 3 × 10 s, the lysates were cold-centrifuged (4°C) at 12,500 *g* for 15 min, and supernatants (S) containing soluble proteins were transferred to new tubes. Detergent-insoluble pellets (P), containing aggregated proteins, were solubilized in urea buffer (2% SDS, 6 M urea, 30 mM TrisHCl, pH 7.6) in the same volume, by shaking at 600 rpm for 10 min at 50°C. Equal volumes of protein extracts from each fraction (S, P) were resolved by SDS-PAGE. Western blotting was performed by transfer to nitrocellulose membrane (Bio-Rad, Hercules, CA) and by blocking in 5% (w/v) skim milk powder (Fluka, Buchs, Switzerland). Blots were probed with antibodies against GFP (20) and Hsp70 (HSPA1A/B; SPA-810; Stressgen, San Diego, CA), HSF1 (Cell Signaling, Danvers, MA, USA), SIRT1 (Cell Signaling), and SIRT6 (Sigma-Aldrich, St. Louis, MO, USA). Immunodetection was performed by ECL (PerkinElmer, Wellesley, MA, USA).

Estimation of misfolded protein expression levels

Cells were trypsinized and split, and the ratio of GFP^+ cells was determined by flow cytometry (FACSCalibur; BD Biosciences, Heidelberg, Germany) using the FL1 (530/30 BP) channel. The rest of the cells were lysed in urea buffer, and protein concentration was determined by detergent-compatible BCA protein assay (Pierce, Rockford, IL, USA). Protein extracts (10 μg) were loaded on SDS-PAGE along with known quantities of recombinant GFP (Roche). GFP expression levels (% of total protein) were determined by densitometric analysis of the GFP Western blots using Image J (U.S. National Institutes of Health, Bethesda, MD, USA), normalized to the corresponding GFP^+ cell ratio.

Immunofluorescence microscopy

Cells were grown on poly-L-lysine-coated (0.1 mg/ml; Sigma) glass coverslips. At 2 d after transfection, cells were fixed and permeabilized in prechilled (−20°C) absolute methanol for 5 min. After being washed twice in PBS, cells were blocked in 1% BSA, then incubated in an anti-Hsp70 mouse monoclonal antibody solution (BD Biosciences) recognizing both inducible (HSPA1A/B) and constitutive (HSPA8) Hsp70 for 1 h at room temperature. Following washing, cells were stained by Cy3-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch Labs, West Grove, PA, USA) for 30 min at room temperature. After washing, cells were counterstained with DAPI (Molecular Probes, Eugene, OR, USA). Coverslips were mounted using Vectashield (Vector Laboratories, Burlingame, CA, USA) and visualized by epifluorescence microscope (Nikon Eclipse E400; Nikon, Tokyo, Japan), employing ×10 × 100 (oil immersion) power with the appropriate filter sets.

HSF1 transactivation and luciferase folding assays

Cells were cotransfected with a reporter plasmid harboring the *hsp70* promoter region upstream of the firefly luciferase gene (hsp70.1pr-luc) and a β-galactosidase plasmid driven by the cytomegalovirus promoter (β-gal) for an internal control. For luciferase folding, a firefly luciferase plasmid harboring the thymidine kinase minimal promoter (TK-luc) was used. Cotransfections were done at a ratio of 4:1:1. Cells were lysed in 1X Reporter Lysis Buffer (Promega) for 20 min at room temperature with occasional rocking. For luciferase folding assay, cells were immediately cooled on ice to prevent renaturation of luciferase, and cell lysis was performed on an ice bed for 40 min. Then cells were centrifuged (4°C) at 12,500 *g* for 3 min. Cell lysates were processed for β-galactosidase

and luciferase (Bright-Glo; Promega) assays according to the manufacturer's protocols. Absorbances and luminescence were measured by a plate reader (Thermo Varioskan Flash; Thermo Scientific, Wiesbaden, Germany). Data were expressed by dividing luminescence values by β-gal activities (luc/β-gal).

BrdU incorporation

Cells grown on poly-L-lysine-coated coverslips were pulse-labeled with bromodeoxyuridine (BrdU; Molecular Probes) at 100 μM for 3 h. After being washed once in PBS, cells were fixed in 4% paraformaldehyde (Sigma) at 4°C for 30 min. Following washing in PBS containing 0.5% (w/v) BSA (wash buffer), cells were permeabilized in 1% Triton-X in PBS for 20 min at room temperature, then stained with FITC-conjugated anti-GFP antibody (Abcam, Cambridge, MA, USA) for 40 min at room temperature. Following washing, genomic DNA was denatured in 2 M HCl containing 0.5% Tween-20 for 30 min at 37°C. Cells were stained with Alexa Fluor 546-conjugated anti-BrdU antibody (Molecular Probes) for 40 min at room temperature. Following multiple washes, cells were counterstained with DAPI for 5 min and analyzed by an epifluorescence microscope (DMI6000B; Leica Microsystems, Wetzlar, Germany), employing ×10 × 10 power with the appropriate filter sets (DAPI: A4, FITC: L5, Alexa Fluor 546: TX2). Image analysis was performed by counting 600–700 cells/ area for 6 different areas randomly selected from each sample.

Annexin assay

Cells were treated with the proteasome inhibitor MG-132 (2 μM; Calbiochem, San Diego, CA, USA) on the same day of transfection, for a total duration of 42 h. Cells were trypsinized and cold-centrifuged (4°C) at 2000 rpm for 5 min. Following washing in ice-cold PBS, cells were resuspended in 100 μl annexin buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$) and stained with 5 μl annexin V-Alexa Fluor 647 conjugate (Molecular Probes–Invitrogen) for 20 min at room temperature. After the addition of 900 μl annexin buffer, cells were mixed gently and immediately analyzed by flow cytometry. Cells were counted (10,000) and gated in an FSC/SSC dot plot to eliminate cell debris. Acquisition and analysis were done by BD CellQuest Pro software (BD Biosciences). Native GFP and annexin signals were measured in a dual-parameter dot plot by FL1 (530/30 BP) *vs.* FL4 (661/16 BP) channels, respectively. The percentage of annexin$^+$ cells out of the GFP$^+$ subpopulation was calculated by the formula UR/(UR+LR)×100, as obtained from quadrant statistics.

Analysis of cellular DNA content

Cells were treated with 2 μM MG-132 1 day after transfection for 20 h. For concomitant heat-stress experiments, cells were heat-shocked at 43°C or kept 37°C at for 30 min in a circulating water bath. Cells were collected and cold-centrifuged (4°C) at 1800 rpm for 3 min. The resulting cell pellet was resuspended in 1 ml prechilled (at −20°C) 70% ethanol at room temperature for 30 min, then stored at −20°C overnight. Fixed cells were pelleted at 2200 rpm for 5 min at 4°C and resuspended in 1 ml extraction buffer (200 mM Na_2HPO_4, pH 7.8, adjusted with 200 mM citric acid) containing 10 μg/ml final concentration of RNase A (Sigma). Following incubation at room temperature for 30 min, the remaining nuclear DNA was stained by propidium iodide (10 μg/ml) for 10 min at room temperature. Gated total cell population was immediately analyzed by a flow cytometer (FACSCalibur; BD Biosciences) in a 2-parameter dot plot with FL1 (530/30 BP) *vs.* FL3 (650 LP) channels, measuring the native GFP and PI fluorescence, respectively. For data analysis (BD CellQuest Pro), only the percentage of GFP$^+$ cells with an intact cell cycle (*i.e.*, combination of G_1-S-G_2/M peaks) out of the gated total population (*i.e.*, UR values obtained from the quadrant statistics) was taken into consideration.

Statistical analysis

Data were analyzed using SPSS 15.0 software (SPSS, Chicago, IL, USA) and compared by Student's *t* test. All data are presented as means ± SE of indicated numbers of independent experiments. Values of $P < 0.05$ were considered significant.

RESULTS

GFP-degron and GFP-Δ9CAT display characteristics of misfolding

First, we generated GFP-fusions of wild-type (wtCAT) and Δ9CAT and used GFP and GFP-degron and CAT variants to transiently transfect COS-7 cells and investigated their solubility by sequential extraction into detergent-soluble and insoluble fractions. While GFP and GFP-wtCAT showed an equal distribution, GFP-degron and GFP-Δ9CAT sedimented predominantly in detergent-insoluble pellet fractions, respectively (**Fig. 1*A***). This sedimentation was not due to higher-level overexpression, since both mutants were detected at a level 2- to 5-fold less than their wild-type counterparts (see the sum of S+P fractions in Figs. 1*A* and **2*A***). Moreover, we observed GFP-reactive bands at higher molecular weight in the pellet fractions of GFP-degron and GFP-Δ9CAT, indicating the presence of insoluble oligomeric and/or ubiquitinylated species, both of which indicate the exposure of buried regions in misfolded ensembles. Spectrophotometric CAT assays (21) revealed that deletion of the 9 C-terminal amino acids also caused total loss of CAT enzymatic activity (Supplemental Fig. S1). Thus, destabilizing modifications induced the loss of native conformation and converted both GFP and GFP-wtCAT into a less soluble state.

Next, we examined the subcellular distribution of GFP-degron and GFP-Δ9CAT by fluorescence microscopy. Both GFP-degron and GFP-Δ9CAT prevailed predominantly as deposits of perinuclear aggregates resembling aggresomes, while GFP and GFP-wtCAT distributed diffusely (Fig. 1*B*). These observations have been confirmed in live cells (data not shown) and demonstrated that the decreased solubility was not an artifact caused by cell lysis, but was due to the formation of aggregated ensembles inside living cells, a hallmark of protein misfolding (3). Our findings on the perinuclear deposition of GFP-degron are consistent with those of Link *et al.* (18).

Expression of both GFP-degron and GFP-Δ9CAT increased the level of inducible Hsp70 (HSPA1A/B; Fig. 1*A*, bottom panel). Equally increased Hsp70 levels were observed in the soluble and pellet fractions,

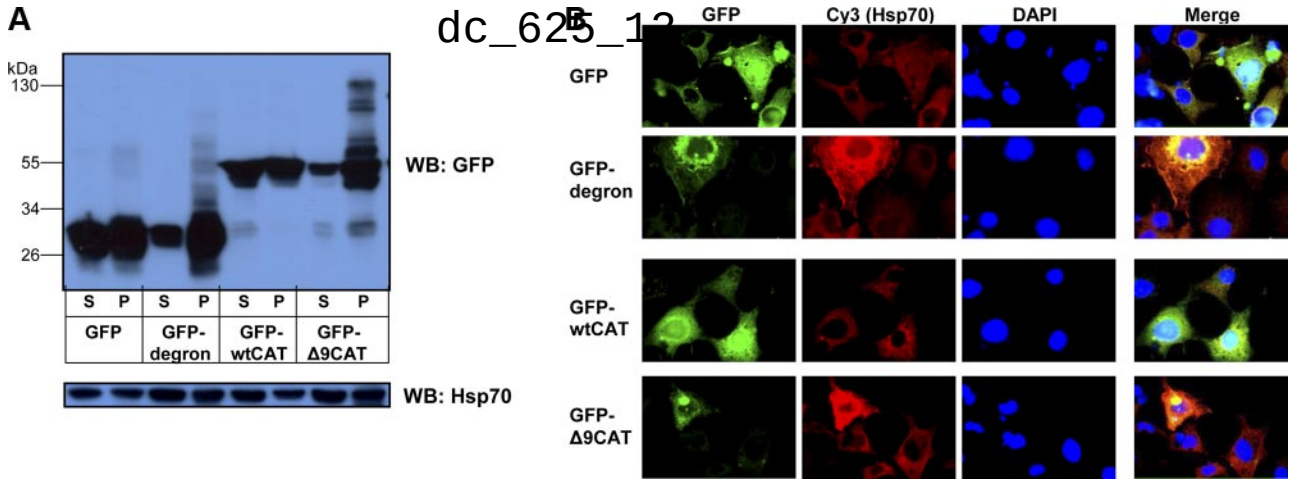

Figure 1. GFP-degron and GFP-Δ9CAT display characteristics of misfolding. *A*) Misfolding GFP-degron and GFP-Δ9CAT proteins sediment in detergent-insoluble aggregates and increase Hsp70 (HSPA1A/B) protein level. After transient transfections with the indicated constructs (GFP, GFP-degron, GFP-wtCAT, GFP-Δ9CAT), COS-7 cells were lysed, and cell lysates were separated into soluble (S) and detergent-insoluble pellet (P) fractions by centrifugation and analyzed by Western blotting for GFP and Hsp70, respectively. *B*) Misfolding proteins form perinuclear aggregates and accumulate total Hsp70 protein *in vivo*. Transfected cells grown on coverslips were fixed in methanol; stained by an antibody recognizing both inducible (HSPA1A/B) and constitutive (HSPA8) Hsp70, followed by a Cy3-conjugated secondary antibody, and DAPI for nuclear DNA; and were visualized under a fluorescence microscope. Images are representatives of 5 independent experiments.

respectively, of both mutants, indicating that Hsp70 did not show a strong preference toward insoluble species after cell lysis. Moreover, immunofluorescence analyses showed that GFP-degron and GFP-Δ9CAT led to a robust constitutive and inducible Hsp70 (HSPA8 and HSPA1A/B, respectively) accumulation only in cells where they were expressed, in contrast to the soluble controls GFP and GFP-wtCAT (Fig. 1*B*). Remarkably, Hsp70 was strongly enriched in both GFP-degron and GFP-Δ9CAT aggregates. The Western blot and immunofluorescence data together suggest a transient association of Hsp70 with the misfolded/aggregated species of GFP-degron and GFP-Δ9CAT and are consistent with a previous report showing a dynamic interaction of Hsp70 with polyglutamine proteins (22). These results demonstrate that both GFP-degron and GFP-Δ9CAT exhibit decreased solubility and aggregation, induce and transiently associate with the chaperone Hsp70, and establish GFP-degron and GFP-Δ9CAT as *bona fide* misfolded proteins.

Misfolded proteins undergo proteasomal degradation

Both Link *et al.* (18) and our group observed GFP-degron as perinuclear aggregates (Fig. 1), though GFP-degron$^+$ cells were always only a modest fraction (5–15%) of the total cell population. Hence, we tested whether a proteolytic degradation of misfolded proteins occurred. Using the proteasome inhibitor MG-132, we detected an increased level of both GFP-degron and GFP-Δ9CAT by flow cytometry. MG-132, at a concentration as low as 2 μM (a concentration without severe cytotoxicity), induced a significant increase in GFP-degron$^+$ and GFP-Δ9CAT$^+$ cells, approaching the ratio of GFP$^+$ and GFP-wtCAT$^+$ cells (Fig. 2*A*). It is important to note that neither GFP-degron nor GFP-Δ9CAT was detected in soluble forms, and proteasome inhibition did not significantly change the appearance and topology of aggregates examined by fluorescent microscopy, which suggests that aggregation is a direct consequence of the presence of misfolded, undegradable ensembles. We found that 3-methyladenine, a specific inhibitor of autophagy, did not increase GFP-fluorescence (data not shown), suggesting that the stabilization observed with MG-132 was not due to an unspecific inhibition of the cellular proteolytic capacity. Altogether, these results define the proteasome responsible for the degradation of these misfolded proteins.

Flow cytometric histograms of MG-132-treated cell populations show that proteasome inhibition stabilizes GFP-degron and GFP-Δ9CAT at fluorescence intensities exceeding 10^2 and 3×10^2, respectively, suggesting that misfolded species are tolerated below these tresholds (Fig. 2*B*). We determined the corresponding expression levels by densitometry of anti-GFP Western blots of total cell lysates (Fig. 2*C, D*). While traces of misfolded proteins were detected using these settings, inhibition of their turnover increased the average expression of GFP-degron and GFP-Δ9CAT to 2.2 *vs.* 0.9% of total cellular protein, respectively. These results are consistent with the higher peak of GFP-degron observed in flow cytometry (Fig. 2*B*). Considering the logarithmic scale of fluorescence and the abovementioned thresholds, we assume that maximal expression levels cannot exceed 1–2% of total cellular proteins, and that misfolded proteins in the range of ~0.02–0.04% of total cellular proteins are efficiently sensed and disposed.

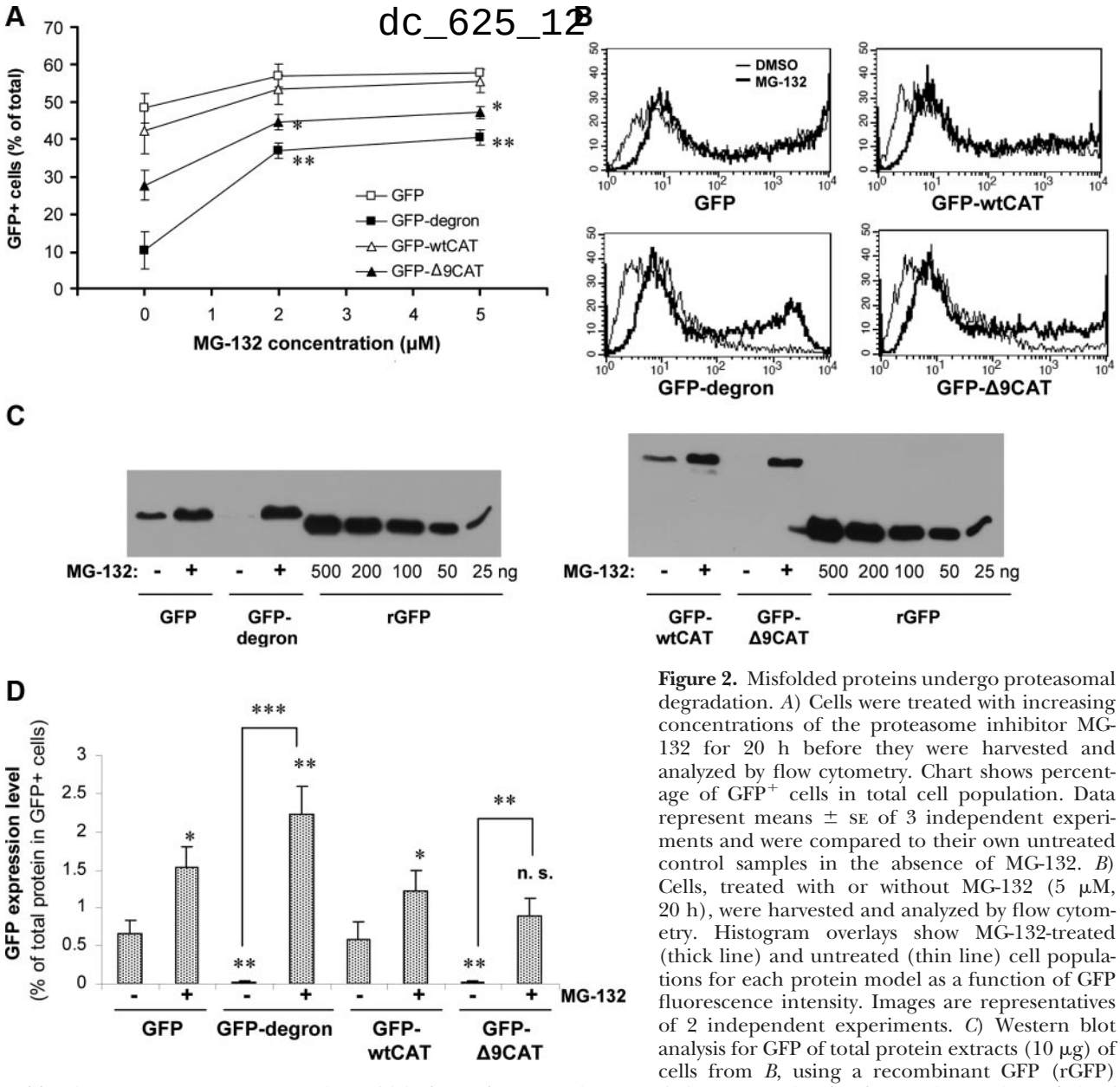

Figure 2. Misfolded proteins undergo proteasomal degradation. *A*) Cells were treated with increasing concentrations of the proteasome inhibitor MG-132 for 20 h before they were harvested and analyzed by flow cytometry. Chart shows percentage of GFP^+ cells in total cell population. Data represent means ± SE of 3 independent experiments and were compared to their own untreated control samples in the absence of MG-132. *B*) Cells, treated with or without MG-132 (5 μM, 20 h), were harvested and analyzed by flow cytometry. Histogram overlays show MG-132-treated (thick line) and untreated (thin line) cell populations for each protein model as a function of GFP fluorescence intensity. Images are representatives of 2 independent experiments. *C*) Western blot analysis for GFP of total protein extracts (10 μg) of cells from *B*, using a recombinant GFP (rGFP) calibration. Images are representatives of 2 independent experiments. *D*) GFP expression levels. Densitometric analysis of Western blots from *C*, normalized to their corresponding GFP positivities. Data represent means ± SE of 2 independent experiments and were compared to untreated GFP and GFP-wtCAT controls (asterisks on columns) and to GFP-degron/GFP-Δ9CAT in the absence or presence of MG-132, respectively (linked bars). n.s., not significant. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Misfolded proteins activate the *hsp70* promoter

Since we could see an accumulation of Hsp70 protein with both misfolded proteins, we were interested whether they would induce transactivation of heat-shock factor 1 (HSF1), responsible for the induction of *hsp* genes. To accomplish this, we used a reporter construct harboring the *hsp70* promoter fused to firefly luciferase (23). GFP-degron expression resulted in a moderate induction of the reporter at 37°C by up to 4-fold compared to that of GFP (**Fig. 3*A***). GFP-Δ9CAT was able to induce the reporter gene at a smaller, comparable extent (3-fold increase compared to the induction by GFP-wtCAT). Induction of the reporter by misfolded proteins was completely blunted by cotransfection with HSF1 siRNA, confirming that the induction was entirely HSF1-dependent, consistent with previous results (data not shown; ref. 24). GFP-degron and GFP-Δ9CAT did not activate the endoplasmic reticulum chaperone, *grp78* promoter (data not shown), suggesting that misfolding of cytosolic proteins does not result in the activation of the endoplasmic reticulum unfolded protein response, consistent with recent studies in yeast (25–26).

