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In Vivo Analysis of the Hsp90 Cochaperone Sti1 (p60)

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Hsp90 interacts with Sti1 (p60) in lysates of yeast and vertebrate cells. Here we provide the first analysis of their interaction in vivo. *Saccharomyces cerevisiae* mutations that eliminate Sti1 or reduce intracellular concentrations of Hsp90 individually have little or no effect on growth at normal temperatures. However, when combined, the mutations greatly reduce or eliminate growth. Furthermore, overexpression of Sti1 has allele-specific effects on cells carrying various *hsp90*^{ts} point mutations. These genetic interactions provide strong evidence that Hsp90 and Sti1 interact in vivo and that their functions are closely allied. Indeed, deletion of *STI1* reduces the in vivo activity of the Hsp90 target protein, glucocorticoid receptor (GR). Mutations in GR that eliminate interaction with Hsp90 also eliminate the effects of the *sti1* deletion. Examination of GR protein complexes in the *sti1* deletion mutant reveals a selective increase in the concentration of GR-Ydj1 complexes, supporting previous hypotheses that Ydj1 functions at an early step in the maturation of GR and that Sti1 acts at an intermediate step. Deletion of *STI1* also reduces the in vivo activity of another, unrelated Hsp90 target protein, v-Src. Our data indicate that Sti1 is a general factor in the maturation of Hsp90 target proteins and support earlier suggestions that Hsp90 matures even very different target proteins by a similar mechanism.

Hsp90 is a highly conserved protein chaperone whose molecular functions are just beginning to be deciphered. In vitro, purified Hsp90 can function as a general chaperone, preventing the aggregation of casein kinase II (30) and certain denatured proteins such as citrate synthase (60). Hsp90 also maintains previously denatured substrates such as β-galactosidase (15), dihydrofolate reductase (DHFR) (62), and luciferase (23) in folding-competent states. Unlike Hsp70, which interacts with unfolded polypeptide chains (14), Hsp90 is believed to interact with substrates at the terminal stages of folding (22, 27). In vivo, Hsp90 displays a high degree of specificity for particular target proteins (37). It associates with certain signaling factors such as steroid hormone receptors (6, 41, 44, 48), basic helix-loop-helix transcription factors (49), oncogenic tyrosine kinases (5), and normal cellular serine/threonine kinases (30, 42, 58, 59) yet shows little or no tendency to interact with closely related proteins (9, 10, 61).

In association with these specific target proteins, Hsp90's chaperone activity has acquired regulatory functions. When glucocorticoid receptor (GR) is isolated from cells that have not been treated with hormone, it is complexed with two molecules of Hsp90 (3, 28). In this state, the receptor cannot bind DNA (39). When the Hsp90-GR interaction is disrupted, either by the addition of hormone (39) or by physical means (high salt) (41), the receptor acquires DNA-binding activity. Moreover, heat disrupts Hsp90's interaction with GR in vitro (11, 29) and at least partially activates GR in vivo (32). Mutations in GR which abolish GR-Hsp90 interactions lead to activation of receptors in the absence of hormone (17). These findings argue that Hsp90 helps to maintain GR in a repressed state until hormone triggers receptor activation.

Hsp90 does not, however, simply serve to repress the receptor. When mammalian GR is expressed in the yeast *Saccharomyces cerevisiae*, reducing the concentration of Hsp90 greatly reduces the ability of newly synthesized GR to undergo hormone-dependent activation (35). Thus, Hsp90 is required for GR to achieve a hormone-activatable conformation. Experiments with an Hsp90 temperature-sensitive mutant revealed that even after GR has been synthesized and matured into its hormone-activatable conformation, Hsp90 function is continuously required to maintain the receptor in this state (32). Furthermore, when Hsp90-GR interactions are disrupted in vitro, the receptor loses the ability to bind ligand (4). Ligand-binding activity is restored upon reconstitution of Hsp90-receptor interactions in reticulocyte lysate (46, 47). Apparently, Hsp90 not only prevents premature activation of receptors but also potentiates their hormone-dependent activation.

Hsp90 has similar effects on the oncogenic tyrosine kinase, pp60$^{v\text{-}src}$ (v-Src), a very different type of target protein. While newly synthesized v-Src is in transit to the plasma membrane, it associates with Hsp90 and is inactive as a tyrosine kinase. The kinase activity of v-Src appears coincidentally with dissociation from Hsp90 and insertion into the membrane (5), suggesting that Hsp90 keeps the kinase inactive during transport. However, genetic analysis in yeast demonstrates that the tyrosine kinase activity of v-Src is absolutely dependent on Hsp90 function (32, 61). Thus, as with GR, Hsp90 appears to play a dual role in v-Src function, maintaining it in a repressed but activatable state.