We next addressed how misfolded proteins would interfere with the induction of the heat-shock response by a moderate proteotoxic heat stress (43°C). The presence of GFP-degron and GFP-Δ9CAT, neither

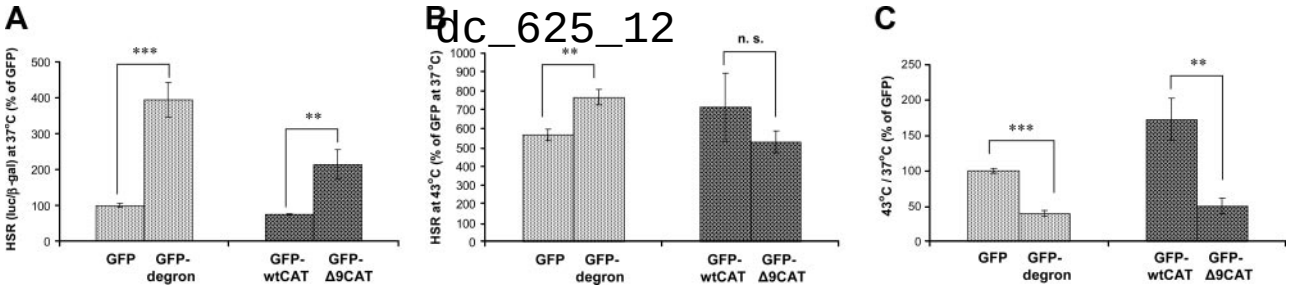

Figure 3. Misfolded proteins activate the *hsp70* promoter. HSF1-transactivation at 37°C (*A*), heat-induced HSF1-transactivation (*B*), and the ratio of induced and basal outputs (*C*). *A*, *B*) Along with the indicated constructs, cells were cotransfected with hsp70.1pr-luc and β-galactosidase (β-gal) plasmids. A second set (*B*) was given a heat shock at 43°C for 30 min. Luciferase and β-gal assays were performed 18 h later, and their ratios were expressed as percentages of the value obtained in the GFP sample. *C*) Alternatively, fold inductions in the heat-shock response were calculated by dividing the normalized (luc/β-gal) heat-shocked (43°C) values by their corresponding basal (37°C) values and are displayed in the chart as a percentage of the value obtained in the GFP sample. Data represent means ± SE of 7 independent experiments for GFP and GFP-degron and 3 independent experiments for GFP-wtCAT and GFP-Δ9CAT, respectively. Values were compared to the respective wild-type controls. n.s., not significant. $^{**}P < 0.01$; $^{***}P < 0.001$.

failed to synergize with nor compromised the heat-induced activity of the reporter gene construct, respectively, suggesting that the induction of the heat-shock response was not significantly changed by a transient expression of a single misfolded protein (Fig. 3*B*). However, misfolded proteins led to a higher basal level as well as a reduced reserve capacity of the heat-shock response, suggesting that the induction of heat-shock proteins was under a constant strain at basal conditions (Fig. 3*C*). We observed the same phenomenon with bovine serum albumin misdirected to the cytosol (GFP-cBSA) and with the coiled-coil region of the Golgi complex protein 170 fused to GFP (GFP170*), a nonpolyQ protein reported to form nuclear aggregates and colocalize with Hsp70, consistent with a previous reports (refs. 9, 24 and data not shown). Hence, we conclude that a transient expression of a single misfolded protein induces HSF1-dependent transactivation without a severe compromise of heat inducibility along with a reduced reserve capacity of the heat-shock response.

Opposing effects by GFP-degron and GFP-Δ9CAT on chaperone-mediated folding

Chronic expression of polyQ proteins was previously shown to interfere with protein folding in *C. elegans* (17). To test this possibility in replicative cells, we asked how acute expression misfolded proteins would affect the activity of firefly luciferase, a metastable multidomain protein commonly used as a model substrate to monitor chaperone activity/function (27). Overexpression of GFP-degron caused a 25% decrease of luciferase activity compared to that of wild-type GFP-cotransfected cells at 37°C (**Fig. 4*A***). Surprisingly, GFP-Δ9CAT enhanced luciferase activity by 20% compared to that of GFP-wtCAT at 37°C (Fig. 4*B*). With increasing periods of time at 42°C heat shock, the difference between luciferase activities of both GFP *vs.* GFP-degron and GFP-wtCAT *vs.* GFP-Δ9CAT pairs disappeared. Similar phenomena were observed with MG-132, consistent with the proteasomal clearance of an extensive amount of mistranslated-misfolded proteins already at nonstress conditions (28). Hsp70 overexpression promoted luciferase folding by ~25% for all constructs even in the presence of MG-132 (data not shown), establishing that global manipulations of the proteostatic buffer were efficiently monitored by firefly luciferase. None of the interventions affected the activity of β-galactosidase (data not shown). We conclude that in contrast to global proteotoxic stresses (heat shock and MG-132), short-term expression of single misfolded proteins exerts a modest effect on chaperone-mediated protein folding.

Misfolded proteins inhibit cell proliferation

We next investigated the consequences of protein misfolding on cell growth. Incorporation of the thymidine analog bromodeoxyuridine (BrdU) into genomic DNA is an indicator of cell division. To analyze rates of BrdU uptake in transfected and untransfected subpopulations we immunostained the cells with both anti-GFP and anti-BrdU antibodies. Image analyses of dual immunofluorescence data revealed that both GFP-degron$^+$ and GFP-Δ9CAT$^+$ cells incorporated less BrdU than their wild-type counterparts (**Fig. 5**). Induction of cell proliferation arrest was more pronounced with GFP-degron, suggesting a stronger effect of GFP-degron on cell growth. MG-132 (2 μM, 20 h) treatment completely blocked BrdU uptake (data not shown). Thus, similarly to proteasome inhibition, misfolded proteins arrest cell proliferation.

Misfolded proteins promote stress-induced cell death

Although misfolded proteins inhibited cell proliferation (Fig. 5*B*), we detected neither any necrosis by propidium iodide exclusion nor apoptosis by sub-G_1 peak of DNA (data not shown). However, treatment of transfected cells with MG-132 resulted in an altered histogram with a distorted sub-G_1 peak containing cell

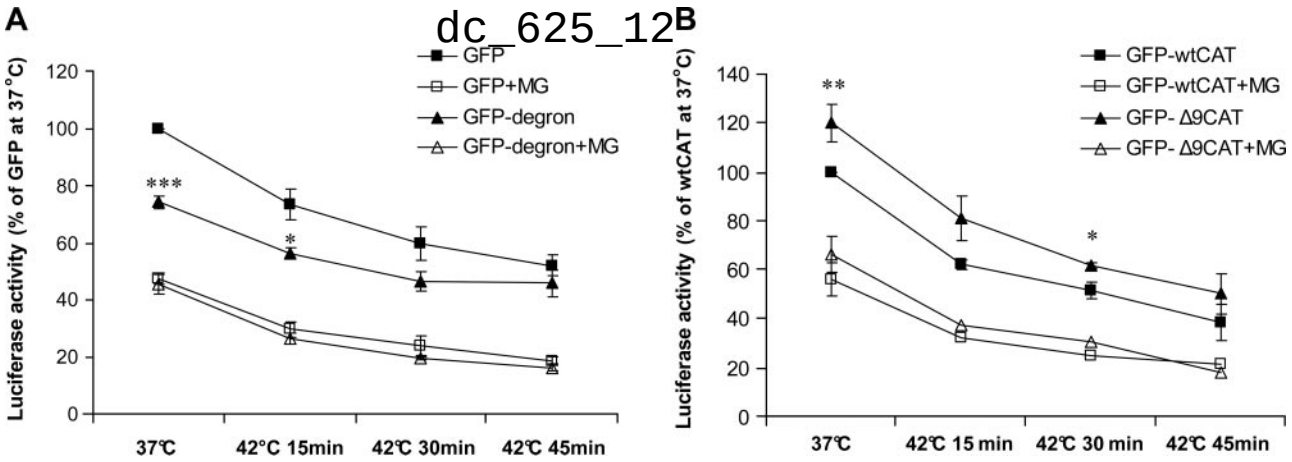

Figure 4. Opposing effects by GFP-degron and GFP-Δ9CAT on chaperone-mediated folding. GFP-degron compromises (*A*), while GFP-Δ9CAT enhances (*B*) firefly luciferase folding at 37°C. Along with the indicated protein models, cells were cotransfected with TK-luc and β-gal plasmids. After treatment with or without MG-132 (2 μM, 20 h), cells were either incubated at 37°C or given a heat shock at 42°C for the indicated periods of time. Immediately after the heat shock, cells were cooled on ice, lysed, and analyzed, as described in Materials and Methods. Charts show normalized luciferase (luc/β-gal) activities as a percentage of the value obtained in the respective wild-type controls at 37°C without MG-132 treatment. Data represent means ± SE of 3 independent experiments and were compared to the respective wild-type controls subjected to the same treatment. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

debris along with a marked decrease in the number of intact cells (**Fig. 6*A***). Therefore, as a more appropriate marker of the number of intact (live) cells, a joint measure of cell proliferation and death, we calculated the area below the G_1-S-G_2/M peaks of GFP^+ cells (labeled by peaks in Fig. 6*A*). GFP-degron and GFP-Δ9CAT transfection combined with MG-132 treatment resulted in a lesser amount of intact GFP^+ cells compared to their wild-type controls already at 37°C (Fig. 6*B*). Notably, there was a further sharp decrease in the number of GFP-degron- or GFP-Δ9CAT-transfected intact cells when they were given a moderate heat shock (43°C, 30 min) that did not affect GFP and GFP-wtCAT controls.

To obtain an independent measure of cell death, we performed annexin staining, detecting cell death from early apoptosis through late necrosis. GFP^+ cells displayed enhanced annexin staining compared with GFP-negative population and MG-132 treatment resulted in

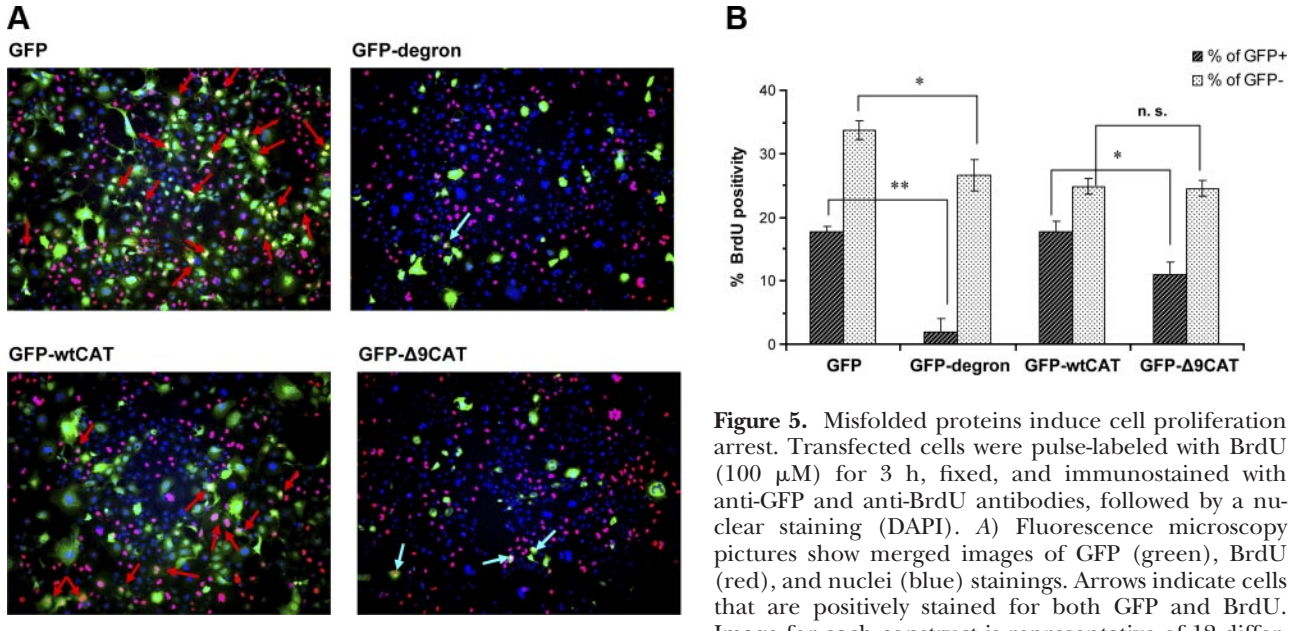

Figure 5. Misfolded proteins induce cell proliferation arrest. Transfected cells were pulse-labeled with BrdU (100 μM) for 3 h, fixed, and immunostained with anti-GFP and anti-BrdU antibodies, followed by a nuclear staining (DAPI). *A*) Fluorescence microscopy pictures show merged images of GFP (green), BrdU (red), and nuclei (blue) stainings. Arrows indicate cells that are positively stained for both GFP and BrdU. Image for each construct is representative of 12 different regions from 2 independent experiments. *B*) Quantification of dual immunofluorescence data. Six different areas randomly selected from each protein model with an average of 600–700 cells/area were first counted for single DAPI, GFP, and BrdU signals. Double positives for both GFP and BrdU signals were then counted, and calculations were done accordingly to determine percentages of BrdU positivity in GFP^+ (dark bars) and GFP-negative (light bars) subpopulations. Data represent means ± SE of 2 independent experiments and were compared to the respective wild-type controls. n.s., not significant. $*P < 0.05$; $**P < 0.01$.

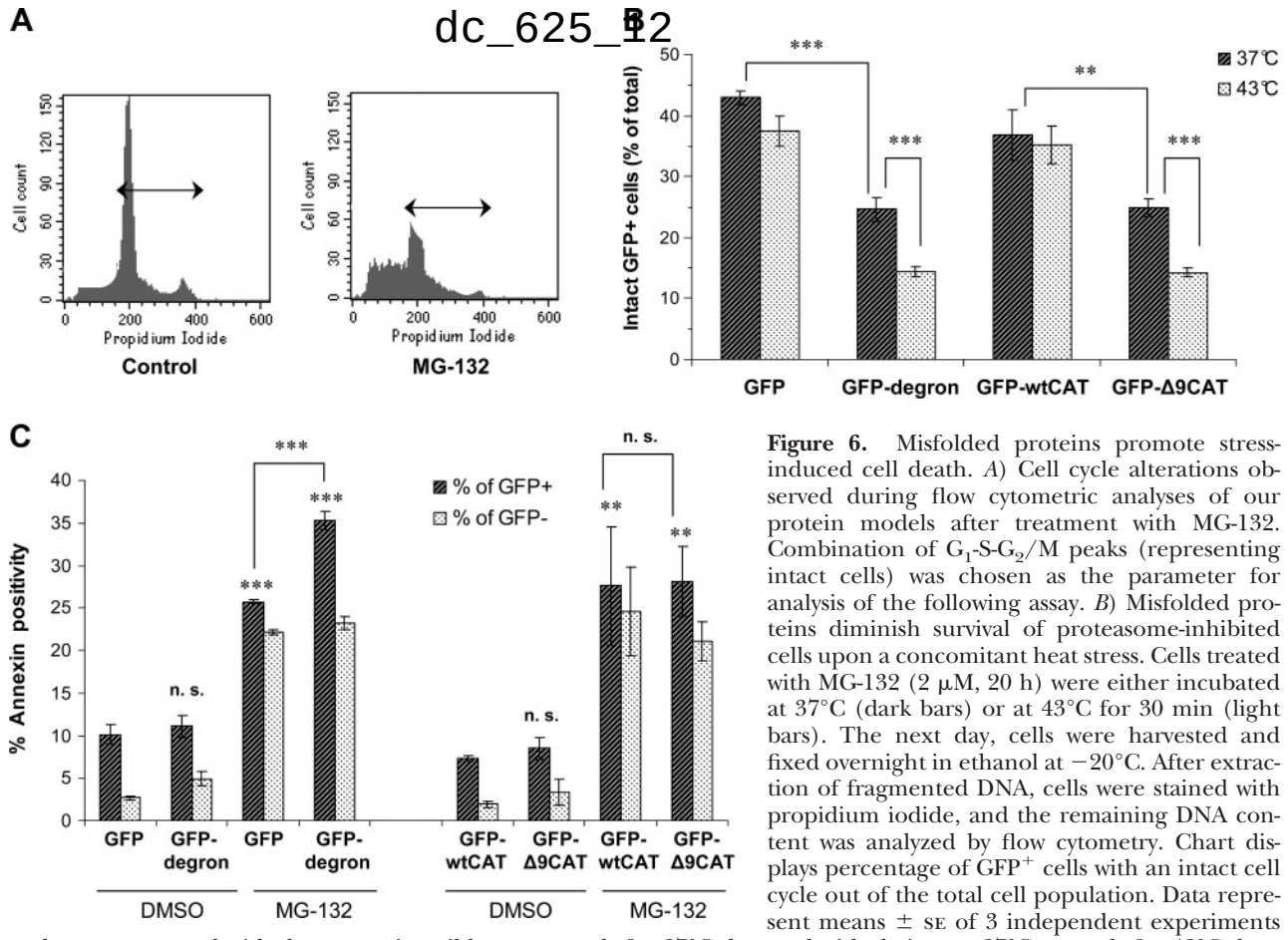

Figure 6. Misfolded proteins promote stress-induced cell death. *A*) Cell cycle alterations observed during flow cytometric analyses of our protein models after treatment with MG-132. Combination of G_1-S-G_2/M peaks (representing intact cells) was chosen as the parameter for analysis of the following assay. *B*) Misfolded proteins diminish survival of proteasome-inhibited cells upon a concomitant heat stress. Cells treated with MG-132 (2 μM, 20 h) were either incubated at 37°C (dark bars) or at 43°C for 30 min (light bars). The next day, cells were harvested and fixed overnight in ethanol at −20°C. After extraction of fragmented DNA, cells were stained with propidium iodide, and the remaining DNA content was analyzed by flow cytometry. Chart displays percentage of GFP^+ cells with an intact cell cycle out of the total cell population. Data represent means ± SE of 3 independent experiments and were compared with the respective wild-type controls for 37°C data and with their own 37°C controls for 43°C data. *C*) Misfolded GFP-degron, but not GFP-Δ9CAT, induces annexin positivity upon proteasome inhibition. Cells treated with or without MG-132 (2 μM, 42 h) were stained with annexin V, and analyzed by flow cytometry. The chart shows the percentage of annexin positivity in GFP^+ (dark bars) and GFP-negative (light bars) subpopulations for each protein model. Data represent means ± SE of 3 independent experiments, and GFP^+ values were compared to GFP and GFP-wtCAT controls (asterisks on columns) and GFP-degron/GFP-Δ9CAT to their respective MG-132-treated wild-type control (linked bars). n.s., not significant. $^{**}P < 0.01$; $^{***}P < 0.001$.

an increase in annexin-positivity from 10 to 25%, consistent with a general proteotoxicity induced by proteasome inhibition (Fig. 6*C*). Similarly to the previous propidium iodide data, there was no significant difference between annexin staining of GFP-degron- and GFP-Δ9CAT-transfected cells compared to that of their wild-type counterparts. However, MG-132 treatment of cells expressing GFP-degron, but not GFP-Δ9CAT, yielded a significantly higher annexin positivity compared to controls (Fig. 6*C*). These results confirm the GFP-degron-related findings presented in Fig. 6*B* and suggest that some form of GFP-Δ9CAT-induced cell death may escape detection by annexin in our experimental conditions. Nevertheless, when GFP-Δ9CAT cells underwent a moderate heat shock, they also displayed an increased annexin positivity comparable to that of GFP-degron (data not shown). Moreover, we obtained similar results with 2 other misfolded protein models, GFP-cBSA and GFP170*, upon MG-132 treatment (Supplemental Fig. S2). Thus, diverse misfolded proteins sensitize cells to manifest enhanced cytotoxicity in response to stress.

HSF1, Hsp70, and SIRT1 protect from GFP-degron-induced cytotoxicity

We next addressed how genetic activation of stress-responsive mechanisms would combat the cytotoxicity induced by GFP-degron. HSF1 and Hsp70 are important determinants of the heat-shock response, ameliorating toxicity of neurodegenerative models (10, 29–30). SIRT1 is an NAD^+-dependent protein deacetylase, which was reported to control HSF1 activity by prolonging its DNA binding through deacetylation (31). It was also shown to be protective in initial studies against polyglutamine-induced cytotoxicity in both *C. elegans* and mammalian neurons (32), though its general role in invertebrate longevity has been questioned (33). SIRT6 is a nuclear sirtuin paralog implicated in dietary restriction and the metabolic syndrome; however, there are no data on its role in proteostasis (34).

All the HSF1, Hsp70, SIRT1, and SIRT6 constructs were strongly overexpressed upon transfection (**Fig. 7*A***). None of the constructs caused any significant difference in the number of GFP-degron$^+$ cells (*i.e.*,

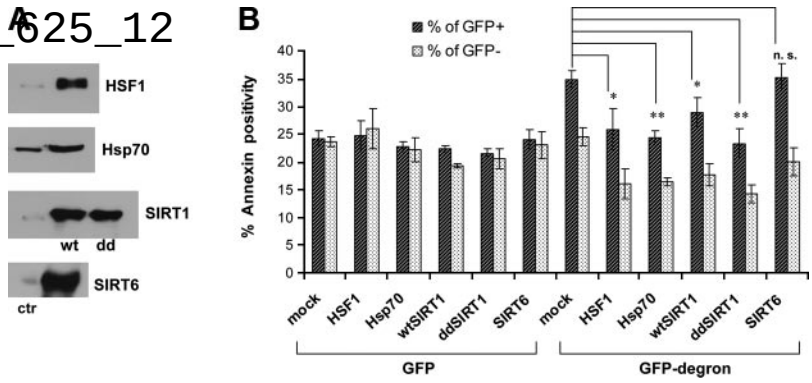

Figure 7. HSF1, Hsp70, and SIRT1 protect from GFP-degron-induced cytotoxicity. *A*) HSF1, Hsp70, SIRT1s, and SIRT6 are strongly overexpressed on transfection. Cells were transfected with mock (ctr), HSF1, Hsp70, wtSIRT1, deacetylase-deficient H363Y mutant SIRT1 (ddSIRT1), and SIRT6 constructs, respectively, for 24 h, lysed, and analyzed by Western blotting for the respective proteins. Images are representatives of 2 independent experiments. *B*) Cell death of MG-132-treated (2 μM, 42 h) cells. Cotransfections of the constructs were done at a ratio of 1:1 (misfolded protein construct:modulator). Annexin assay was performed as described in Materials and Methods. Chart shows the percentage of annexin positivity in GFP^+ (dark bars) and GFP-negative (light bars) subpopulations for each cotransfection. Renilla luciferase plasmid served as mock. Data represent means ± SE of 3 independent experiments. Statistical comparisons were made between the respective GFP^+ samples compared to mock-cotransfected sample. n.s., not significant. $*P < 0.05$; $**P < 0.01$.

cells expressing and not degrading GFP-degron) in the absence or presence of MG-132 (data not shown), suggesting they did not alter the turnover of GFP-degron. HSF1, Hsp70, and SIRT1 constructs were all found to prevent cell death in MG-132-treated GFP-degron-expressing cells (Fig. 7*B*). Interestingly, SIRT1-mediated recovery from GFP-degron-induced cytotoxicity was independent of the deacetylase activity of SIRT1, as both wild-type (wtSIRT1) and the deacetylase-deficient H363Y mutant (ddSIRT1) were protective. In contrast, SIRT6 did not exert a protective effect, giving an independent evidence for the specific action of SIRT1. This finding is in line with a recent study where SIRT1-induced neuroprotection was found to be independent of its deacetylase activity (35), suggesting a novel, noncatalytic mechanism of cytoprotection for SIRT1.

DISCUSSION

Conformational diseases share common features: the accumulation of non-native protein species and a consequent cytotoxicity. Here we have isolated the general effects elicited by single misfolded polypeptides by expressing 2 *bona fide* misfolded proteins not occurring in the mammalian cytosol. Both GFP-fused degron and C-terminally truncated chloramphenicol acetyltransferase lost solubility and exhibited aggregation, transiently associated with and induced the expression of the chaperone Hsp70, underwent a proteasomal degradation in the majority of cells, induced growth arrest, and caused cytotoxicity in response to stress, which was ameliorated by stress-responsive mechanisms (**Fig. 8**). These features obtained with diverse misfolded proteins are consistent with an inherent toxicity of the misfolded ensembles *per se*.

Loss of a stabilizing interaction mediated by a C-terminal residue in Δ9CAT disrupts its stability and results in inclusion body formation in *E. coli* (19, 36). Our results indicate that GFP-Δ9CAT instability also induces its misfolding in mammalian cells (Figs. 1 and 2, and Supplemental Fig. S1). The 16-residue degron peptide supplements proteins with a C-terminal amphipathic α helix conferring destabilization to the extended proteins (18, 37). GFP-degron appears to be a Janus-faced molecule, *i.e.*, both an *in vivo* ubiquitin-proteasome system (UPS) reporter (6, 38) and a misfolded protein (Fig. 1 and ref. 18). This dual behavior can be explained by considering a primary misfolding of GFP-degron and its chaperone-mediated disposal by the UPS (Fig. 8 and ref. 18), a pathway identical to that of global protein misfolding (13). This model is supported by the similar phenotype (exhibiting aggregation, chaperone induction, growth inhibition) of GFP-degron to several misfolded proteins: YFP point mutants in yeast (26), GFP170* (present study and ref. 9), and GFP-Δ9CAT (present study), respectively. In addition, yeast Ura3p fused to degron requires the Hsp70/Hsp40 orthologs Ssa1p/Ydj1p for both its proteasomal degradation and solubility (39), in agreement with a degron-induced misfolding of Ura3p.

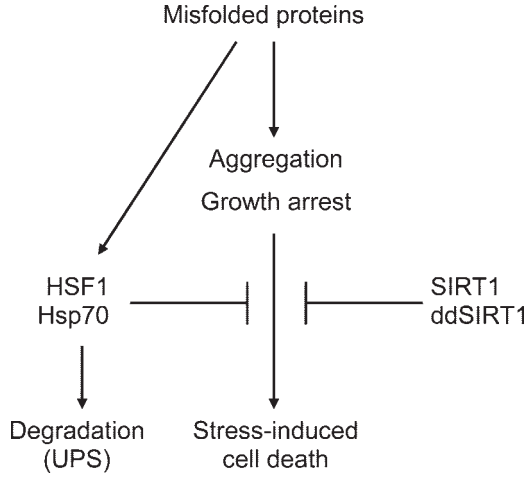

Figure 8. Misfolded proteins in mammalian cells. Misfolded proteins induce the heat-shock response and are turned over by the UPS in the majority of cells. In cells that are incompetent to degrade them, they form aggregates and cause growth arrest. Upon various stresses, such as the inhibition of UPS or heat shock, misfolded proteins promote cell death. The genetic up-regulation of the heat-shock response (HSF1 or Hsp70) and the sirtuin SIRT1, independently of its deacetylase activity (ddSIRT1), protects from stress-induced cytotoxicity.

Our findings suggest that aggregate formation proceeds if the level of misfolded proteins exceeds the proteostatic capacity of the cell. Upon transient transfection in our study (Fig. 1*B*) and in that of Link *et al.* (18) misfolded proteins were efficiently cleared from the majority of cell population and formed aggregates in GFP^+ cells. These effects were augmented by proteasome inhibition (Fig. 2). Overexpression of Hsp70 or HSF1 did not decrease the number of GFP-degron$^+$ cells (*i.e.*, did not increase the turnover of the protein), which, along with the yeast study on Ura3p-degron (39), suggests that chaperones are required for but do not limit GFP-degron degradation. These findings suggest that the UPS is a rate-limiting step in the chaperone-mediated disposal of misfolded proteins and offer a rationale for GFP-degron as an *in vivo* marker measuring UPS capacity.

The roles of Hsp70 and HSF1 in cells expressing misfolded proteins (Fig. 7) corroborate the well-documented role of the heat-shock response in the recognition and defense against proteotoxicity (22, 40). The decreasing order of intensity of Hsp70 induction by GFP-degron > GFP-Δ9CAT > GFP-cBSA = GFP170* indicates a proportional release of HSF1 from its repressing chaperone complex in response to misfolding. This effect is consistent with the previously reported, dose-dependent effect of cytosolic BSA on HSF1 activation (24). Considering the ~1–2% maximal level of misfolded proteins induced by proteasome inhibition and an induction of Hsp70 in the heat-shock range, we speculate that misfolded ensembles at the 0.02–0.04% threshold level may elicit a signal potent enough to provoke a significant threat, namely, cell cycle arrest. This threat, however, appears to be compensated in our experimental conditions by a highly efficient recognition/disposal (demonstrated by the negligible effects of misfolded protein expression on thermotolerance and on cytotoxicity). Recent studies demonstrate a specific, HSF1-mediated cytosolic unfolded protein response within the wider heat-shock response/proteostatic network in yeast and plants (25–26, 41–42). Our results, by showing a selective activation of hsp70 *vs.* the ER stress marker grp78 promoter (Fig. 3*A* and data not shown), extend the existence of this specific cytosolic response to mammalian cells. The misfolded proteins used in this study provide a tractable model to identify the cytosolic unfolded protein response at the systems level in mammalian cells.

We observed a differential, but modest, effect of GFP-degron and GFP-Δ9CAT on firefly luciferase folding (Fig. 4). The reason for this difference is unknown. It may be that GFP-degron and GFP-Δ9CAT differentially affect protein transcription/translation. Though we did not test luciferase mRNA and protein levels, the similar β-galactosidase activities and the equal residual activities after heat shock or MG-132 treatment exclude such a possibility. The GFP-Δ9CAT-induced luciferase activation may be explained by its less pronounced effects (*i.e.*, higher ratio of GFP^+ cells, slower turnover, smaller *hsp70*-promoter induction, and decreased inhibition of cell proliferation), suggesting less toxic misfolded species formed by GFP-Δ9CAT, which in turn may activate chaperone-mediated folding. Though these data do not allow a clear conclusion, the modest effects and the clear dissection from toxicity (Figs. 5 and 6) suggest chaperone-mediated folding is not the major determinant of short-term action of individual misfolded proteins.

Our results in COS-7 cells recapitulate the proliferation arrest obtained with single misfolded mutants, including polyQ103-GFP in HEK-293 cells (6), GFP170* in COS-7 cells (9), YFP mutants (26), and azetidine-carboxylate in yeast (43), which suggests a conserved, general effect of protein misfolding. This occurs in GFP^+ cells (*i.e.*, cells that are unable to degrade the misfolded proteins; Fig. 8). The almost complete loss of BrdU incorporation may be consistent with a G_1 arrest. As in COS-7 cells, the SV40 large T-antigen inactivates both Rb and p53; it is tempting to speculate that mechanisms distinct from these checkpoint responses may inhibit proliferation. Indeed, such mechanisms arising from the inherent toxicity of misfolding have been demonstrated, such as sequestration of the growth-promoting CBP into aggregates (5, 9). Alternatively, growth inhibition may be due to the adaptive response, *e.g.*, HSF1 mediating reversible cell cycle arrest in yeast (43) or increased Hsp70 inhibiting growth (44–45). Though the molecular pathways induced by misfolded proteins remain to be analyzed, our findings on transformed cells might have a potential effect in nontransformed mitotic cells or stem cells.

A number of studies demonstrated extensive apoptosis using disease-associated mutants in cell (mostly neuronal) culture and in animal models (1, 7). However, we have not been able to detect death of immortalized COS-7 cells expressing misfolded proteins in nonstress conditions during the time scale of our experiments. Whether the long-term expression of a single misfolded protein leads to cell loss requires further studies. We also consider the possibility that misfolded proteins may induce cytotoxicity in neurons, where proteostatic defenses, such as the heat-shock response, are inherently weaker (46–47). Moreover, paralysis in the absence of apoptosis in *C. elegans* muscle expressing GFP-degron (18) and proliferation arrest (our study) suggest that severe dysfunctionality does not necessarily need cell death. The augmentation of death observed upon proteasome inhibition or heat shock by 4 misfolded mutants (Fig. 6 and Supplemental Fig. S2) indicates that already a short-term expression of misfolded ensembles compromise cells to mount an efficient adaptive response to proteotoxic stress. Defective stress tolerance seems to be a deleterious consequence of misfolding, as up-regulation of 2 stress-responsive mechanisms, the heat-shock response (HSF1 and Hsp70), as well as the sirtuin SIRT1, conferred protection (Fig. 7). Our findings extend earlier studies on the beneficial role of chaperones in various models (18, 40). The cytoprotective effect by chaperones may either lie in the restoration of protein homeostasis and/or the negative regulation by Hsp70 of death signals *via* p38 and JNK (48–49).

Our findings on the SIRT1 overexpression protecting

from GFP-degron-induced proteotoxicity confirm previous results involving neurodegenerative models (32, 50–51). Moreover, the lack of effect of SIRT6 overexpression indicates divergent roles for sirtuin paralogs in mammals. SIRT1-dependent deacetylation of key signaling molecules including HSF1 and consequent cellular events have been extensively reported (31, 52). However, our study did not demonstrate a requirement of deacetylase activity, suggesting the SIRT1-mediated response did not demand an increased deacetylation/activation of HSF1 in our model (Fig. 7*B*). Recently, growing body of evidence shows deacetylase-independent functions of SIRT1 (53–55) including neuroprotection (35); however, the molecular mechanism of SIRT1 remains enigmatic. Our results extend these functions to protein misfolding and recall tests on the involvement of SIRT1 deacetylase activity in protection from neurodegenerative disease models (32, 50–51). Finding the proper role for SIRT1 is especially important in the light of our recent demonstration of the inability of SIRT1 orthologs to extend life span in invertebrates (33), which has implications on the development and use of SIRT1 activators as potential drug candidates (56).

Our results show that the action of misfolded proteins depends on the proteostatic balance and cellular heterogeneity. Protein misfolding is not limited to single genes/proteins, but it rather involves a significant fraction of the proteome probably due to destabilizing SNPs (57), mistranslation (2, 28, 58), and post-translational modifications. We note that the level of misfolded proteins in our study is comparable to protein carbonyls in aged tissues (3–4). Lack of dilution of damage as well as the age-associated collapse and/or intrinsic weakness of proteostasis precipitate symptoms particularly in postmitotic neurons during aging (16, 46–47). Our findings raise a question whether protein misfolding would induce damage to the replicative compartment *in situ*, which may have implications for aging of regenerating tissues. Indeed, dystrophy of hair follicles has been reported in a mistranslation mouse model (2). Detrimental outcome of misfolding may also shape the evolution of tumors, where intensive growth (translation) and high stress are associated with the absolute reliance on HSF1 (59). These hypotheses, as well as the long-term effects of misfolded proteins on cellular fitness, are subjects of future studies. As a conclusion, our study provides a mammalian cellular model of misfolded proteins and reveals that short-term expression of single misfolded proteins independently of sequence and function inhibit proliferation and stress adaptation in replicating cells. FJ

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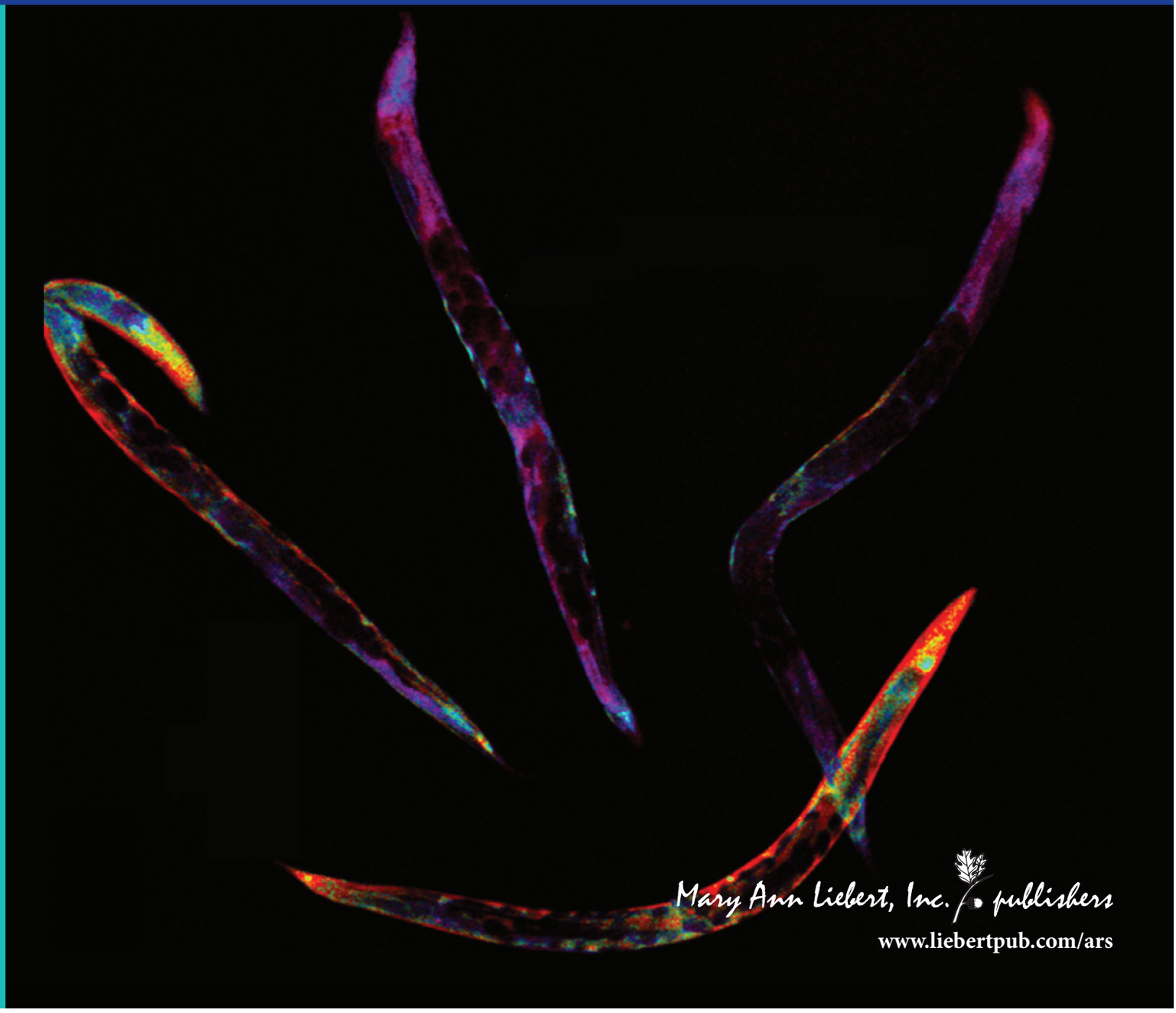

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RNA Interference Links Oxidative Stress to the Inhibition of Heat Stress Adaptation

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Abstract

Increased oxidative stress is associated with various diseases and aging, while adaptation to heat stress is an important determinant of survival and contributes to longevity. However, the impact of oxidative stress on heat resistance remains largely unclear. ***Aim:*** In this study we investigated how oxidative stress impinges on heat stress responses. ***Results:*** We report that hydrogen-peroxide (H_2O_2) pretreatment inhibits both acquired thermotolerance and heat-induced Hsp70 expression in mammalian cells, as well as acquired thermotolerance in the nematode *Caenorhabditis elegans*, *via* RNA interference. Moreover, we demonstrate that elimination of RNA interference by silencing key enzymes in microRNA biogenesis, *dcr-1* or *pash-1*, restores the diminished intrinsic thermotolerance of aged and H_2O_2-elimination compromised (catalase-2 and peroxiredoxin-2 deficient) worms. ***Innovation and Conclusion:*** These results uncover a novel post-transcriptional element in the regulation of heat stress adaptation under oxidative conditions that may have implications in disease susceptibility and aging. *Antioxid. Redox Signal.* 17, 890–901.

Introduction

Basic physiological processes such as metabolism, cellular signaling, and immunity are associated with the production of reactive oxygen species (ROS) (16). An accumulation of ROS, called oxidative stress, plays a critical role in various diseases and in aging (13, 16, 34). Although an excess of ROS generates diverse molecular and cellular damages and evokes a plethora of signaling events, how it is involved in the induction or aggravation of these pathological states is not entirely understood.

Increased resistance to heat stress protects against degenerative diseases in mammals (9, 32) and associates with longevity in *Caenorhabditis elegans* (10, 26). Intrinsic thermotolerance is maintained by multiple mechanisms. A preconditioning (*i.e.*, heat) stress induces acquired thermotolerance, mediated by the heat shock response *via* heat shock factor (HSF1)-dependent induction of heat shock proteins (Hsp-s) (30, 47). Previous studies reported contrasting results of oxidative stress on HSF1 activation (2, 28) and Hsp70 levels (14, 22, 43). However, the effect of oxidative stress on thermotolerance remains largely unexplored.

RNA interference is a powerful post-transcriptional regulator of gene expression that operates *via* ~22 nt microRNAs (miRNAs) (27). Genomic miRNA precursors are processed by highly specific RNases: the nuclear Drosha/PASH-1 produces hairpin pre-miRNAs, which are transported to the cytoplasm and cleaved to mature miRNAs by Dicer/DCR-1 (capital names indicate the respective nematode orthologs). Hence, Dicer/Drosha knockout is a reliable tool to investigate the general role of miRNAs (5, 41, 44). miRNAs bind to the mRNA 3′ untranslated region (3′UTR), repress translation, or promote mRNA degradation (27). miRNAs modulate diverse

Innovation

Oxidative stress is a serious cause of cell and tissue damage associated with many human diseases. Our observations beyond demonstrating a novel crosstalk between various types of stresses *via* RNA interference extend our understanding on how oxidative stress may debilitate physiological function. As RNA interference exhibits a significant functional conservation from nematodes to humans, we anticipate that the mechanism identified herein may be involved in human diseases and aging.

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biological processes. Their connection with stress is exemplified by imparting robustness to gene expression networks in response to environmental change (24) and by the profound alterations of miRNA expression upon heat and oxidative stresses (25, 42, 49) [reviewed in (23)]. Heat and ischemic preconditioning-induced miRNAs induce Hsp70 and are cardioprotective during ischemia-reperfusion in mice (48, 49). Moreover, miRNAs modulate the life span and stress resistance of *C. elegans* involving DAF-16 and HSF1 (6, 11), underscoring a vital role of RNA interference in stress responses.

In this study we focused on the impact of oxidative stress on heat stress adaptation and found that hydrogen-peroxide (H_2O_2) pretreatment inhibited acquired thermotolerance in both COS-7 mammalian cells and in *C. elegans*. As an underlying mechanism, H_2O_2 inhibited the heat-induction of Hsp70 in cells, consistent with a recent study (1). Moreover, H_2O_2 prevented the heat-induction of an Hsp70 3'UTR reporter. H_2O_2-induced effects required Dicer, a key enzyme in miRNA biogenesis, in both cells and worms. We further found that RNAi against Dicer and Drosha orthologs restored the compromised thermotolerance of two worm strains deficient in H_2O_2 disposal. Finally, Dicer silencing delayed the decline of thermotolerance in aging worms and phenocopied the effect of the antioxidant *N*-acetyl-L-cysteine (NAC). Our results reveal RNA interference as a mediator of oxidative stress-induced inhibition of heat stress responses.

Results

H_2O_2 inhibits acquired thermotolerance and Hsp70 induction at the post-transcriptional level in COS-7 cells

The effect of a transient H_2O_2 exposure on thermotolerance of COS-7 cells was determined by subjecting cells to a lethal heat stress 24 h after H_2O_2 and/or preconditioning heat treatments. Heat preconditioning elicited a large increase in survival (acquired thermotolerance, Fig. 1A). A prior H_2O_2 treatment slightly increased intrinsic thermotolerance. Importantly, it potently inhibited acquired thermotolerance in a concentration-dependent manner (Fig. 1A).