In addition to various target proteins, Hsp90 binds to other cellular proteins, Hsp90-associated proteins (Hsp90APs), which are thought to act with Hsp90 to modulate target protein function (1, 40, 56). Hsp90APs can be divided into three subgroups: (i) other known chaperone proteins (e.g., Hsp70), (ii) peptidylprolyl *cis-trans* isomerases (immunophilins of both the FKBP and cyclophilin classes), and (iii) proteins whose biochemical functions are just beginning to be characterized (e.g., p60, p50, p48, and p23). Like Hsp90, these Hsp90APs are ubiquitous and well conserved throughout the eukaryotic lineage (40, 56). In fact, not only are individual Hsp90APs conserved, but the general composition of Hsp90 chaperone complexes is conserved from yeast to human (7).

Our understanding of Hsp90APs derives largely from analysis of their roles in the in vitro reconstitution of salt-disrupted aporeceptor complexes (46, 53). Complexes reconstituted in reticulocyte lysates contain the same proteins as complexes

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isolated from cells not treated with hormone, namely, Hsp90, Hsp70, FKBP52 (FKBP59), and p23 (19, 52, 53). The minimum components required for the maturation of receptors to a ligand-binding conformation in vitro are Hsp90, Hsp70, p60, and p23 (12). Apparently, Hsp70 is required for Hsp90-receptor interactions to occur (18, 54) and p23 is required to maintain receptors in a hormone-activatable conformation (20, 57). The p60 protein is found in intermediate complexes and is thought to play a transient role in receptor maturation (51), joining Hsp70 and Hsp90 (8).

Here we analyze the function of Sti1, the *S. cerevisiae* homolog of p60 (55), in vivo. *STI1* was originally cloned by Nicolet and Craig in a screen for genes that in high copy number enhance the transcription of a *lacZ* reporter under the control of an Hsp70 (*SSA4*) promoter (33). Because *sti1* null mutants exhibit normal regulation of *SSA4*, its role in Hsp70 regulation is unclear (33). Recently, we demonstrated that Sti1 is a major component of the Hsp90 chaperone complex in yeast (7). Here we examine genetic interactions between Sti1 and Hsp90 and investigate the effects of Sti1 on the maturation of Hsp90 target proteins in vivo.

MATERIALS AND METHODS

Plasmid and strain construction. All strains used in this study are derivatives of CN11 (Δ*trp1 lys1 lys2 ura3-52 leu2-3,112 his3-11,15 sti1-1*::*HIS3*) (33) (a gift of E. Craig), ΔCLD82 (*ade2-1 trp1-1 ura3-1 leu2-3,112 his3-12,16 hsc82*::*LEU2*) (32), and ΔPLD82 (*ade2-1 trp1-1 ura3-1 leu2-3,112 his3-12,16 hsp82*::*LEU2*) (32). The *sti1* mutation in CN11 was created by insertion of the *HIS3* gene into the *Kpn*I site at amino acid 521 of Sti1 (33). It appears to be a null mutation, since no protein can be detected in CN11 with an antibody (gift of D. Toft) that recognizes amino acids 28 to 520 of Sti1 (data not shown).

Strains JC-21 (*HSC82 HSP82 STI1*), JC-22 (*HSC82 HSP82 sti1-1*::*HIS3*), JC-23 (*HSC82 hsp82*::*LEU2 STI1*), JC-24 (*HSC82 hsp82*::*LEU2 sti1-1*::*HIS3*), and JC-25 (*hsc82*::*LEU2 HSP82 STI1*) are the meiotic products of JC-20 (CN11 × ΔCLD82) or JC-19 (CN11 × ΔPLD82). These strains transformed with GR, FLAG GR, GR truncation mutant, v-Src, c-Src, and Sti1 expression plasmids (below) are designed JC-21/GR, JC-21/GR^{FLAG}, JC-21/GR^{T}, JC-21/v-src, JC-21/c-src, JC-21/pUSti1 (low-copy-number plasmid), and JC-21/YEpSti1 (high-copy-number plasmid). JC-S1 is a meiotic product of JC-26 (JC-22 × $GPDHsp82^{FP}$ [7]). Its only source of Hsp90 is a histidine-tagged Hsp82 fusion protein expressed from the glyceraldehyde-3-phosphate dehydrogenase (*GPD*) promoter on a low-copy-number *TRP1* marked plasmid (pTGPD-H-$Hsp82^{FP}$). This strain also contains the *sti1-1*::*HIS3* and *pep4*::*HIS3* mutations. JC-S2 was created by transforming JC-S1 with pUSti1. LEPHsp82, P82a, and the *hsp82*^{ts} strains T22Ia, A41Va, G81Sa, T101Ia, G170Da, G313Sa, E381Ka, and A587Ta have been described elsewhere (7, 32).