To examine whether the decrease in acquired thermotolerance is due to the inhibition of the heat shock response, we pretreated COS-7 cells with a series of H_2O_2 concentrations and monitored the heat induction of Hsp70 by flow cytometry (Fig. 1B). Cells, exposed to heat shock, exhibited an ~10-fold induction of Hsp70, concordant with the induction of thermotolerance (cf. Fig. 1A). H_2O_2 treatment did not affect basal

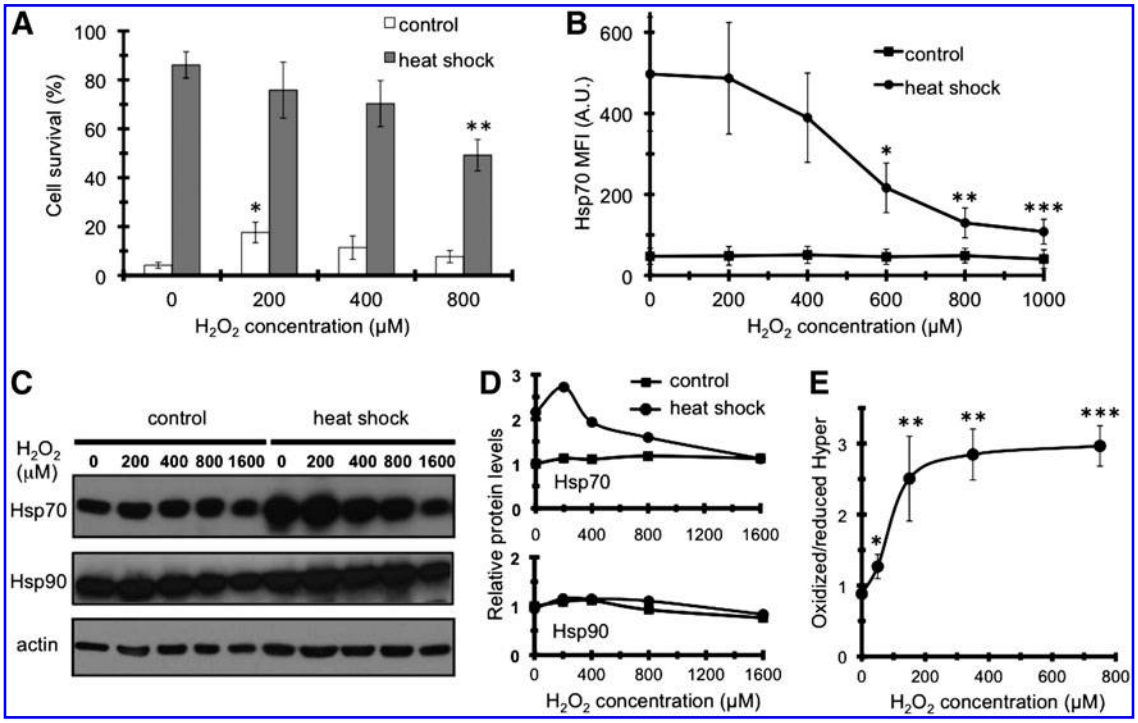

FIG. 1. Hydrogen-peroxide (H_2O_2) impairs heat-preconditioned thermotolerance and Hsp70 heat-induction in COS-7 cells. (A) Effect of H_2O_2 on thermotolerance. Cells were treated by the indicated concentrations of H_2O_2 for 2 h, then kept at 37°C (control) or at 43°C for 30 min (heat shock). About 24 h later cells were subjected to a lethal heat stress (45°C, 60 min). Cell survival was analyzed 24 h later by Trypan blue exclusion. Values are means ± standard deviations (SDs) of three experiments. **(B, C)** Effect of H_2O_2 on Hsp70 and Hsp90 protein levels. Cells were treated by H_2O_2 and heat shock as in panel A. Five hours later Hsp70 levels were analyzed by flow cytometry using a monoclonal antibody **(B)** or by Western blot using a polyclonal anti-Hsp70 and monoclonal anti-Hsp90 and anti-actin antibodies, respectively **(C).** Values are means ± SDs of five experiments compared to their respective controls, and image is a representative of three experiments. **(D)** Densitometric analysis of relative Hsp70 and Hsp90 levels from **(C)**. **(E)** H_2O_2 titration curve of cytosolic HyPer-C in COS-7 cells. The 490/420-nm fluorescence excitation ratio of HyPer was calculated after background fluorescence subtraction from two experiments. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Hsp70 level, but inhibited Hsp70 heat induction in a concentration-dependent manner (Fig. 1B). Western blots using a polyclonal anti-Hsp70 antibody showed a similar inhibition of Hsp70, but not of the specific chaperone Hsp90 (Fig. 1C, D). These results exclude an H_2O_2-induced modification or degradation of Hsp70 as well as a general, stress-induced transcriptional or translational block. The efficacy of H_2O_2 was verified by cells expressing the H_2O_2-sensor Hyper-C (Fig. 1E) (12). Thus, H_2O_2 pretreatment compromises both acquired thermotolerance and Hsp70 heat-induction in COS-7 cells.

Next, we investigated the site of action of H_2O_2 along the heat shock regulon. Upon heat, misfolded proteins activate HSF1, which binds to heat shock promoter elements and induces *hsp* gene transcription (32). To assess the level of HSF1-dependent transactivation, we transfected COS-7 cells with a *hsp70pr*/luciferase vector and performed reporter gene assays after cells had either been oxidatively stressed and/or heat-shocked. Heat shock markedly induced reporter activity, while H_2O_2 treatment significantly affected neither basal nor heat-induced transactivation (Fig. 2A). Likewise, H_2O_2 treatment did not decrease *hsp70* mRNA level (Fig. 2B). Thus, a transcriptional inhibition does not seem to underlie the H_2O_2-induced decrease in Hsp70 protein expression.

To assess, if H_2O_2 could down-regulate Hsp70 post-translationally, we changed the order of stresses (*i.e.*, employed H_2O_2 after heat shock) and followed the Hsp70 protein level by flow cytometry (Fig. 2C). H_2O_2 did not change the heat shock-induced sustained elevation of Hsp70, which excluded the possibility of an accelerated Hsp70 turnover.

The 3′UTR is intimately connected with the post-transcriptional regulation of mRNAs. To investigate the molecular events at the Hsp70 3′UTR, we took use of a reporter harboring the mouse *hsp70.1* 3′UTR fused to Firefly luciferase (18). Monitoring luciferase activity provided an estimate of the impact of the *hsp70* 3′UTR on the translation of luciferase mRNA following H_2O_2 and/or heat shock treatments. 3′UTR reporter activity displayed a time-dependent increase after heat-shock peaking at 6 h (Fig. 2C). This finding is consistent with early reports in *Drosophila* and mammalian cells on the role of the 3′UTR in the regulation of Hsp70 protein synthesis during heat shock (33, 38). Neither H_2O_2 nor the combination of H_2O_2 and heat shock increased luciferase activity above the

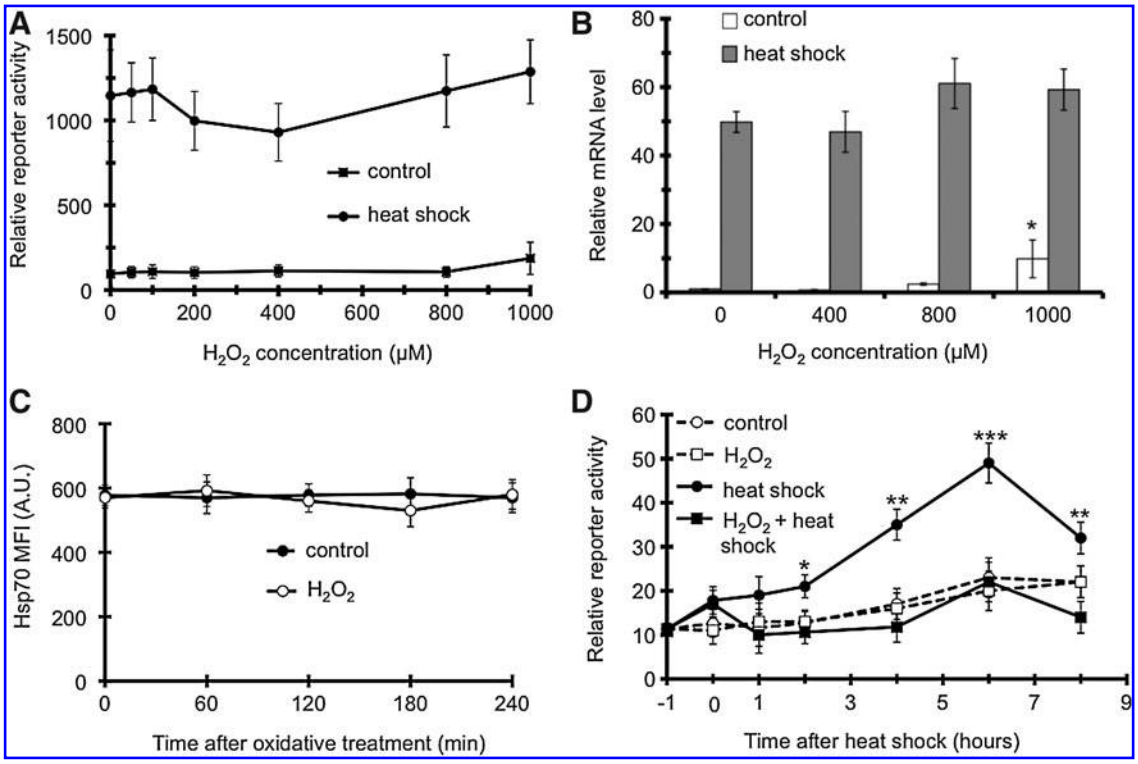

FIG. 2. H_2O_2 inhibition of Hsp70 involves a potential post-transcriptional regulation. (A) H_2O_2 does not affect *hsp70* promoter activation. Cells transfected with the *hsp70.1pr*/luc and control plasmids were treated as in Figure 1. Enzyme activities were measured 18 h later, and their ratios were expressed. **(B)** H_2O_2 does not diminish *hsp70* (HSPA1A) mRNA expression. Cells were treated as in Figure 1. mRNA levels were determined 1-h after treatments by quantitative reverse transcriptase–polymerase chain reaction and expressed relative to *β*-actin. **(C)** H_2O_2 does not affect Hsp70 protein turnover. Cells were heat shocked as above, after 2 h at 37°C cells were incubated in the absence (control), or presence of 800 μM of H_2O_2 for 2 h, and then harvested at the indicated timepoints, and analyzed by flow cytometry. **(D)** H_2O_2 inhibits the heat-induced luciferase reporter translation mediated by the Hsp70 3′ untranslated region (3′UTR). Cells transfected with a pGL3/luc/*hsp70.1* 3′-UTR and control plasmids were treated by 650 μM H_2O_2 for 2 h, and then kept at 37°C or heat shocked. At the indicated timepoints enzyme activities were determined, and expressed as a ratio. Values are means ± SDs of three experiments. n.s., non-significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

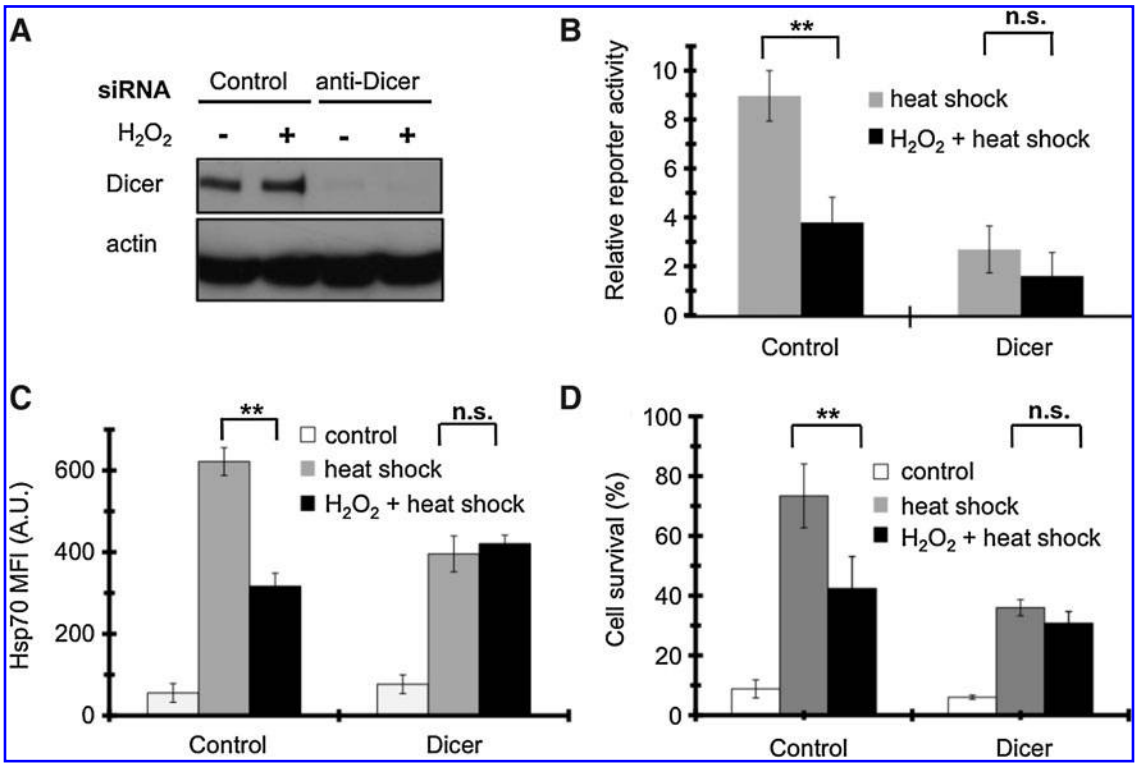

FIG. 3. RNA interference mediates H_2O_2-induced inhibition of Hsp70 induction and acquired thermotolerance in COS-7 cells. (A) Effect of H_2O_2 treatment and anti-Dicer siRNA on Dicer protein level. Two days after transfecton by anti-Dicer or control siRNA, respectively, cells were treated with 800 μM H_2O_2 for 2 h. Protein levels were analyzed by Western blot. Image is a representative of three experiments. **(B)** Effect of Dicer siRNA and H_2O_2 on the Hsp70 3′UTR activation. Cells undergoing a 2-day co-transfection with a control/Dicer siRNA and the 3′UTR reporter plasmids were treated by 650 μM H_2O_2 for 2 h, and then heat shocked. About 6 h later enzyme activities were determined and expressed as a ratio. **(C)** Effect of Dicer siRNA and H_2O_2 on Hsp70 protein expression. Cells transfected with a control/Dicer siRNA were treated by 800 μM H_2O_2 for 2 h, and then kept at 37°C or heat shocked. Five hours later Hsp70 levels were analyzed by flow cytometry. **(D)** Effect of Dicer siRNA and H_2O_2 on heat preconditioned thermotolerance. Cells transfected with a control/Dicer siRNA were treated by 800 μM H_2O_2 for 2 h, and then kept at 37°C or heat shocked. Lethal heat stress and survival assay was performed as in Figure 1A. Values are means ± SDs of three experiments. n.s., non-significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

baseline demonstrating that H_2O_2 entirely prevented the heat-induced activation by the *hsp70* 3′UTR (Fig. 2C).

RNA interference mediates H_2O_2-induced inhibition of Hsp70 induction and acquired thermotolerance in COS-7 cells

RNA interference is a powerful modulator of stress responses (23). To address whether RNA interference may mediate the events involving the Hsp70 3′UTR, we blocked miRNA maturation by anti-Dicer siRNA transfection. Only the siRNA, but not H_2O_2 led to a knock-down of Dicer (Fig. 3A). Intriguingly, anti-Dicer siRNA led to a large decrease in heat-induced 3′UTR reporter activity, suggesting that Dicer was necessary for the 3′UTR-mediated translational activation of the luciferase mRNA upon heat shock. This inhibition was comparable to that induced by H_2O_2, and a combination of anti-Dicer siRNA and H_2O_2 was not additive (Fig. 3B). Thus, H_2O_2 prevents the heat-induced Hsp70 3′UTR activation primarily *via* RNA interference.

To investigate how the inhibition of the 3′UTR by RNA interference is reflected in Hsp70 translation, we determined Hsp70 protein levels in anti-Dicer siRNA-transfected cells undergoing H_2O_2 and heat shock treatments. Dicer silencing inhibited Hsp70 heat-induction to approximately two-thirds of the control siRNA transfected value, comparable to the effect of Dicer silencing (Fig. 3C). Remarkably, H_2O_2 could not further reduce Hsp70 expression in Dicer-silenced cells, suggesting that the effect of H_2O_2 required an intact RNA interference.

These results suggested that RNA interference might play a role in the H_2O_2-induced inhibition of acquired thermotolerance. Indeed, we found that Dicer siRNA reduced acquired thermotolerance in heat-preconditioned cells, which was similar to the effect of H_2O_2 (Fig. 3D). Moreover, H_2O_2 did not further diminish thermotolerance in Dicer-silenced cells, in agreement with our observations on Hsp70 induction (cf. Fig. 3C). Hence, we conclude that RNA interference mediates the H_2O_2-induced inhibition of heat stress adaptation in COS-7 cells.

H_2O_2 inhibits aquired thermotolerance through DCR-1 in C. elegans

To address if the effect of H_2O_2 on heat stress adaptation was conserved during evolution, we used *C. elegans*, a

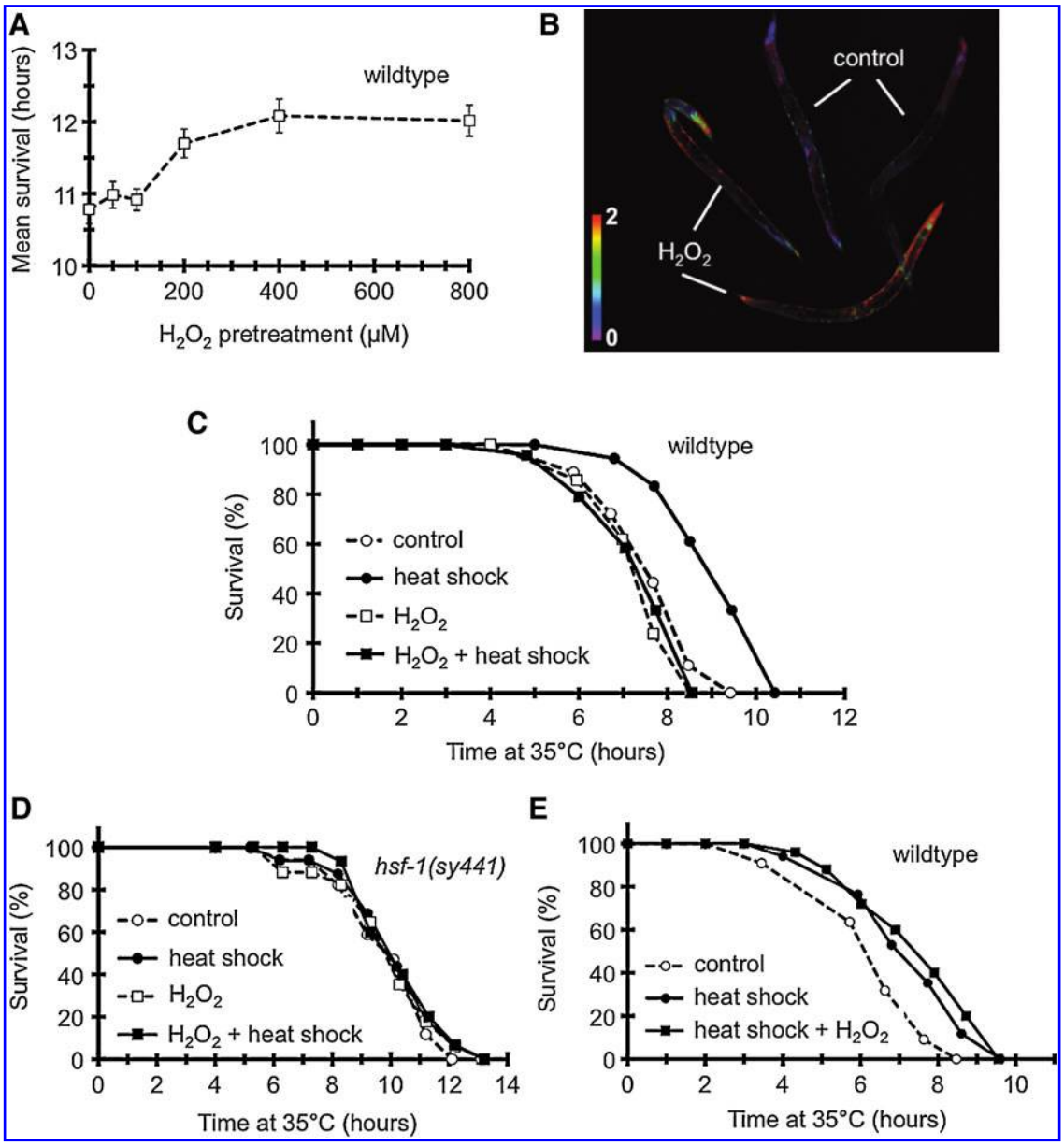

FIG. 4. A prior H_2O_2 treatment inhibits aquired thermotolerance in an HSF1-dependent manner in *Caenorhabditis elegans*. (A) Effect of preconditioning H_2O_2 treatments on oxidative tolerance. Oxidative stress was applied in the liquid nematode growth medium (NGM) for 1 h at 20°C, 12–14 h before a lethal oxidative challenge. Data are means ± SDs of two separate experiments. **(B)** Intensity-normalized ratio image demonstrating a rapid rise of oxidized/reduced HyPer ratio in jrIs[Prpl-17::HyPer] worms in response to a 1-min challenge by 100 μM H_2O_2 in liquid NGM. Representative image from five independent experiments. **(C)** Effect of H_2O_2 (100 μM for 1 h) on intrinsic and acquired thermotolerance induced by a preconditioning heat shock (heat shock, 30° for 2 h). Lethal heat stress was employed 12 h later. Only heat shock induces a significant difference in survival ($p < 0.0001$). **(D)** No change in thermotolerance by a preconditioning heat shock and/or an H_2O_2 treatment in *hsf-1(sy441)* mutant worms ($p > 0.1$). **(E)** H_2O_2 employed after the preconditioning heat shock does not abrogate acquired thermotolerance ($p > 0.1$ compared to heat shock). (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

powerful model system exhibiting an organismal complexity. In search of an H_2O_2-exposure that did not cause significant damage in nematodes, we found that a treatment by 100 μM for 1 h was below the threshold to induce oxidative tolerance to a lethal H_2O_2 challenge (Fig. 4A). This concentration induced a rapid signal elevation in the pharynx and intestine of worms ubiquitously expressing HyPer (Fig. 4B) (3). We used the 100-μM pretreatment to investigate its effect on nematodal thermotolerance. A preconditioning heat shock at 30°C for 2 h resulted in a 20%–40% increase in thermotolerance (Fig. 4C). A prior H_2O_2 treatment did not affect intrinsic thermotolerance of worms; however, it entirely abolished acquisition of thermotolerance by the preconditioning heat shock.

To address whether H_2O_2 would affect an HSF1-dependent process, we employed the *hsf-1(sy441)* point mutant strain

harboring a truncated transactivation domain that prevented the heat-induction of HSF1-target genes (15). In line with recently published data of McColl and colleagues (29), HSF1 was required for aquired, but not for intrinsic thermotolerance (Fig. 4D). H_2O_2 treatment was not additive to the *hsf-1(sy441)* background; it affected neither basal nor heat-preconditioned survival. Moreover, in wild-type worms, H_2O_2, if applied after the preconditioning heat shock, was unable to inhibit acquired thermotolerance (Fig. 4E), suggesting that H_2O_2 needs to precede heat preconditioning. Thus, H_2O_2 specifically inhibits the acquisition of HSF1-dependent thermotolerance in *C. elegans*.

If, similarly to mammalian cells, H_2O_2 inhibited the heat shock response *via* RNA interference in *C. elegans*, then worms deficient in miRNA synthesis would escape from the H_2O_2-dependent inhibition of thermotolerance. Investigating this hypothesis we found that silencing the Dicer ortholog by *dcr-1(RNAi)* restored the acquired thermotolerance of H_2O_2-treated worms to levels comparable to heat shock alone (Fig. 5A, B). *dcr-1(RNAi) per se* did not affect thermotolerance (Fig. 5B). We obtained similar results using loss-of-function *dcr-1* mutant nematodes (Fig. 5C). The efficiency of *dcr-1* silencing and the lack of a general disruption of RNA interference by H_2O_2, respectively, were demonstrated by an RNA interference reporter strain (20) (Fig. 5D). We made attempts to investigate an analogous involvement of Hsp70 regulation. Unfortunately, a number of antibodies were unable to detect nematode Hsp70. Quantitative polymerase chain reaction (PCR) measurements revealed a tendency of H_2O_2 preconditioning to augment heat-induced *hsp-70* mRNA expression. However, *dcr-1(RNAi)* significantly altered neither heat-induced mRNA level nor the H_2O_2-induced elevation (Supplementary Fig. S1; Supplementary Data are available online at www.liebertonline.com/ars). Despite the unclear involvement of Hsp70, RNA interference is required for H_2O_2 to inhibit acquired thermotolerance in worms.

Inhibition of RNA interference restores thermotolerance in endogenous models of oxidative stress

Next we asked how thermotolerance might be affected by chronic genetic disturbances in antioxidant defense. Antioxidant enzymes provide protection against oxidative stress

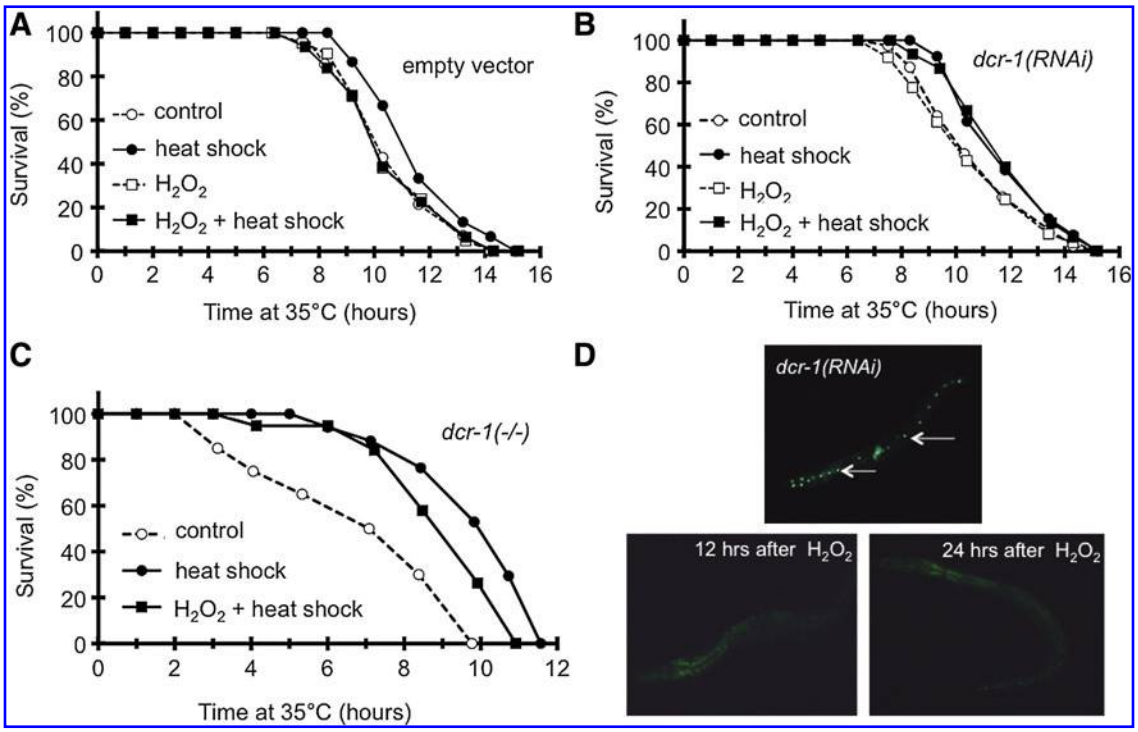

FIG. 5. DCR-1 mediates the H_2O_2-induced inhibition of thermotolerance in *C. elegans*. Effect of H_2O_2 on intrinsic and acquired thermotolerance in worms fed by empty (EV, **A)** or *dcr-1(RNAi)* **(B)** vectors, respectively. Treatments were as in Figure 4. Note that the activatory effect of preconditioning heat shock was less pronounced on RNAi plates. In EV-fed worms **(A)** only heat shock, while in *dcr-1(RNAi)*-fed worms **(B)** both heat shock as well as H_2O_2 + heat shock induced a significantly higher survival compared with controls ($p < 0.001$). **(C)** Both heat shock and H_2O_2 + heat shock induces a significant increase in thermotolerance in *dcr-1(ok247);unc-32(e189)* nematodes ($p < 0.0001$ *vs.* control). Survival curves are representatives of three experiments yielding similar results. **(D)** H_2O_2 does not compromise RNA interference. Epifluorescence image demonstrating increased expression of p*ajm*::GFP (harboring an anti-GFP hairpin siRNA in addition to the GFP sequence) in the GR1401 RNA interference reporter strain fed by *dcr-1(RNAi). Arrows* point to specific dots localized to epithelial seam cells. In contrast, H_2O_2 (100 μM for 1 h) treatment followed by a 12- or 24-h recovery did not inhibit GFP silencing. Please note the autoflurescence of oxidatively stressed worms. Representative image from three independent experiments. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

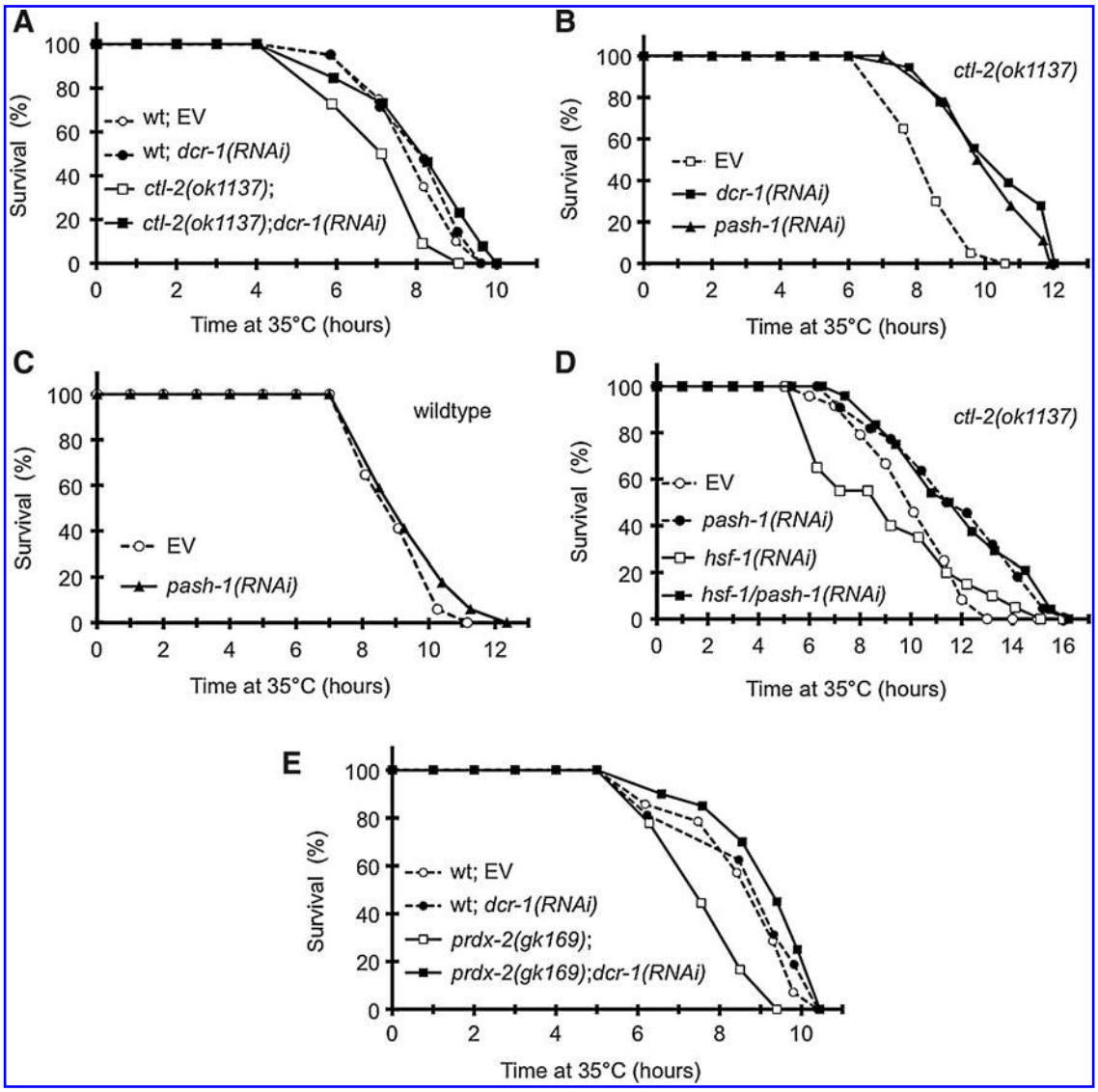

FIG. 6. Loss of RNA interference rescues thermotolerance in nematodes with genetic defects of H_2O_2 disposal. (A) Effect of *dcr-1(RNAi)* on thermotolerance of N2 and *ctl-2(ok1137)* worms. *ctl-2(ok1137)* worms exhibited significantly shorter survival ($p < 0.001$), while other survivals were not significantly different ($p > 0.2$), compared to N2 control. **(B)** *pash-1(RNAi)* phenocopies *dcr-1(RNAi)* by inducing a significant increase in thermotolerance of *ctl-2(ok1137)* ($p < 0.0001$) compared to that of the EV control. **(C)** *pash-1(RNAi)* does not change thermotolerance of wild-type worms ($p > 0.1$) compared to that of the EV control. **(D)** *pash-1(RNAi)* extends thermotolerance independently of *hsf-1* in *ctl-2(ok1137)* worms [$p < 0.01$ *vs. pash-1/hsf-1(RNAi)*]. **(E)** Effect of *dcr-1(RNAi)* on thermotolerance of N2 and *prdx-2(gk169)* worms. *prdx-2(gk169)* worms fed by EV exhibited significantly shorter ($p < 0.0001$), while those fed by *dcr-1(RNAi)* exhibited slightly longer survival ($p < 0.05$) compared to N2 control. Survival curves are representatives of three independent experiments giving similar results.

by removing ROS. Catalase-2 is a peroxisomal enzyme involved in H_2O_2 elimination accounting for ~80% of total catalase activity in the worm (39). *ctl-2* loss of function elevates endogenous H_2O_2 levels (3), decreases oxidative tolerance and shortens lifespan (39). We observed that *ctl-2(ok1137)* animals exhibited impaired intrinsic thermotolerance compared to wild type, which was completely restored by *dcr-1(RNAi)* (Fig. 6A). Silencing the Drosha ortholog PASH-1, the other key enzyme in miRNA biogenesis, phenocopied the effect of *dcr-1(RNAi)* in the *ctl-2(ok1137)* strain (Fig. 6B) without affecting wild-type thermotolerance (Fig. 6C). Neither the survival decrease in *ctl-2(ok1137)* nor the amelioration by *pash-1(RNAi)* was prevented by *hsf-1(RNAi)* (Fig. 6D). Hence, *ctl-2* loss of function modulates intrinsic thermotolerance, not involving the HSF1-Hsp axis.

To test whether the observed phenomena might be attributed to the general impairment of H_2O_2 elimination, we examined the lack of peroxiredoxin-2, involved in H_2O_2 reduction in the cytosol. *prdx-2(gk169)* worms, similarly to the *ctl-2(ok1137)* strain, are susceptible to H_2O_2 injury, and display a shortened lifespan (37). We found that *prdx-2* knockout also markedly decreased *C. elegans* thermotolerance (Fig. 6E). Importantly, *dcr-1(RNAi)* prevented thermotolerance inhibition in *prdx-2(gk169)* worms. Together these data suggest that genetic defects in H_2O_2 elimination compromise heat stress adaptation *via* RNA interference.

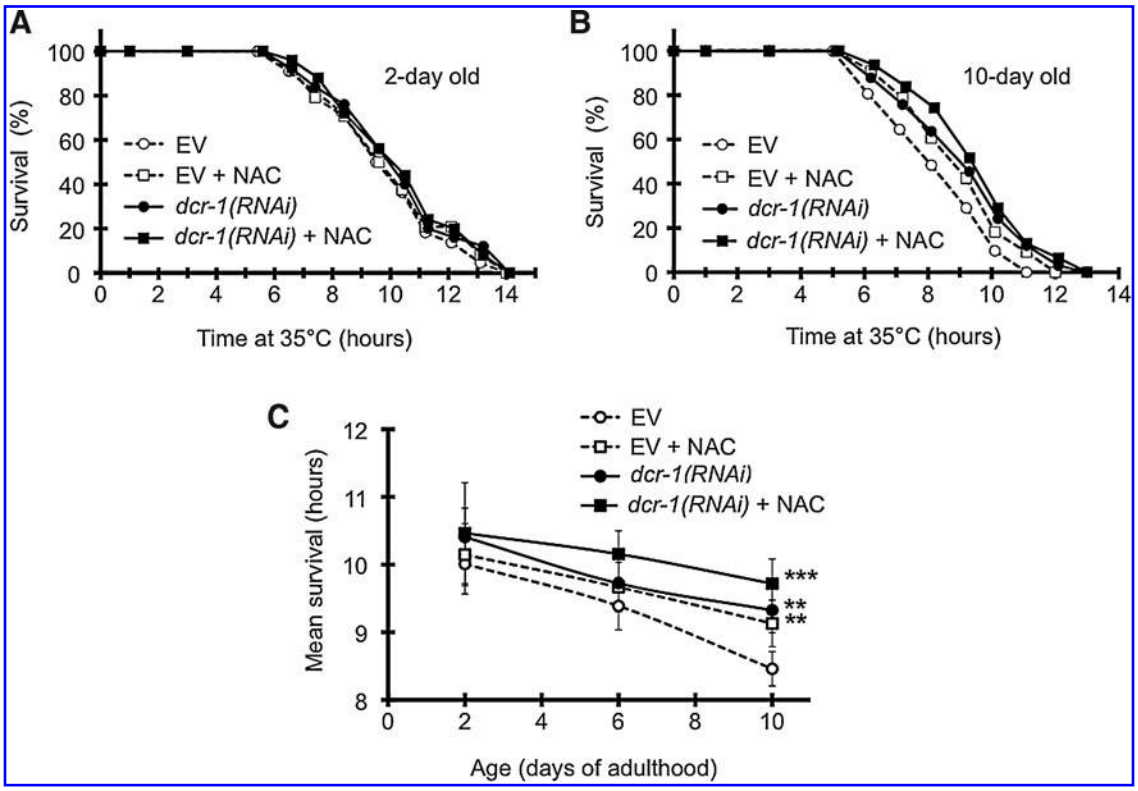

FIG. 7. Loss of RNA interference and the antioxidant *N*-acetyl-L-cysteine ameliorate age-associated decline of thermotolerance in *C. elegans*. Thermotolerance of 2-day **(A)** and 10-day **(B)** old nematodes treated by *dcr-1(RNAi)* and/or 5 m*M* *N*-acetyl-L-cysteine (NAC; from day 1 of adulthood). There was no significant difference in survival between treatments at day 2 ($p > 0.1$). The 10-day old control (EV) exhibited a significantly shorter survival ($p < 0.001$ *vs.* 2-day EV). All *dcr-1(RNAi)* and/or 5 m*M* NAC induced a significant increase in survival ($p < 0.001$ *vs.* 10-day old EV), which approached ($p = 0.028$ 10-day EV + NAC *vs.* 2-day EV), and became non-significant ($p > 0.05$ 10-day *dcr-1(RNAi)* and *dcr-1(RNAi)* + NAC *vs.* 2-day EV) compared with the survival of the 2-day old EV control. Survivals of 10-day *dcr-1(RNAi)* and *dcr-1(RNAi)* + NAC strains were not significantly different ($p > 0.4$). **(C)** Mean thermotolerance of nematodes treated by *dcr-1(RNAi)* and/or 5 m*M* NAC as a function of age. *Panels* are representatives of two independent experiments yielding similar results. $^{**}p < 0.01$, $^{***}p < 0.001$.

Inhibition of RNA interference delays age-dependent decline of thermotolerance

Aging is characterized by a collapse of proteostasis and an impairment of the heat shock response in *C. elegans* (4). Consistent with this, we observed a decline in *C. elegans* thermotolerance during aging (Fig. 7A–C). Oxidative stress and H_2O_2 increases during aging and ROS are considered a major cause of aging (3, 34). To address if oxidative stress affected thermotolerance during aging, we treated worms with the small molecular antioxidant, NAC. Intriguingly, NAC was able to reduce the decline of thermotolerance during aging resulting in a milder slope and a significant difference at the old worms at day 10 of age (Fig. 7A–C).

Then we asked, whether RNA interference was involved in the age-associated decline of thermotolerance of worms. *dcr-1(RNAi)* did not significantly influence the thermotolerance of young animals at day 1 (Fig. 5), but efficiently suppressed the age-induced decline of thermotolerance similarly to NAC treatment (Fig. 7A–C). Moreover, the combination of *dcr-1(RNAi)* with NAC was not significantly different from the effect of *dcr-1(RNAi)* at any time points tested. These findings indicate that RNA interference is involved in the oxidative stress-induced age-dependent decline of heat stress adaptation in *C. elegans*.

Discussion

In this study, we have presented evidence that oxidative stress inhibits the adaptive responses to heat stress in both mammalian cells and *C. elegans*. Silencing Dicer and Drosha orthologs, key enzymes specific to miRNA maturation reveals a conserved role for RNA interference. In mammalian cells H_2O_2 abolishes a positive action of RNA interference on acquired thermotolerance. Inhibition of RNA interference does not alter thermotolerance in young nematodes, suggesting that H_2O_2 may induce miRNA(s) that inhibit the acquisition of thermotolerance. Intrinsic thermotolerance decrease of *prdx-2* and *ctl-2* knockouts and aged worms might require accumulation of miRNA(s) inhibiting HSF1-independent processes. Despite species-specific and context-dependent mechanisms, our results provide support to the modulation of stress responses by RNA interference (Fig. 8) (23).

Our findings on the post-transcriptional inhibition of Hsp70 expression offer a potential molecular mechanism underlying the H_2O_2-induced compromise of acquired thermotolerance. Early reports demonstrating a heat-induced stabilization of *hsp70* mRNA by its 3′UTR (33, 38) and the decrease in heat-induced *hsp70* mRNA by H_2O_2 in glioma cells (1) suggested that H_2O_2 may prevent mRNA stabilization. However, our results showing no impact of H_2O_2 on

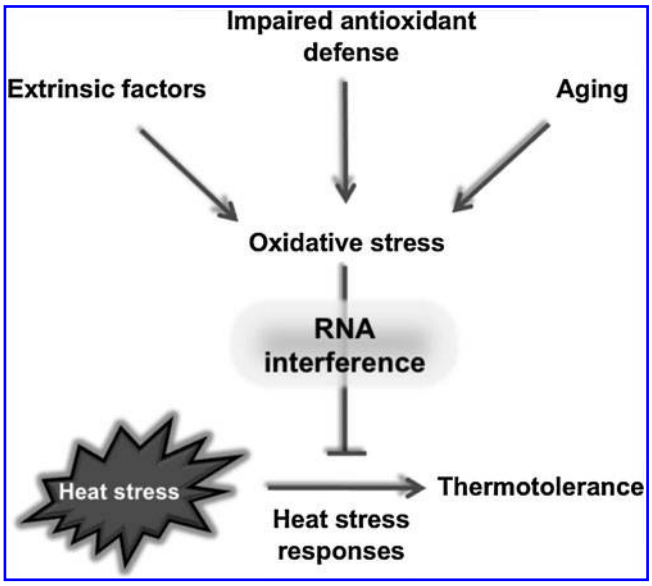

FIG. 8. Model for the role of RNA interference in the modulation of heat stress responses by oxidative stress. Oxidative stress may be induced by various sources, such as increased production/extrinsic factor (H_2O_2), decreased elimination (impaired defense, endogenous mutants), or a combination of the two (aging). RNA interference mediates an inhibitory action of oxidative stress and reduces heat resistance.

hsp70 mRNA and inhibition of 3′UTR reporter, respectively, are consistent with a compromised translation by H_2O_2. Interestingly, inflammatory cytokines inhibit colonic Hsp70 translation by recruiting its mRNA to stress granules (17, 18). Although it may be one plausible mechanism, our results using Dicer knockdown suggest the involvement of miRNA(s). Possible scenarios include an H_2O_2-induced decrease of activatory miRNA(s), or displacement/domination of heat-induced activatory miRNA(s) by H_2O_2-induced inhibitory/neutral miRNA(s) from the *hsp70* mRNA. Both mechanisms are generally employed by RNA interference (23, 27). Moreover, recent articles provide evidence on miRNAs either inhibiting (miR-378*, miR-711, miR-146a, miR-146b-5b) (35, 46), or ischemic preconditioning-induced miRNAs (miR-1, miR-21, miR-24 or others) (48, 49) activating Hsp70 expression. Identification of the exact mechanism(s) and miRNA(s), as well as an analogous Hsp70 regulation in nematodes requires further studies. Nevertheless, our study raises the idea that pathophysiological oxidative conditions (inflammation, wound healing, aging) might employ RNA interference to post-transcriptionally regulate Hsp70 in various tissues (7, 18, 36).

Our use of mutants deficient in H_2O_2 elimination demonstrates that a chronic disturbance in ROS metabolism impairs intrinsic thermotolerance, independently of HSF1 (Fig. 6). This defect can entirely be reversed by blocking miRNA maturation, suggesting a profound post-transcriptional remodeling of heat stress adaptation by RNA interference in response to oxidative stress. McColl *et al.* elegantly showed that increased intrinsic thermotolerance in *daf-2* mutant worms is mediated by a *daf-16*-dependent translational response (29). A common motif in the two studies is that RNA interference or translation do not limit survival in young worms; however, they differentially condition heat resistance in both long-lived insulin-like signaling mutants and short-lived oxidative defense-deficient mutants and aged worms, respectively [(29) and our study]. It is tempting to speculate that the *daf-16*-regulated response of insulin signaling mutants might involve miRNAs. The clarification of a possible interaction of the translational response and RNA interference in the regulation of stress resistance remains the task of future studies.