The expression vector pUGPD was constructed by inserting the 0.7-kb *Eco*RI-*Bam*HI fragment containing the *GPD* promoter from p2Hg (35) into pRS316 (50). The Sti1 expression plasmid pUSti1 was constructed by inserting the 2.0-kb *Eco*RI-RV fragment of Sti1 from YEp-Sti1 (33) first into pVZ1 (Stratagene) and then into the *Xba*I site of pUGPD. The FLAG GR expression plasmid pMTFLAG/GR/pGR was constructed by inserting the 3.0-kb *Xho*I-*Bam*HI fragment containing the *GPD* promoter and FLAG GR from pCHFLAGGR (24) into pG-N795 (45) (cut with *Sma*I and *Bam*HI). The *src* expression plasmids Y314v-src and Y314c-src were obtained from D. Morgan (61). The GR truncation mutant expression plasmid pGN556 and the GR reporter plasmid pSX26.1 were obtained from K. Yamamoto (35). The GR expression plasmid p2A/GRGZ, the histidine-tagged Hsp82 protein expression plasmid pTGPD-H-$Hsp82^{FP}$, and the wild-type Hsp82 protein expression plasmid pTGPD-Hsp82 have been described elsewhere (7, 32).

Yeast and bacterial transformants were obtained by the lithium acetate (21) and calcium chloride (26) methods, respectively, or by electroporation with a Gene Pulser (Bio-Rad). Control vectors were introduced as needed to ensure that all strains in an experiment had the same auxotrophic markers.

Yeast media and culture conditions. Cells were cultured at 25°C with vigorous shaking in YPDA (1% yeast extract, 2% Bacto Peptone, 2% glucose, 10 mg of adenine sulfate per ml) or, when plasmid selection was required, in SD, SR, or SGal (2% dextrose, raffinose, or galactose, respectively, 0.7% yeast nitrogen base without amino acids, and 0.5% ammonium sulfate, supplemented with essential amino acids and nucleotides). To activate GR, deoxycorticosterone (DOC; 20 μM unless otherwise indicated) was added to mid-log-phase, SD-minus-adenine cultures for 1 h. To induce $pp60^{src}$ expression, cells were grown to 2×10^6 per ml in SR minus uracil and tryptophan, collected by centrifugation, and transferred to SGal minus uracil and tryptophan for 7 h.

β-Galactosidase assays. Cells (5×10^7 per sample) were collected by centrifugation (3,000 × *g*, 5 min), washed twice with H_2O, and resuspended in 300 μl of LBPi [0.1 M potassium phosphate, 20% glycerol, 1 mM dithiothreitol, 2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 2 μg each of leupeptin, aprotinin, and pepstatin A per ml (pH 7.8)]. An equal volume of glass beads (425 to 600 μm) was added, and cells were lysed by agitation on a Biospec bead beater for 1 min at 4°C. Lysates were cleared by centrifugation (14,000 × *g*, 2 min). β-Galactosidase activity was measured with the Galactolight kit (Tropix) and normalized to the protein concentration of the lysate as determined by the Bio-Rad protein assay, using bovine serum albumin as a standard.