Aging in the worm is characterized by an increased accumulation of ROS as well as a collapse of protein homeostasis (4). Our results on the age-induced decline of intrinsic thermotolerance support these observations, and use of the antioxidant NAC demonstrates a progressive causal role for ROS in decline of stress resistance during aging (Fig. 7). Importantly, both the comparable pattern of NAC and *dcr-1(RNAi)* protection and the lack of significant synergism imply a substantially overlapping mode of action. Moreover, increased protection by *dcr-1(RNAi)* suggests that RNA interference adversely affects heat resistance with aging. Single miRNAs do not seem to play an essential role in *C. elegans* development and growth, but both RNA interference and single miRNAs are indispensable to ensure proper development during environmental stress (20, 24, 31). Likewise, there is an extensive change in miRNA expression during *C. elegans* aging (11, 19) and several individual miRNAs similarly modulate longevity and stress resistance in *C. elegans* (11). Inhibition of the entire RNA interference in adulthood provides strong evidence to the general dysregulation of miRNAs in aging and in oxidative stress with a negative impact on stress resistance (Figs. 6 and 7). It remains to be seen whether RNA interference would pose a trade-off between fine-tuning developmental programs and growth during stress in exchange for a self-maintenance later in life. Our results imply that beyond well-characterized stress-responsive HSF1 and DAF-16 pathways, RNA interference may offer a novel target to alleviate decline of stress responses during aging.

Materials and Methods

Materials

Reagents for cell culture were from Invitrogen. Solutions for flow cytometry were from BD Biosciences. Electrophoresis and blotting reagents were from Bio-Rad. *N*-acetyl-L-cysteine and H_2O_2 were from Sigma. All other reagents were from either from Sigma or Fluka.

Cell culture and survival

COS-7 cells were obtained from the ATCC. Cells were cultured as described (40). Cell survival was analyzed by Trypan Blue exclusion 24 h after challenge.

Determination of protein levels

Flow cytometry using a fluorescein-isothiocyanate-conjugated monoclonal anti-Hsp70 antibody (StressGen), cell lysis, and Western blotting using a polyclonal anti-Hsp70 antibody (21), or antibodies against Hsp90 (Stressgen), Dicer (CST), actin (Sigma) was carried out as previously described (40).

Transfection and reporter gene assays

Cells were transfected at a density of 40% using Lipofectamine (Invitrogen). Control/anti-Dicer siRNA (Quiagen)

was introduced at 100 nM. Further treatments were applied at 48 h post-transfection. For the hsp70-promoter reporter gene assay, cells were transfected with 0.35 μg *hsp70.1pr*/Firefly luciferase plasmid (Rick Morimoto, Northwestern University) and cytomegalovirus/β-galactosidase plasmids, while for the 3′UTR reporter assay 0.35 μg pGL3 basic or pGL3/*hsp70.1 3′-UTR* plasmid (Eugene Chang, University of Chicago) and thymidine kinase/Renilla luciferase plasmids were employed, respectively. Treatments were performed 24 h post-transfection. About 18 h post-treatment reporter activities were measured using commercial assay kits (Promega) and expressed.

C. elegans *strains and RNA interference*

Strains were obtained from the CGC, if not otherwise specified. The following strains were used in this study: wild type (N2), jrIs[Prpl-17::HyPer], PS3551 *hsf-1(sy441)*, BB1 *dcr-1(ok247);unc-32(e189)* III, VC289 *prdx-2(gk169)* II, VC574 *ctl-2(ok1137)* II, and the GFP RNAi-reporter GR1401 (Gary Ruvkun, Harvard University). Strains were backcrossed to the wild type at east three times to clear potential background mutations, and were maintained as described (8). RNAi was performed as described by feeding worms with HT115(DE3) bacteria transformed with empty vector, *dcr-1(RNAi)* Gary Ruvkun (Harvard University) or *pash-1(RNAi)* (Source BioScience) vectors, repectively (45). Experiments were carried out in the second generation. Experiments were performed in the second generation with synchronized young 1-day-old adults, except for age-related thermotolerance.

Thermotolerance assay

Thermotolerance was performed on nematode growth medium plates at 35°C till complete extinction of the population using 25 animals per condition in at least two independent trials. Viability was determined hourly by assaying for movement in response to gentle prodding.

mRNA expression analysis

mRNA was prepared using the GeneJET RNA Purification Kit (Fermentas). mRNA was reverse transcribed using the RevertAid^{TM} cDNA Synthesis Kit (Fermentas). Quantitative PCR was performed in an ABI 7300 System by Taqman Gene Expression Assays: HSPA1A: Hs_00359147_s1; β-actin: Hs_99999903_m1 (Applied Biosystems). Relative amounts of *hsp70* mRNA were determined using the Comparative Cycle Treshold Method for quantitation and normalized to actin mRNA levels. Please see Supplementary Materials and Methods for the analysis of hsp70 mRNA expression in nematodes.

Analysis of H_2O_2 levels and fluorescence microscopy

Fluorescence measurements in COS-7 cells transfected by HyPer-C (Miklós Geiszt, Semmelweis University) were performed as described (12). HyPer titration was achieved by sequential addition of increasing concentrations of H_2O_2. Mean fluorescence intensities over individual cells were calculated from 3-min recordings. H_2O_2 in worms was monitored using the jrIs[Prpl-17::HyPer] strain, ubiquitously expressing the H_2O_2-biosensor HyPer, was used. Worms were immobilized and imaged as described (3).

Statistical analysis

Data were analyzed using SPSS software 15.0 (SPSS, Inc.). Survival curves were compared by the log-rank test. If not stated otherwise, all experiments were repeated at least three times. Variables were expressed as mean ± standard deviation. Statistical significance was indicated as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

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Author Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

3′UTR = 3′ untranslated region
H_2O_2 = hydrogen-peroxide
HSF1 = heat shock transcription factor 1
Hsp = heat shock protein
miRNA = microRNA
NAC = *N*-acetyl-L-cysteine
NGM = nematode growth medium
PCR = polymerase chain reaction
ROS = reactive oxygen species
MFI = mean fluorescence intensity

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PLoS PATHOGENS

A Role for SKN-1/Nrf in Pathogen Resistance and Immunosenescence in *Caenorhabditis elegans*

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Abstract

A proper immune response ensures survival in a hostile environment and promotes longevity. Recent evidence indicates that innate immunity, beyond antimicrobial effectors, also relies on host-defensive mechanisms. The *Caenorhabditis elegans* transcription factor SKN-1 regulates xenobiotic and oxidative stress responses and contributes to longevity, however, its role in immune defense is unknown. Here we show that SKN-1 is required for *C. elegans* pathogen resistance against both Gram-negative *Pseudomonas aeruginosa* and Gram-positive *Enterococcus faecalis* bacteria. Exposure to *P. aeruginosa* leads to SKN-1 accumulation in intestinal nuclei and transcriptional activation of two SKN-1 target genes, *gcs-1* and *gst-4*. Both the Toll/IL-1 Receptor domain protein TIR-1 and the p38 MAPK PMK-1 are required for SKN-1 activation by PA14 exposure. We demonstrate an early onset of immunosenescence with a concomitant age-dependent decline in SKN-1-dependent target gene activation, and a requirement of SKN-1 to enhance pathogen resistance in response to longevity-promoting interventions, such as reduced insulin/IGF-like signaling and preconditioning H_2O_2 treatment. Finally, we find that *wdr-23(RNAi)*-mediated constitutive SKN-1 activation results in excessive transcription of target genes, confers oxidative stress tolerance, but impairs pathogen resistance. Our findings identify SKN-1 as a novel regulator of innate immunity, suggests its involvement in immunosenescence and provide an important crosstalk between pathogenic stress signaling and the xenobiotic/oxidative stress response.

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Introduction

A proper immune response ensures survival in a hostile environment and contributes to longevity. The nematode *Caenorhabditis elegans* provides a valuable genetic tool for studying innate immunity and various aspects of host-pathogen interactions. During infection, both bacterial virulence factors and host antimicrobial defense mechanisms present oxidative and proteotoxic noxae inducing tissue-damage, especially in the intestine [1–5]. Accordingly, several self-protective stress-response regulators including the forkhead transcription factor DAF-16/FOXO [6], the heat shock transcription factor HSF-1 [7] and the X-box binding protein 1 (XBP-1) [8] are required for robust immunity. Moreover, the DAF-16-regulated antioxidant enzymes SOD-3 and CTL-2 contribute to immunity by protecting intestinal cells from reactive oxygen species during exposure to *Enterococcus faecalis* [9]. Strikingly, hyper-activation of DAF-16 enhances susceptibility to bacterial infection [10].

These data illustrate a critical role of stress response in innate immunity, and raise questions about the co-ordination of antimicrobial and host-defense mechanisms. Antimicrobial responses are mediated by a canonical p38 mitogen-activated protein kinase (MAPK) pathway, which is conserved from nematodes to humans [11,12]. Besides, the insulin/IGF-like signaling (IIS) and TGF-β pathways are also involved in the regulation of the pathogen-specific immune response in *C. elegans* [13,14]. Both p38 MAPK and IIS pathways regulate the Nrf1/2/3 ortholog SKN-1, a transcription factor that orchestrates both oxidative and xenobiotic stress responses in *C. elegans* [15,16]. However, the involvement of SKN-1 in the regulation of pathogen stress response is unknown.

In nematodes, three SKN-1 isoforms exist. While the function of SKN-1A has not been elucidated yet, SKN-1B and C provide distinct biological functions. SKN-1B is expressed in the ASI neurons, and mediates lifespan extension in response to dietary restriction [17]. In contrast, intestinal SKN-1C is required for oxidative stress resistance and contributes to longevity by reduced IIS [15]. SKN-1 activity is regulated by phosphorylation and degradation. Under normal conditions, inhibitory phosphorylations by GSK-3 and IIS kinases, AKT-1/2 and SGK-1, retain SKN-1 in the cytosol [15,18], where it is rapidly targeted to proteasomal degradation by the WD40 repeat protein WDR-23 [19,20]. In response to oxidative stress, the p38 MAPK ortholog PMK-1 phosphorylates SKN-1, which then translocates to the nuclei of intestinal cells and induces transcription of phase 2 detoxification genes [16].

Here we report that SKN-1 is required for pathogen resistance against both Gram-negative *P. aeruginosa* and Gram-positive *E. faecalis* bacteria, consistently with an independent study [21] published after submission of this paper. We further demonstrate a

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Author Summary

Innate immunity promotes survival by combating pathogenic threat. During infection, tissue damage is induced both by invading pathogens and immune effectors such as toxins and free radicals. Therefore, it is important to elucidate by what self-protective mechanisms the host defends itself against pathogenic stress. The conserved SKN-1 protein of the roundworm *Caenorhabditis elegans* directs a detoxification response neutralizing harmful compounds as well as confers tolerance to oxidative stress. Here we identify SKN-1 as a novel regulator of *C. elegans* innate immunity. We show that SKN-1 contributes to resistance against infection caused by two bacterial pathogens. Components of a pathogen-responsive signaling pathway are required to activate SKN-1 in intestinal cells at the site of infection. Moreover, the SKN-1-dependent response to pathogen exposure declines during aging, whereas mild metabolic and oxidative stresses, known to extend lifespan, evoke a SKN-1-dependent boosting of immunity. Finally, we find that elimination of an inhibitory protein leads to an excessive activation of SKN-1 and impairs pathogen resistance. Thus, SKN-1 integrates various chemical, metabolic and microbial signals to elicit a self-protective detoxification response, which promotes innate immunity and may be relevant to diseases and aging of the human immune system.

Toll and Interleukin-1 Receptor domain protein (TIR-1)/PMK-1-dependent SKN-1 activation upon *P. aeruginosa* infection. Moreover, we show a gradual decrease of pathogen resistance and of the activation of SKN-1-dependent targets during aging, and a requirement of SKN-1 to boost immunity in response to longevity-promoting manipulations, such as reduced IIS and preconditioning H_2O_2 treatment. Finally, we find that hyperactivation of SKN-1 impairs pathogen resistance. Our results indicate an intricate regulation of innate immunity by SKN-1 and links pathogenic stress signaling to the xenobiotic stress response.

Results

SKN-1 is required for bacterial pathogen resistance in *C. elegans*

To study the role of SKN-1 in *C. elegans* immunity, we examined the pathogen resistance of animals in the absence of SKN-1. *skn-1(zu135)* allele is considered to be a genetic null mutation as it creates a premature stop codon that affects all SKN-1 isoforms [15]. *skn-1(zu135)* mutant worms were first exposed to the Gram-negative *Pseudomonas aeruginosa* (PA14) strain. As *skn-1(zu135)* mutants are sterile, we eliminated the difference between them and wild-type N2 strain arising from the 'bag of worms' phenotype, a major contributor to killing. To this end, germline development was inhibited by silencing *cdc-25.1*, required for embryonic mitosis and meiosis. *cdc-25.1(RNAi)* animals exhibit extended survival on pathogenic bacteria, as reported previously [21,22]. In these conditions, we observed an increased susceptibility of *skn-1(zu135)* mutants to PA14 (Figures 1A and S1A, Tables S1A and S1E). This result was confirmed by using *skn-1(RNAi)* (Figures 1B and S1B, Table S1A and S1E). Furthermore, when animals were exposed to the Gram-positive *Enterococcus faecalis* SdB262 strain, both *skn-1(zu135)* and *skn-1(RNAi)* exhibited significantly decreased survival, though in this case the absence of SKN-1 exerted a more modest effect (Figures S1C and S1D, Table S1E). These results suggest a requirement of SKN-1 for the efficient immune response against two distinct bacterial pathogens. In subsequent experiments, we focused on further defining the role of SKN-1 in the antibacterial response against *P. aeruginosa*.

P. aeruginosa infection triggers SKN-1 activation

To investigate if SKN-1 nuclear translocation occurs upon PA14 exposure, we incubated *skn-1::gfp* L3 larvae on *P. aeruginosa* lawn for 5 hours. We found a massive accumulation of SKN-1::GFP in intestinal nuclei of infected larvae, compared to control animals fed by the non-pathogenic OP50 *Escherichia coli* strain (Figures 2A and 2B). The specificity of this response was demonstrated by a complete inhibition using a *skn-1*-specific double-stranded RNA. To reveal a SKN-1-dependent transcrip-

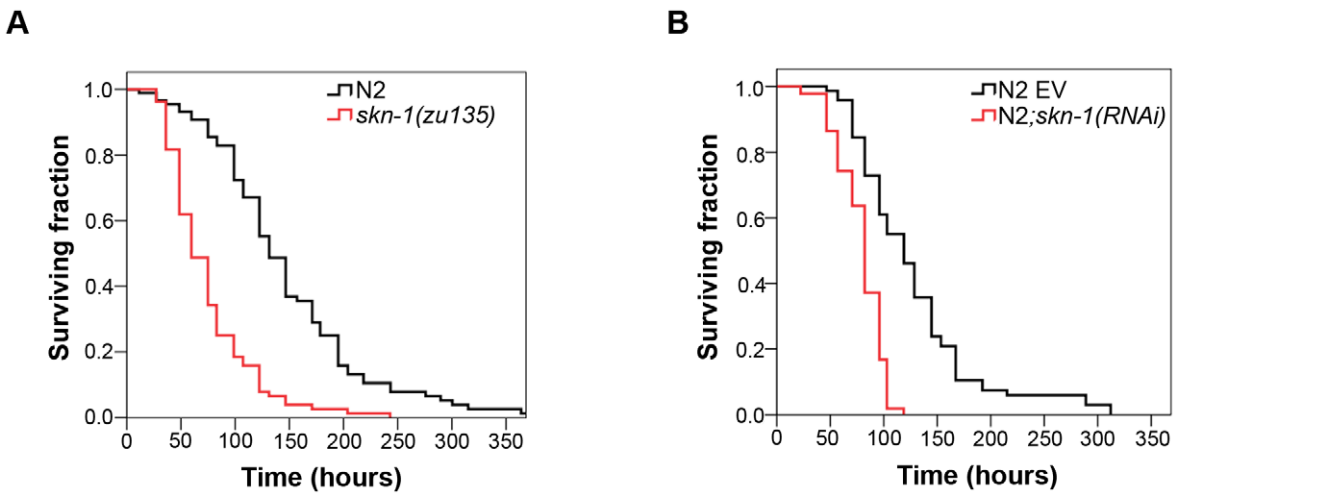

Figure 1. SKN-1 is required for bacterial pathogen resistance. (**A, B**) Increased susceptibility to *Pseudomonas aeruginosa* PA14 occurs in both *skn-1(zu135)* mutant ($p<0.0001$) and *skn-1(RNAi)* nematodes ($p<0.0001$). Killing assays were performed with at least 90 young adult animals in each condition. EV: empty vector RNAi.
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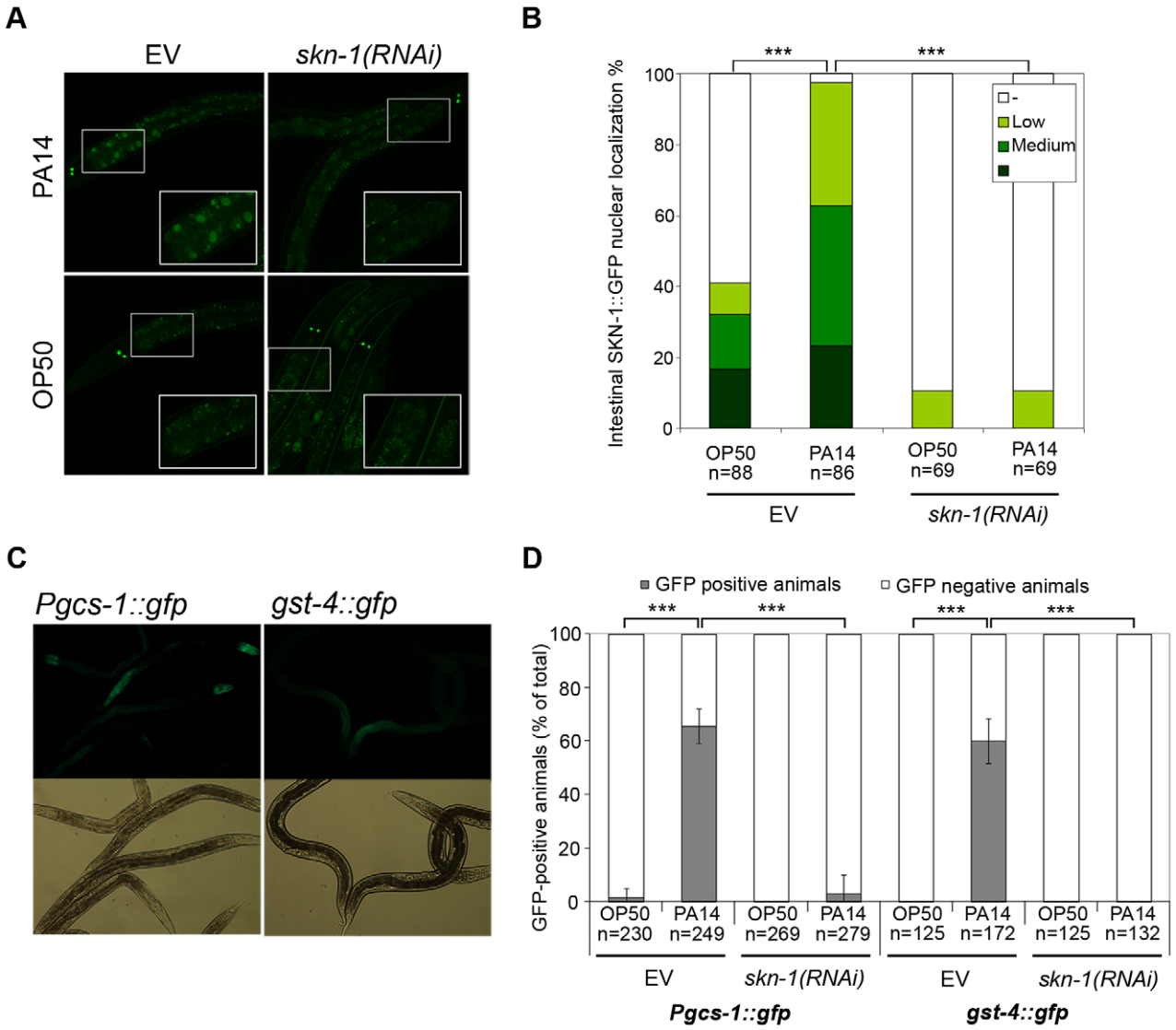

Figure 2. *P. aeruginosa* infection activates SKN-1. (**A**) Representative epifluorescence image demonstrating the translocation of SKN-1::GFP in the *Is007*[SKN-1::GFP] strain to intestinal nuclei in L3 larvae, fed by the empty vector or *skn-1* dsRNA, upon a 5-hour exposure to *P. aeruginosa* PA14. Note that the intestinal tissue displays autofluorescence, and in the ASI neurons SKN-1::GFP is not silenced by *skn-1* RNAi treatment. (**B**) Quantification of SKN-1 nuclear translocation from data shown on panel (A). SKN-1::GFP-positive nuclei were counted in the intestine of 78 animals. "Low" refers to animals in which SKN-1::GFP was detected in less than 5 intestinal nuclei, while "high" indicates that SKN-1::GFP signal was present in more than 15 intestinal nuclei. (**C**) Representative epifluorescence microscopic image showing intestinal expression of *Pgcs-1*::GFP and GST-4::GFP in L3 larvae upon a 24-hour PA14 exposure. Images of control animals incubated on OP50 bacteria are shown in Figure S2. (**D**) Quantification of reporter expression demonstrating the SKN-1-dependence of the response. Data were obtained from panel (C) completed with the data of *skn-1(RNAi)* animals. Microscopic images are representatives of 3 independent experiments. EV: empty vector RNAi.
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tional activation upon PA14 infection, we examined the *Pgcs-1::gfp* and *gst-4::gfp* reporter strains. While *gcs-1* is regulated exclusively by SKN-1, *gst-4* is under the mutual control of both DAF-16 and SKN-1 [15]. We observed an effective intestinal induction of fluorescence to comparable extent in both strains in response to a 24 h-exposure of PA14 (Figures 2C and 2D). Both the *gcs-1* promoter activation and the GST-4 expression were significantly suppressed by feeding worms with *skn-1(RNAi)*, indicating the specific requirement of SKN-1 to elicit these responses. Thus, PA14 infection induces nuclear translocation of SKN-1 and transcriptional activation of its targets.

The TIR-1/PMK-1 pathway controls SKN-1 activation upon *P. aeruginosa* infection

The p38 MAPK ortholog PMK-1 has a fundamental role in *C. elegans* innate immunity [11]. To investigate whether PMK-1 regulates SKN-1 in response to bacterial exposure, we monitored the activity of a *Pgcs-1*::GFP reporter in a wild-type and a *pmk-1(km25)* mutant genetic background (Figures 3A and 3B). We found that silencing *pmk-1* entirely prevented the SKN-1-dependent activation of *gcs-1* in response to PA14 infection. In physiological settings, PMK-1 is inactivated by the dual specificity MAPK phosphatase VHP-1 [23]. Suppression of VHP-1 resulted

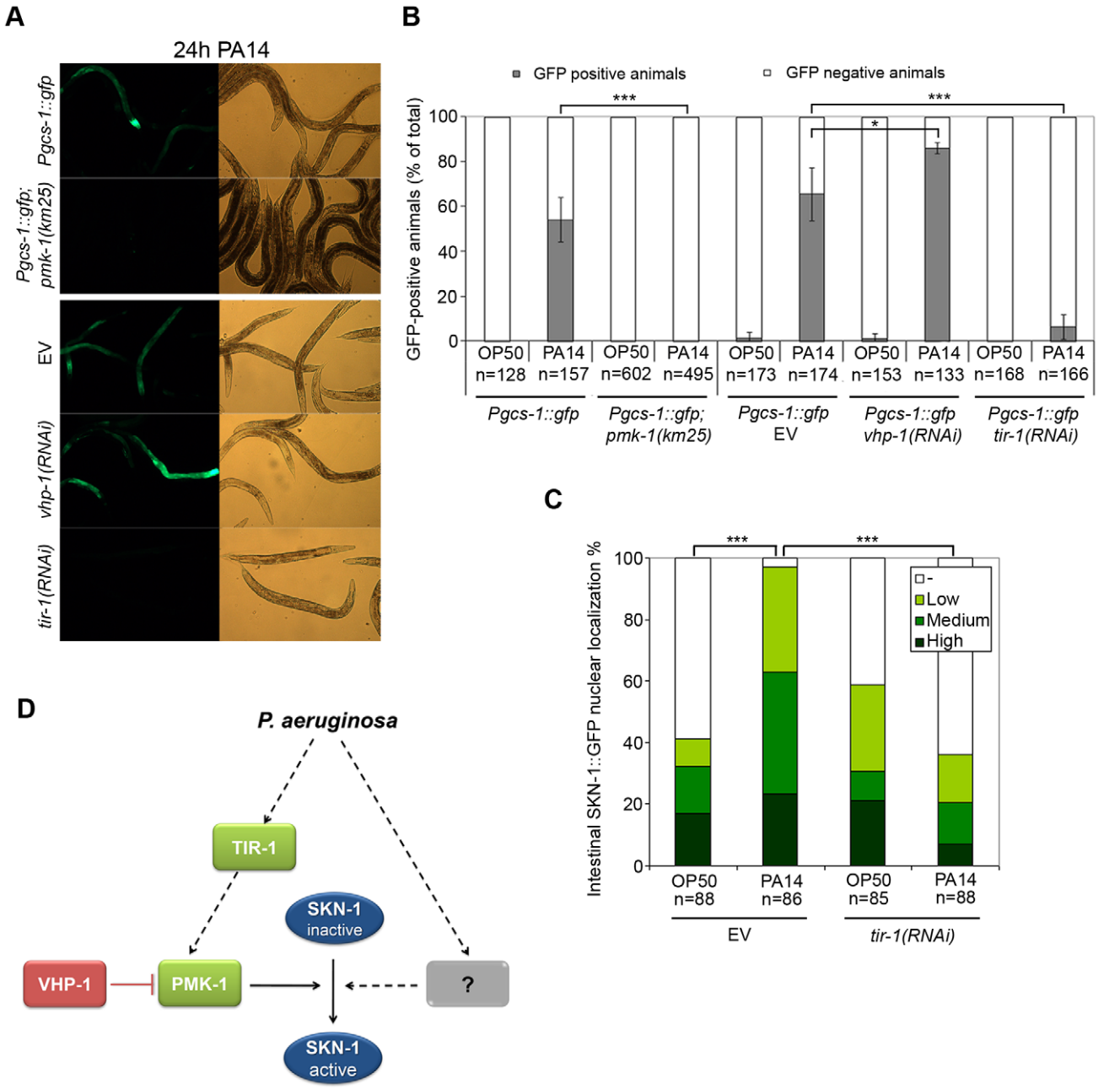

Figure 3. The pathogen response-specific TIR-1 and p38 MAPK PMK-1 are required for SKN-1 activation upon *P. aeruginosa* infection. (**A**) Representative epifluorescence microscopic images showing the expression of *Pgcs-1*::GFP in *pmk-1(km25)* mutants as well as in the p38 MAPK phosphatase *vhp-1(RNAi)*, and the Toll/IL-1 resistance (TIR) domain protein *tir-1(RNAi)* animals in response to *P. aeruginosa* infection. L3 larvae were exposed to PA14 for 24 hours. Microscopic images are representatives from 3 independent experiments. (**B**) Quantification of reporter expression from data shown on panel (A) completed with data of control animals fed by OP50 for 24 h. (**C**) Quantification of SKN-1 nuclear translocation in *tir-1(RNAi)* L3 larvae upon 5 h PA14 exposure. Representative epifluorescence images of *tir(RNAi)* L3 larvae are shown in Figure S3. Please note that data in Figure 2B and 3C were derived from the same set of experiments. (**D**) Suggested model of SKN-1 activation during *P. aeruginosa* infection. Upon exposure to PA14, the TIR-1/PMK-1 pathway is indispensable but insufficient to elicit SKN-1 transactivation. We propose a second, unknown factor/pathway that is required to activate SKN-1. Whether the two pathways act in parallel or consecutively is unclear. Solid arrows indicate a direct, while dashed arrows indicate an indirect/unknown connection. EV: empty vector RNAi.
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in increased PMK-1 phosphorylation and resistance to PA14 [23]. However, *vhp-1(RNAi)* significantly increased *Pgcs-1*::GFP activation upon PA14, but not upon OP50 exposure, suggesting that PMK-1 is an indispensable permissive factor for SKN-1 activation by infection.

TIR-1 is a conserved Toll/IL-1 resistance (TIR) domain protein known to activate p38 MAPK signaling independently of the Toll-like receptor ortholog *tol-1* during PA14 infection [24,25]. Depletion of TIR-1 by RNAi prevented *Pgcs-1*::GFP fluorescence upon PA14 infection (Figures 3A and 3B). A similar inhibition in

Pgcs-1::GFP expression was also observed in *tir-1(qd4)* mutant animals (data not shown). Moreover, silencing *tir-1* prevented the nuclear translocation of SKN-1 induced by PA14 infection, but did not affect its baseline expression levels (Figures 3C and S3.). Altogether, these results suggest that the TIR-1/PMK-1 pathway is necessary to attain activation of SKN-1 by PA14 exposure (Figure 3D).

Involvement of SKN-1 in immunosenescence

Immune function declines with age, leading to compromised immune responses to infections in the elderly. Accordingly, aged nematodes exhibit increased susceptibility to infection by various pathogens, including *P. aeruginosa* [26–28]. As SKN-1 is required for both longevity and for pathogen resistance, we asked if chronological aging affected SKN-1-dependent target gene expression in nematodes exposed to pathogenic stress. To this end we examined the promoter induction of *gcs-1* by PA14 in L3 stage larvae, 4-day and in 9-day old adult worms, respectively (Figures 4A and 4B). We observed a massive age-dependent decrease in the expression of *Pgcs-1*::GFP reporter after 24 h of PA14 infection. To investigate, how SKN-1 activity is involved in immunosenescence, we examined the survival of 1, 4 and 9 day-old adult N2 and *skn-1(zu135)* mutant nematodes exposed to PA14. We observed that pathogen resistance in wild-type animals already declined at day 4 as previously described by Laws *et al.* [26]. Consistent with a premature decline of self defense in the absence of SKN-1 activity, 4 d adult N2 worms showed similar survival on PA14 to 1 d adult *skn-1(zu135)* animals ($p = 0.1429$). Furthermore, we found that *skn-1(zu135)* mutant animals exhibited increased susceptibility to PA14, compared to N2 at all ages ($p>0.0001$) (Figure 4C and Table S1B), indicating that SKN-1 function is also required to survive infection beyond day 9.

To address the potential involvement of SKN-1-dependent gene expression in immunosenescence, we performed a bioinformatics analysis using the microarray data of Youngman *et al.* [27]. From the 379 genes exhibiting the most significant down-regulation during aging (>10 fold down-regulation at d15 *vs.* d6) we identified 46 SKN-1-regulated genes (based on *skn-1(RNAi)* screens [29,30]) (Figure 4D, Table S2). Next, we examined the regulation of these genes with respect to oxidative stress, PA14 and PMK-1 dependent regulation using Wormbase expression data [31]. Strikingly, SKN-1-regulated genes subject to PA14-dependent regulation were over-represented compared to those regulated by either oxidative stress or PMK-1, respectively. These results confirm a progressive age-dependent compromise in pathogen resistance and imply that a decline in SKN-1 function contributes to immunosenescence.

Reduced IIS and oxidative preconditioning require SKN-1 for enhanced pathogen resistance

Loss-of-function mutations in the insulin/IGF-1 receptor gene, *daf-2* enhance stress resistance and extend lifespan, and both processes require DAF-16 and SKN-1 activity [15]. As reduced IIS increases pathogen resistance [6], we investigated the contribution of SKN-1 to pathogen resistance in *daf-2(e1370)* mutant animals. In accordance with previously published data [6], *daf-2(e1370)* mutants exhibited robustly increased pathogen resistance against PA14 (Figure 5A and Table S1C). However silencing *skn-1* by RNAi largely increased their susceptibility to PA14. These data suggest that SKN-1 is required for reduced IIS to bring about enhanced pathogen resistance against *P. aeruginosa*.

Exposure to mild oxidative stress induces tolerance to a lethal challenge, cross-tolerance to other stresses and extends lifespan [32]. To address the impact of oxidative preconditioning on pathogen resistance, nematodes were pretreated with various concentrations of H_2O_2, and then exposed to PA14 infection. H_2O_2 preconditioning induced resistance against PA14 in a concentration-dependent manner, reaching a 2-fold increase in survival by 2 mM H_2O_2, compared to untreated controls (Figure 5B and Table S1D). Intriguingly, the same treatment on *skn-1(zu135)* mutant nematodes not only exhibited a decreased pathogen resistance, but had a strongly suppressed reaction to H_2O_2 (Figure 5C and Table S1D). We also found that the mutation of another major oxidative stress response regulator, DAF-16 (*daf-16(mu86)*), shows an even shorter basal survival, compared to *skn-1(zu135)*, and poorly responded to H_2O_2 (Figure 5C and Table S1D). Thus, oxidative preconditioning requires both SKN-1 and DAF-16 for enhanced pathogen resistance against *P. aeruginosa*.

Excessive activation of SKN-1 by *wdr-23(RNAi)* impairs pathogen resistance

Finally, we investigated whether increased activation of SKN-1 was able to promote pathogen resistance. Stabilization of SKN-1 by RNAi against *wdr-23* has been shown to induce constitutive SKN-1 activation, resistance to oxidative stress and longevity [19]. Feeding worms with *wdr-23(RNAi)* indeed resulted in an unexpectedly robust increase in the expression of *Pgcs-1*::GFP and GST-4::GFP (Figure 6A) compared to the PA14-induced expression (Figure 2C). To our surprise, *wdr-23(RNAi)*, compared to empty vector feeding greatly reduced pathogen resistance to PA14 (Figure 6B and Table S1A). *wdr-23(RNAi)* did not impair survival in a *skn-1(zu135)* mutant background excluding a SKN-1-independent impact of WDR-23 on pathogen resistance. Interestingly, the compromised reactivity of worms to *wdr-23(RNAi)* was confined to pathogenic stress. Determination of oxidative tolerance revealed that *wdr-23(RNAi)* animals exhibited increased survival, whereas *skn-1(RNAi)* nematodes displayed decreased survival, compared to control worms, when exposed to 3 mM or 5 mM H_2O_2, respectively (Figure 6C). Our results suggest that an excessive post-translational stabilization of SKN-1 induces oxidative stress resistance but impairs resistance to bacterial infection.

Discussion

Our present study identified SKN-1 as a novel regulator of pathogen resistance against both Gram-negative *P. aeruginosa* and Gram-positive *E. faecalis* bacteria (Figures 1 and S1). We demonstrated a TIR-1/PMK-1-dependent SKN-1 activation upon *P. aeruginosa* infection (Figures 2 and 3). Moreover, we showed an early onset of immunosenescence with a parallel decline in SKN-1-dependent transcriptional activation (Figure 4) and a requirement of SKN-1 to efficient immunity in response to reduced IIS or preconditioning H_2O_2 treatment (Figure 5). Finally, we found that excessive activation of SKN-1 by blocking its turnover impaired pathogen resistance (Figure 6).

The xenobiotic stress response provides a conserved defense mechanism against oxidative and electrophilic stress via the induction of phase 2 detoxification enzymes [33]. Nrf2 and its nematode ortholog, SKN-1, are transcription factors important in oxidative and xenobiotic stress response [34,35]. Previously, several studies demonstrated the importance of Nrf2 in innate immunity in mammals [36–38]. For example, $Nrf2^{-/-}$ mice exhibit increased susceptibility to bacterial infection and bacterial lipopolysaccharide (LPS)-induced inflammation [39]. Similarly, our present study demonstrates that SKN-1 deficiency in *C. elegans* impairs resistance to infection (Figures 1 and S1). During the revision of our manuscript an independent paper from the Garsin

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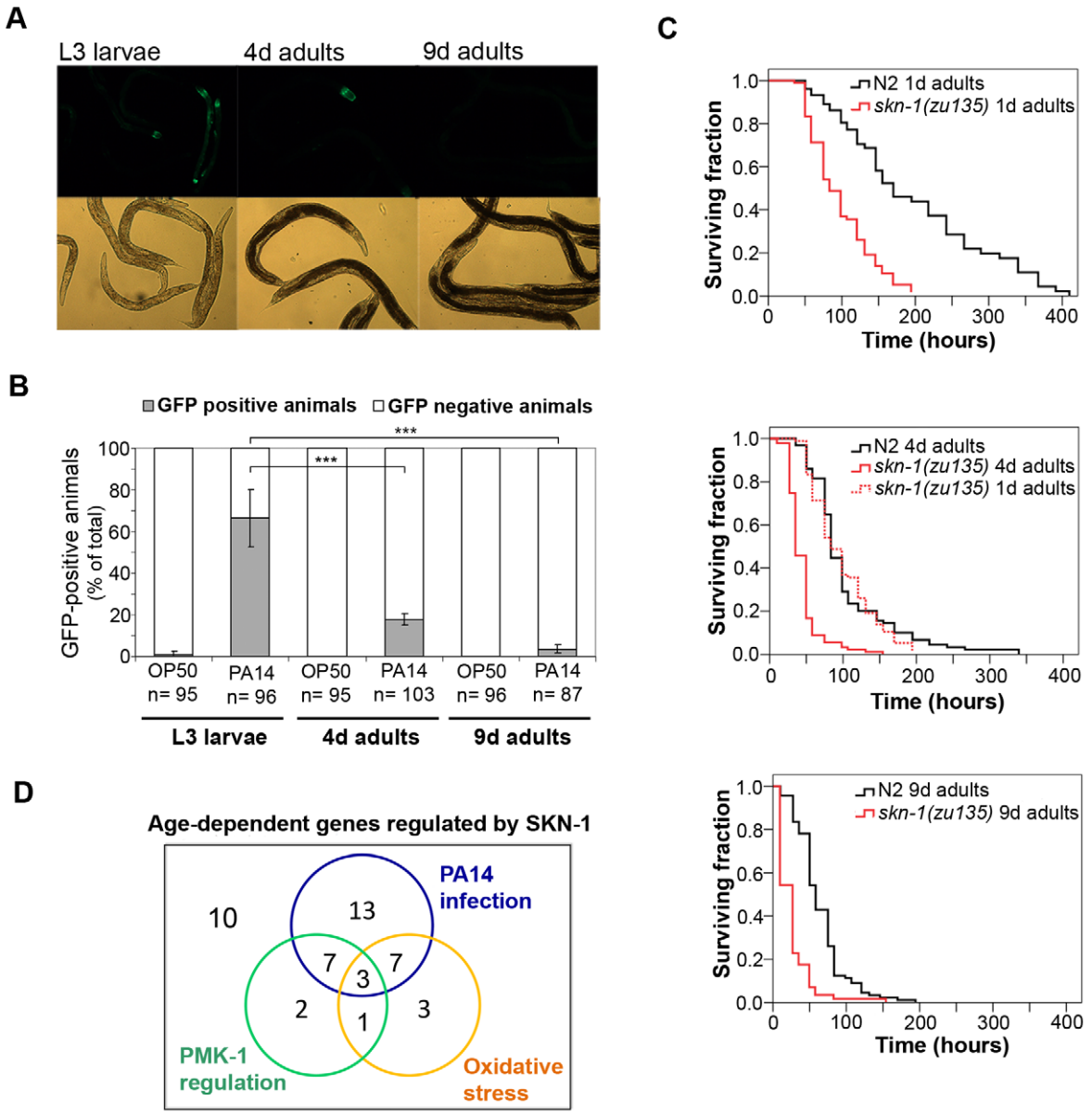

Figure 4. Involvement of SKN-1 in immunosenescence. (**A**) Representative epifluorescence images showing the decreased induction of the *gcs-1* promoter. L3 larvae, 4 d/9 d adult *Pgcs-1::gfp* worms were exposed to PA14 for 24 h. (**B**) Quantification of the epifluorescence images of panel (A). Epifluorescence images are representatives of two independent experiments. EV: empty vector RNAi. (**C**) Pathogen resistance of young adult (1 day-old), 4 day-old and 9 day-old adult N2 and *skn-1(zu135)* mutant animals. *skn-1(zu135)* mutant worms exhibited significantly increased susceptibility to PA14 compared to N2 wild-type animals at all ages ($p<0.0001$). 1 day-old adult *skn-1(zu135)* worms show similar pathogen resistance to 4 day-old N2 worms ($p=0.1429$) (middle graph). Killing assays were performed with 3 parallel plates in each condition in 2 independent trials. (**D**) Venn diagram showing the distribution of age-regulated SKN-1 target genes. Data were analyzed by finding the overlaps between micro-array databases containing the genes down-regulated at least 10-fold in 15 d adult compared to 6 d adult wild-type animals [27] and SKN-1 dependent genes under non-stress [29] or oxidative stress conditions [30] using expression data from Wormbase [31]. Please note that the majority of genes belong to those regulated by PA14 infection. 10 of 46 genes could be assigned to none of the groups. For the detailed gene list please refer to Table S2.

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lab appeared, which obtained similar results [21]. A previous study found no significant impairment of pathogen resistance by *skn-1(zu135)* and *skn-1(zu67)* mutations in the wildtype background [40]. A possible reason of this discrepancy might be the use of *cdc-25.1(RNAi)* by the Garsin lab and our study, suggesting that selective bagging in wild-type *vs.* sterile *skn-1* mutants might have masked the pathogen resistance decrease induced by loss of *skn-1* in the previous investigation. Consistently with this note, the use of *skn-1(RNAi)* from the L1 stage, which did not induce sterility [21], confirmed the decrease in pathogen resistance. A similar finding was also reported as an earlier unpublished result of Evans *et al.* [41].

We observed a nuclear translocation and transcriptional activation of SKN-1 in the intestine, consistent with the primary site of infection (Figures 2 and 3). Furthermore, we could not detect any apparent change in SKN-1 intensity or nuclear localization in ASI neurons upon PA14 exposure (Figure 2). This finding is in agreement with previous reports on constitutive SKN-

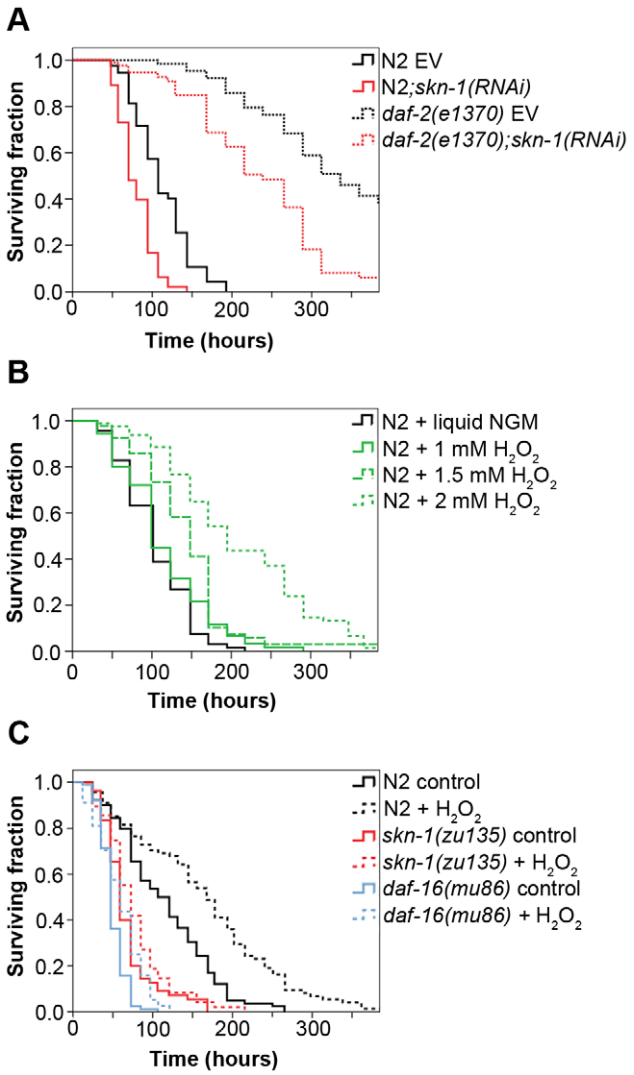

Figure 5. Reduced IIS and oxidative preconditioning require SKN-1 for enhanced pathogen resistance. (**A**) *daf-2(e1370)* mutant nematodes exhibited increased resistance to *P. aeruginosa*, compared to that of wild-type N2 worms ($p<0.0001$). *skn-1(RNAi)* treatment of *daf-2(e1370)* animals increased the susceptibility to *P. aeruginosa* infection ($p<0.0001$). Killing assays were performed with at least 90 young 1-day old adult animals in each condition. (**B**) H_2O_2 pretreatment increased survival on PA14 in a concentration-dependent manner. Survival curves of N2 wild-type worms treated with various concentrations of H_2O_2 in liquid NGM: 1 mM ($p=0.425$), 1.5 mM ($p<0.0001$) and 2 mM ($p<0.0001$) 12 h prior to the killing assay are shown. Killing assay was performed with 90 3-day old adult animals in each condition. (**C**) Oxidative preconditioning-induced pathogen resistance was impaired in the absence of SKN-1 or DAF-16. Increase in survival was less pronounced in either *skn-1(zu135)* ($p=0.0156$) or *daf-16(mu86)* mutant ($p=0.0304$), than in wild-type animals ($p<0.0001$). Survival curves of the same genetic background were compared in the absence and presence of H_2O_2. Data were combined from at least two experiments with 89 animals in average for each group. EV: empty vector RNAi.
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1B activity and a lack of interaction between SKN-1B and WDR-23 in ASI neurons, respectively [19,34]. Together, our data imply an active role of the intestinal SKN-1C isoform and does not allow a conclusion regarding the involvement of the ASI neuronal SKN-1B in the inducible antibacterial response. However, a continuous transcriptional output of SKN-1B and/or a different mode of regulation of SKN-1B in response to infection cannot be excluded. Hence, a tissue-specific analysis of SKN-1 function may give a clue whether SKN-1 isoforms co-operate in immunity.