Immunoblot analysis. Cells (10^8 per sample) were collected by centrifugation (3,000 × *g*, 5 min) and resuspended in ice-cold ethanol containing 1 mM phenylmethylsulfonyl fluoride. An equal volume of glass beads (425 to 600 μm) was added, and cells were lysed by agitation on a Biospec bead beater for 2 min at 4°C. Proteins precipitated at −30°C for 15 min were collected by centrifugation, vacuum desiccated for 10 min, and resuspended in sample buffer (25). After separation by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (7.5 or 10% acrylamide), proteins were transferred to Immobilon-P membranes (Millipore) and stained with Coomassie blue to confirm equal loading. Blots were blocked with 10% nonfat dehydrated milk in phosphate-buffered saline (8 mg of NaCl, 0.2 mg of KCl, 1.44 mg of Na_2HPO_4, and 0.24 mg of KH_2PO_4 per ml [pH 7.4]) and incubated with primary antibody for 1 h, with rabbit anti-mouse immunoglobulin G (Cappel) for 1 h, and with protein A-conjugated horseradish peroxidase (Boehringer Mannheim) for 1 h. Immune complexes were visualized with the Amersham ECL reagent. The following antibodies were used: Ig 62b (a gift of J. Warner) to detect the yeast ribosomal L3 protein, BuGR2 (Affinity Bioreagents) to detect GR, LA074 (Quality Biotech) to detect Src, 4G10 (Upstate Biotechnology Incorporated) to detect phosphotyrosine residues, and ST2 (a gift of D. Toft) to detect Sti1. Antisera against Ssa and Ydj1 were gifts of E. Craig and M. Douglas, respectively. Rabbit antibody (CpC6) was produced for this study by using a peptide encoding amino acids 356 to 371 of Cpr6.

Analysis of protein complexes. Hsp90 protein complexes were collected on a nickel affinity matrix from yeast cells harboring a histidine-tagged Hsp82 derivative as described previously (7). To examine GR and Sti1 protein complexes, cells were grown to 5×10^6 cells per ml in the absence of hormone and lysed with buffer LyB (10 mM Tris, 50 mM NaCl, 50 mM KCl, 20 mM Na_2MoO_4, 10 mM $MgCl_2$, 20% [wt/vol] glycerol, 1 mM phenylmethylsulfonylfluoride, 2 μg each of pepstatin A, aprotinin, and leupeptin per ml [pH 7.3]) as described previously (7). For immunoprecipitation of FLAG GR, lysates (300 μl) were incubated with 100 μl of anti-FLAG M2 affinity gel (5.6 mg/ml; Eastman Kodak) for 1 h at 4°C. For immunoprecipitation of Sti1, lysates were incubated with ST2 antibody for 1 h at 4°C and then with 50 μl of GammaBind Plus Sepharose (3.0 mg/ml; Pharmacia Biotech) for 30 min at 4°C. Immune complexes were collected by centrifugation and washed six times with LyB containing 0.2% Triton X-100. Proteins were released with 40 μl of SDS sample buffer (26) and analyzed by SDS-PAGE.

Images from silver-stained gels and Western blots were scanned with an Arcus II scanner (Agfa) and Fotolook SA software and then cropped and assembled with Adobe Photoshop.

RESULTS

***STI1* has strong genetic interactions with *HSP90*.** Synthetic lethality and allele-specific suppression are hallmarks of genes that function in the same physical complex or in the same biochemical pathway. To investigate the functional relationship between Sti1 and Hsp90 in vivo, we tested for both types of genetic interactions.

S. cerevisiae contains two genes encoding Hsp90 proteins, *HSP82* and *HSC82*. They are nearly identical in sequence, are functionally equivalent, but have distinct patterns of expression. *HSP82* is constitutively expressed at a low level and strongly induced by stress; *HSC82* is constitutively expressed at a high level and slightly induced by stress. Because the regulation of these genes is largely independent, at 25 or 30°C, *hsc82* deletion mutants have dramatically reduced Hsp90 levels (~1/15 of the wild-type level), whereas *hsp82* deletion mutants have only slightly reduced levels (2). Neither gene disruption affects growth at normal temperatures, but disruption of both is lethal.

S. cerevisiae produces only one protein closely related to p60, Sti1. The presence of a single gene in this class was confirmed by a homology search of the newly completed genomic sequence of *S. cerevisiae* (43). A null mutation of *STI1* has no effect on cell growth at 25 or 30°C and causes only a slight inhibition of growth at higher and lower temperatures (34).