Our findings confirm those of van der Hoeven *et al.* [21] on the critical role of the p38 MAPK pathway in SKN-1 activation (Figure 3). However, the inability of *vhp-1(RNAi)* to activate SKN-1 on OP50 suggests that there should be additional, unidentified signals that govern SKN-1 activation in response to PA14 infection, which will certainly prompt additional studies. We showed an absolute requirement of TIR-1 for SKN-1 nuclear translocation and for *gcs-1* promoter induction upon PA14 exposure, while the Garsin lab reported no to minimal involvement of TIR-1 in *gst-4* and *gcs-1* induction in response to *E. faecalis* [21]. Whether the difference between our observations beyond differences in assays and dosage/treatment by *tir-1(RNAi)* may be due to a differential pathogen sensing of *P. aeruginosa* and *E. faecalis* is an exciting possibility to explore. TIR-1 and PMK-1 are related to the mammalian SARM and p38 MAPK proteins, respectively [42,43]. Although the existence of an orthologous pathway in mammals remains elusive, these findings indicate that the SKN-1-mediated response is an ancient component of innate immunity.

Immunosenescence, the age-dependent decline of immune response, is a critical problem impeding healthy ageing [44]. *C. elegans* provides a useful tool to investigate elements of innate immunity contributing to immunosenescence [45]. A recent systematic study reported an age-dependent progressive increase in susceptibility to PA14, detectable at day 6 of adulthood [27]. Our data on a similar age-related decline in survival, with a 45% decrease in pathogen resistance at day 4 (Figure 4C) establishes an earlier, dramatic onset of immunosenescence. Moreover, the loss of SKN-1 function phenocopies the decreased resistance of d4 worms already at day 1, and continues to negatively affect survival at day 9 (Figure 4C). A parallel strong decline in *gcs-1* transactivation on day 4 and the widespread down-regulation of SKN-1 targets, including PA14-regulated genes, between day 6 and 15 of adulthood (Figure 4D, Table S2) are consistent with this observation and indicate SKN-1 as a key player in immunosenescence.

Youngman and colleagues found an involvement of PMK-1 in a decline of the innate immune response, and hypothesized intestinal deterioration as a primary event in immunosenescence [27]. Of note, the dependence of SKN-1 activation on PMK-1 ([21] and our study), the high number of age-dependent SKN-1 targets among PMK-1 targets (13 of 26; Figure 4D, Table S2 and [27]) and the impact of SKN-1 on intestinal homeostasis [30] suggest a dynamic, probably mutual interaction between SKN-1 and PMK-1 in immunosenescence. We propose that SKN-1-dependent stress responses collapse early in adulthood, which manifest in a vicious circle of decreasing intestinal homeostasis, progressive immunosenescence and increasing pathogenic load in *C. elegans*. This hypothesis is consistent both with the short lifespan of worms in natural conditions and with the allocation of resources to maintain the soma until the production of fit progeny (the "disposable soma theory" [46]).

Genetic or environmental interventions that operate via stress-responsive mechanisms extend both lifespan and pathogen resistance [6,15,32,47,48]. Our results on H_2O_2-induced pathogen resistance (Figure 5B), together with previous analogous heat-shock experiments [7] suggest that mild stresses acting early in adulthood confer resistance against pathogenic stress. Furthermore, the demonstration of the requirement of SKN-1 and DAF-16 in the enhanced pathogen resistance of both H_2O_2-preconditioned and *daf-2(e1370)* mutant nematodes suggests a dynamic

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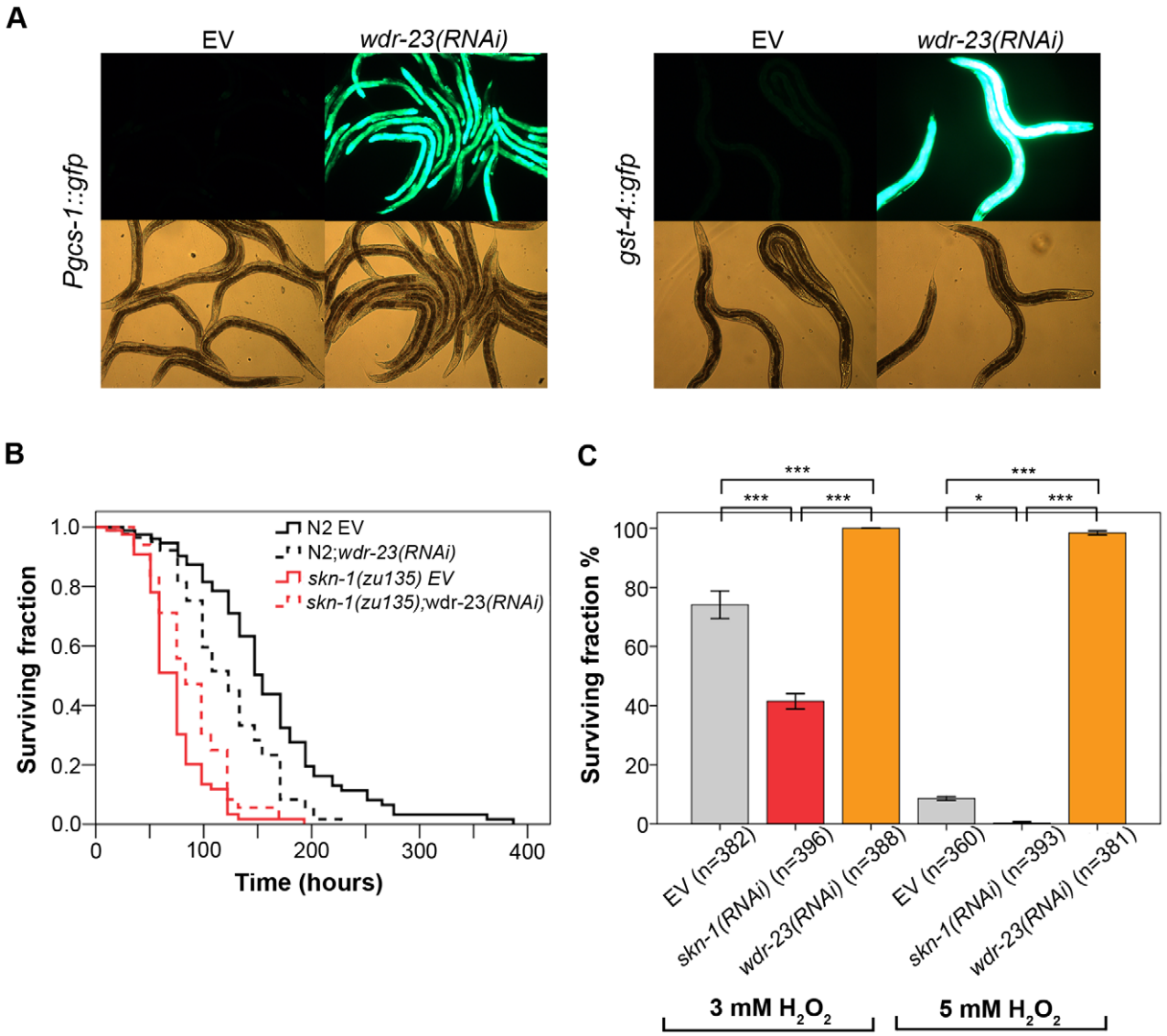

Figure 6. Excessive activation of SKN-1 by *wdr-23(RNAi)* impairs pathogen resistance. (**A**) Robust up-regulation of *Pgcs-1*::GFP and GST-4::GFP in 1 d adult *wdr-23(RNAi)* worms. (**B**) Pathogen resistance of *wdr-23(RNAi)*-fed N2 and *skn-1(zu135)* mutant worms. N2;*wdr-23(RNAi)* exhibited increased susceptibility to *P. aeruginosa* infection ($p<0.0001$). *skn-1(zu135)* mutant nematodes fed by *wdr-23(RNAi)* showed no significant difference in survival on PA14 ($p=0.1992$). Killing assay was performed with at least 90 1-day old adult animals in each condition. Please note that data in Figures 1A and 6B were derived from the same set of experiments. (**C**) *wdr-23(RNAi)* treatment increased ($p<0.0001$, both at 3 mM and 5 mM H_2O_2, respectively), while *skn-1 RNAi* treatment decreased oxidative tolerance to H_2O_2 ($p<0.0001$ at 3 mM H_2O, $p<0.05$ at 5 mM H_2O_2). Worms were treated with 3 mM or 5 mM H_2O_2 for 1 hour, and 24 h after challenge survival was scored. Data were combined from three experiments with 120 animals in average for each group. EV: empty vector RNAi.
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cross-talk of these stress-responsive transcription networks tipping the balance between responses to nutrient availability, oxidative and pathogen stress. Though our data do not allow a clear conclusion, the recent prediction of a DAF-16-dependent regulation of SKN-1 [49] is in line with the proposed functional interaction between SKN-1 and DAF-16 and is a subject of future interesting studies.

Evidence on mammals indicates a defensive role of Nrf2 against inflammation-induced tissue damage [36–39]. An analogous nematode model raises the question, whether SKN-1 affects immunity independently of its impact on aging. Indeed, longevity, stress resistance and pathogen resistance are intimately linked in short-lived *C. elegans*. However, a greater reduction of survival in *skn-1(zu135)* mutants on PA14 than on non-pathogenic OP50 (51% *vs.* 19% compared to N2, Figures 1 and S4, Tables S1 and S3) suggests a stronger impact of SKN-1 on pathogen resistance than on longevity. The pathogen-induced activation of SKN-1 and the large number of PA14-regulated SKN-1-targets including immune-related CUB-like domain proteins (Figure 4D, Table S2) [29] support SKN-1's active involvement in the pathogen response. Finally, it has previously been shown that knock-down of *skn-1* in the *daf-2(e1370)* mutant selectively suppresses stress resistance but not lifespan [15]. Thus, our findings demonstrating a SKN-1-dependent increase of pathogen resistance by this allele (Figure 5A), suggest an immune-specific effect of SKN-1. Studies investigating SKN-1-dependent responses on OP50 *vs.* pathogens

would help reveal the downstream mediators of SKN-1 and to determine the immune-specific and other branches of SKN-1 action.

SKN-1 activation by *wdr-23(RNAi)* impairs pathogen resistance, a result in contrast with those of the Garsin lab [21]. The reason may lie in the use of *cdc-25.1(RNAi)* by us, or in the different dosage/duration of RNAi treatment in the two experimental protocols. Nevertheless, our findings on the adverse effects of excessive SKN-1 activity are consistent with those reporting that loss of WDR-23 activity slows growth via SKN-1 [19], expression of SKN-1 from high-copy arrays is toxic [15], and that SKN-1 mediates increased susceptibility to PA14 in the absence of BLI-3 [21]. Combining the two *wdr-23(RNAi)* results ([21] and our study) clearly shows that this type of activation can dissociate immunity from oxidative stress resistance. As a potential mechanism, excessive SKN-1 activation may remodel the transcriptional response in favor of anti-oxidative defense and/or may repress pathogen-specific defenses. Indeed, differential SKN-1 transcriptional outputs were demonstrated [29]. It is logical to assume that negative and positive inputs regulating SKN-1 allow fine-tuning of stress resistance, growth and immunity. An analogous deterioration of pathogen resistance by the excessive activation of DAF-16 [10] underscores the necessity of tight control of stress responses to avoid deleterious consequences during infection.

Taken together, we propose that an optimal enhancement of SKN-1 activity in proper time-frame may enhance immune responses and delay immunosenescence without compromising longevity. In recent years, *C. elegans* has become a versatile model not only for studying innate immunity, host-pathogen interactions, but for testing pharmacological interventions in drug discovery [50]. The results presented herein may prompt studies on drugs targeting SKN-1/Nrf2 to modulate the innate immune response. In conclusion, our findings indicate an intricate regulation of innate immunity by SKN-1, and link pathogenic stress signaling to xenobiotic and oxidative stress responses.

Materials and Methods

C. elegans strains and maintenance

Nematodes were maintained and propagated on *E. coli* OP50 as described by Brenner [51] at 20°C. The following *C. elegans* strains were obtained from the Caenorhabditis Genetics Center and were used in this study: N2, EU31 *skn-1(zu67)IV/nT1[unc-?(n754) let-?](IV;V)*, KU25 *pmk-1(km25)IV.*, ZD101 *tir-1(qd4)III.* Further strains were used: LD001 *Is007 [skn-1::gfp]*, CF1038 *daf-16(mu86)I.*, CB1370 *daf-2(e1370)III.* (Tibor Vellai, Eötvös Loránd University, Budapest, Hungary), LD1171 *Is003 [Pgcs-1::gfp]* (T. Keith Blackwell, Harvard Medical School, Boston MA, USA) and MJCU017 *kIs17[gst-4::gfp, pDP#MM016B]X.* (Johji Miwa, Chubu University, Kasugai, Japan). Nematodes were treated with *cdc-25.1(RNAi)* to avoid the bacterial infection induced 'bag of worms' phenotype in all experiment.

Crossing and genotyping by PCR

Pgcs-1::gfp;pmk-1(km25) and *Pgcs-1::gfp;tir-1(qd4)* strains were created by mating male *pmk-1(km25)* or *tir-1(qd4)*, respectively, with LD1171 *Is003 [Pgcs-1::gfp]* hermaphrodites. Transgenic *rol* progeny was isolated with the correct genotype as scored by PCR. PCR primers were obtained from Sigma. The primers *pmk-1*-OF (5′-GGATACGGAAGAAGAGCCAATG-3′) and *pmk-1*-OR (5′-CAACAGTCTGCGTGTAATGC-3′) were used to detect the *pmk-1(km25)* deletion allele. The wild-type *pmk-1* allele amplified a 1195-bp fragment compared to a 882-bp fragment from *pmk-1(km25)* allele. Homozygous *pmk-1(km25)* mutants were identified by PCR using primers *pmk-1*-IF (5′-TCCTATAAGTTGCCAT-GACCTCAG-3′) and *pmk-1*-IR (5′-CCCGAGCGAGTACATT-CAGC-3′) from inside the deletion region. Wild-type animals generated a 469-bp fragment, while the homozygous *pmk-1(km25)* allele did not produce any fragment. The primers *tir-1*-OF (5′-TGGGTAAATGAGGAAGAGAGAGAG-3′) and *tir-1*-OR (5′-TCGGTTGACGAGTCGAATTTGG-3′) were used to detect the *tir-1(qd4)* deletion allele. The wild-type *tir-1* allele amplified a 1368-bp fragment compared to a 228-bp fragment from *tir-1(qd4)* allele. Homozygous *tir-1(qd4)* mutants were identified by PCR using primers *tir-1*-OF and *tir-1*-IR (5′-CACAAGAACGTGCAA-CATCG-3′) from inside the deletion region. Wild-type animals generated a 327-bp fragment, while the homozygous *tir-1(qd4)* allele did not produce any fragment.

RNA interference (RNAi)

The HT115(DE3) *E. coli* bacteria producing dsRNA against *cdc-25.1* (Andy Golden NIDDK/NIH, Bethesda MD, USA), *skn-1* (T. Keith Blackwell, Harvard Medical School, Boston MA, USA), *wdr-23* (Keith P. Choe, University of Florida, Gainesville FL, USA), *vhp-1* and *tir-1* (Source BioScience Geneservice, Cambridge, United Kingdom) were used in our study. RNAi feeding *E. coli* clones were grown overnight in LB medium containing 100 μg/ml ampicillin. RNAi treatment was performed as described by Shapira *et al.* [22]. Worms were grown on RNAi bacteria from hatching till young adult stage. If several RNAi constructs were used in one condition, ON cultures of the feeding bacteria strains were mixed equally. Empty vector containing HT115(DE3) bacteria (EV) was used as a control in all cases.

Preparation of pathogenic bacteria

Different human opportunistic bacteria, such as *Pseudomonas aeruginosa* and *Enterococcus faecalis* [28,52] are ubiquitously used as pathogen models. Gram-negative *Pseudomonas aeruginosa* PA14 (David W. Wareham, Queen Mary University of London, London, UK) and Gram-positive *Enterococcus faecalis* SdB262 (Jonathan J. Ewbank, Centre d'Immunologie de Marseille-Luminy, Marseille, France) bacteria were maintained and prepared for experiments as described by Powell and Ausubel [53]. BHI agar was supplemented with 100 μg/ml rifampicin for *E. faecalis* killing assay.

Killing assay

Killing assays were performed with young adult animals at 25°C on slow killing plates (*P. aeruginosa*) or rifampicin BHI plates (*E. faecalis*), or otherwise as it was noted in the figure legend. Dead worms were scored every 12 hours till complete extinction of the population. Viability was determined by assaying for movement in response to gentle prodding. Worms died on the wall of the Petri dish or crawled into the gel were censored. 30 animals per condition were tested with 3 parallel plates in at least two independent trials, except that an H_2O_2-concentration dependence of preconditioned pathogen resistance was established in one trial. For studying pathogen resistance of *daf-2* mutants, nematodes were grown at 15°C. For oxidative preconditioning 2-day old adult animals were treated with 0 (control), 1 mM, 1.5 mM and 2 mM H_2O_2 (Sigma) in liquid NGM for 2 hours at 20°C. Before the killing assay worms were transferred to OP50 seeded NGM plates for a 12-hour recovery period. To test the effect of aging on pathogen resistance, worms were maintained on OP50-seeded NGM plates before the challenge. To avoid 'bag of worms' phenotype, animals were fed by *cdc-25.1(RNAi)*.

Fluorescence microscopy

Nematodes were treated as indicated in the figure legends (Figures 2, 3, 4 and 6). After treatments at least 40 worms per condition were placed on a 2% agarose pad, and immobilized by adding 40 mM levamisole in M9 buffer. Images were taken by a Leica DMI6000B epifluorescence microscope with a DFC480 camera. Epifluorescent microscopic images are representatives of at least 3 experiments. To analyze *Pgcs-1*::GFP and GST-4::GFP expression upon PA14 infection, two groups of animals were determined depending on the detected GFP level in the intestine: GFP positive and GFP negative animals. To study the nuclear localization of SKN-1 in response to PA14 infection minimum 15 *skn-1::gfp* worms per condition were analyzed in at least 3 independent trials. Images were captured by a Zeiss LSM510 confocal laser scanning microscope equipped with a 40×/1.3 oil immersion objective (Plan-Neofluar, Zeiss).

Analysis of SKN-1 dependent targets amongst genes down-regulated by aging

A list of 379 genes exhibiting the most significant age-dependent decline in their expression (>10-fold at d6 *vs.* d15) was acquired from [27]. Data were analyzed by finding the overlaps between genes subject to SKN-1 dependent genes under non-stress [29] or oxidative stress conditions [30]. Then the expression of the identified genes was analyzed based on Wormbase data, focusing on PA14-, oxidative stress- or PMK-1-dependent regulation [31].

Oxidative stress tolerance

Young, 1-day old adult worms were incubated in liquid NGM for 1 hour at 20°C with 3 mM and 5 mM H_2O_2 (Sigma). After oxidative challenge animals were transferred to OP50 seeded NGM plates and viability was tested 24 hours later. 35 animals per plate were examined in each condition with 3 parallel plates in 3 independent trials.

Statistical analysis

Data were analyzed by using the SPSS software 15.0 (SPSS Inc., Chicago, IL, USA). Survival curves were compared by Kaplan-Meyer log-rank test. To compare the means of survival (oxidative tolerance assay) or the GFP expression of the *Pgcs-1::gfp, gst-4::gfp, skn-1::gfp* strains variables were analyzed by one-way ANOVA test. Results are expressed as mean ± standard deviation (SD). Statistical significance was indicated as follows: * $p<0.05$, ** $p<0.001$, *** $p<0.0001$.

Accession numbers

C. elegans proteins/genes: Q17941, Q9XTG7, Q2MGF0, Q17450, O61213, P54145, Q9U3Q6, O02215, G5EC10, Q18198, Q9XUH3, Q9XUF9, O44552, Q27487, Q18938, O17725, O16849, Q968Y9, Q9XVB4, Q9XVA9, Q19223, O62146, G5EGH6, Q8MNR8, Q19774, O02357, Q09321, Q9UAQ9, P91316, Q20770, Q20840, G5EC22, P90893, Q20968, Q21009, Q20117, Q9U2Q9, Q21355, Q9XW45, Q21381, Q09991, Q94269, Q94271, P34528, Q17446, Q2PJ68, P34707, P41977, O02364, Q9XUC0, Q86DA5, Q10038, P90794, Q8WRF1, Q9U309, G5EFR9, Q9GR66, G5ECJ8, Q86S61, O76725, Q9N4X8, Q9NAB1, Q23498, Q23564

Human proteins/genes: Q12778, Q16656, Q16236, Q9Y4A8

Supporting Information

Figure S1 SKN-1 is required for pathogen resistance against both *P. aeruginosa* and *E. faecalis*.
(DOC)

Figure S2 PA14-induced activation of *Pgcs-1*::GFP and GST-4::GFP expression.
(DOC)

Figure S3 Suppression of PA14-induced SKN-1 nuclear localization by *tir-1(RNAi)*.
(DOC)

Figure S4 Lifespan of N2 and *skn-1(zu135)* mutant worms.
(DOC)

Table S1 Statistical analysis of killing assays.
(DOC)

Table S2 List of SKN-1-dependent genes down-regulated by aging.
(DOC)

Table S3 Statistical analysis of lifespan assays.
(DOC)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: DP CS. Performed the experiments: DP. Analyzed the data: DP CS. Contributed reagents/materials/analysis tools: PC. Wrote the paper: DP CS.

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