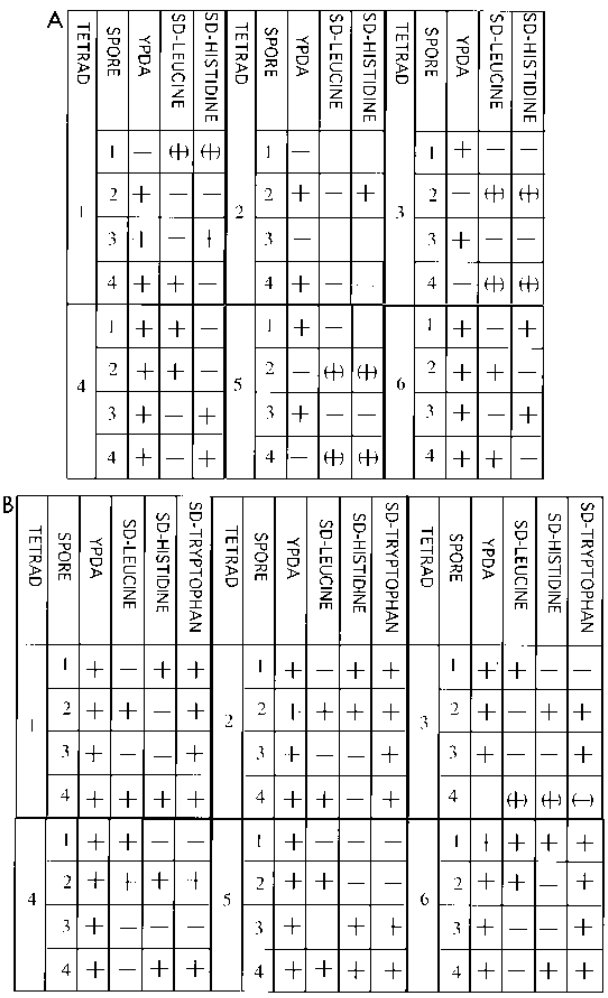

FIG. 1. Synthetic lethality of *sti1* and *hsc82* mutations. Meiotic products of the *sti1 hsc82* heterozygote (JC-20) (A) or JC-20 transformed with a *TRP1*-marked *HSP82* expression plasmid (pTGPD-Hsp82) (B) were scored for growth on rich medium (YPDA), SD minus leucine (to score *hsc82*::*LEU2*), SD minus histidine (to score *sti1-1*::*HIS3*), and SD minus tryptophan (to score pTGPD-Hsp82). +, growth; −, no growth; (+) and (−), inferred markers of dead spores. All spores inferred to carry both *hsc82*::*LEU2* and *sti1-1*::*HIS3* (from expected 2:2 segregation of markers) failed to produce colonies. Spores carrying *hsc82*::*LEU2*, *sti1-1*::*HIS3*, and the expression plasmid (pTGPD-Hsp82) were readily obtained.

To combine mutations in these genes, cells carrying a *sti1* disruption marked with a gene for histidine auxotrophy (*sti1-1*::*HIS3*) were mated with cells carrying either an *hsp82* or an *hsc82* disruption marked with a gene for leucine auxotrophy (*hsp82*::*LEU2* or *hsc82*::*LEU2*). The heterozygous diploids (strains JC-19 and JC-20) were sporulated, and individual meiotic products were examined. Since *STI1*, *HSP82*, and *HSC82* are located on different chromosomes (*STI1* on chromosome XV, *HSP82* on chromosome XVI, and *HSC82* on chromosome XIII), they are expected to sort independently in meiosis. Viable cells containing mutations in any one of the genes or in both *STI1* and *HSP82* were readily obtained (data not shown). In contrast, no viable cells containing mutations in both *STI1* and *HSC82* were recovered in the 20 tetrads analyzed (Fig. 1A shows representative results of six tetrads). When an *HSP82* expression plasmid was transformed into the *hsc82 sti1* heterozygous diploid, viable spores containing both the *sti1* mutation and the *hsc82* mutation were readily obtained. All of these cells, however, also contained the exogenous *HSP82* plasmid (Fig. 1B). Thus, with this combination of markers, *sti1* and *hsc82* exhibit synthetic lethality. When the *hsc82* gene was replaced with *URA3* instead of *LEU2*, pinpoint colonies of *sti1 hsc82* double mutants were observed after 10 days (data not shown). On restreaking, these cells continued to grow very slowly, demonstrating that the double mutant affected growth, not merely germination (data not shown). Thus, the lethality of *sti1 hsc82* mutations depends on the auxotrophic state of the cell. In any case, a simple reduction in Hsp90 levels produced an extreme enhancement of *sti1* phenotypes at 25°C.

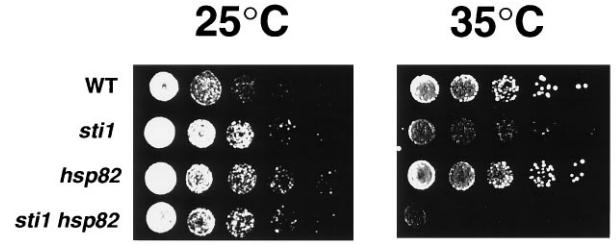

FIG. 2. Synthetic enhancement of *sti1* and *hsp82* mutations. Logarithmically growing wild-type (WT; JC-21), *sti1* (JC-22), *hsp82* (JC-23), and *sti1 hsp82* (JC-24) cells were adjusted to a density of 10^6 cells per ml, serially diluted (fivefold at each step), spotted onto YPDA medium, and grown at 25 or 35°C for 2 days.

At high temperatures, Hsp82 is strongly induced, accumulating to approximately the same level as Hsc82 (2). Thus, at 35°C, disruption of *HSP82* reduced Hsp90 levels to approximately 50% of the wild-type level (data not shown). This mutation had no effect on growth at 35°C (Fig. 2), and a null mutation of *STI1* caused only a slight reduction in growth. In marked contrast, when the *sti1* and *hsp82* mutations were combined, growth was greatly reduced at 35°C. Thus, lowering the cellular concentration of Hsp90 as little as twofold dramatically increased the requirement for Sti1.

Next we asked whether overexpression of *STI1* would suppress the growth defects of temperature-sensitive Hsp90 mutants. Mutant and wild-type strains were transformed either with a high-copy-number plasmid carrying *STI1* or with a control vector. Growth was assessed at permissive, nonpermissive (data not shown), and semipermissive (Fig. 3) temperatures. Overexpression of Sti1 had no effect on the growth of wild-type cells at any temperature tested. Surprisingly, in cells expressing growth-limiting concentrations of Hsp90 (LEPHsp82) (7), overexpression of Sti1 caused a subtle reduction in growth. Strikingly, Sti1 overexpression affected the temperature-sensitive growth of eight broadly distributed *hsp82* point mutants in different ways. In four of the mutants (T22I, G170D, G313S, and A587T), overexpression of Sti1 enhanced growth at 35 to 36°C. It did not, however, rescue growth above 37°C. In two of the mutants (G81S and E381K), overexpression of Sti1 had no effect on growth, and in two others (A41V and T101I), it reduced growth. Thus, the effects of *STI1* overexpression on *hsp82*^{ts} mutants were allele specific.

The synthetic lethality and allele-specific suppression observed between *HSP90* and *STI1* demonstrate that their functions are closely allied. Thus, the physical interaction previously demonstrated for these proteins in vitro has important biological consequences in vivo. To further characterize the function of Sti1, we assessed its role in the maturation of two different Hsp90 target proteins, GR and v-Src.

Sti1 affects Hsp90 target protein function. Wild-type and *sti1* mutant cells were transformed with a plasmid that consti-

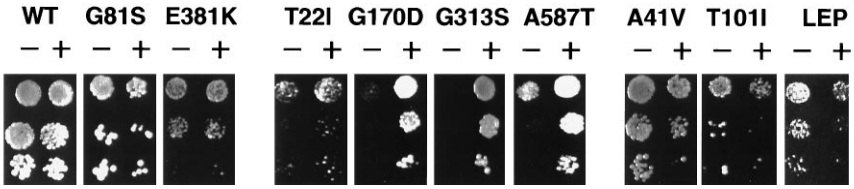

FIG. 3. Allele-specific effects of Sti1 overexpression on *hsp82*^{ts} point mutations. Logarithmically growing cells were adjusted to a density of 10^6 cells per ml, serially diluted (fivefold at each step), spotted onto YPDA medium, and grown at semipermissive temperatures (34°C for T22I, A41V, and E381K; 35°C for G81S, G170D, and G313S; 36°C for the wild type [WT], LEP, and A587T). +, cells transformed with a multicopy *STI1* expression plasmid; −, cells transformed with the control vector. WT, T22I, A41V, G81S, T101I, G170D, G313S, E381K, and A587T are *hsp82 hsc82* strains carrying a low-copy-number plasmid expressing wild-type *HSP82* or the indicated *hsp82*^{ts} allele from the *GPD* promoter (32). LEP (Hsp82LEP), *hsp82 hsc82* cells carrying a low-copy-number *HSP82* expression plasmid that expresses extremely low levels of Hsp82 due to a promoter deletion (7).

tutively expresses GR and carries a glucocorticoid-regulated *lacZ* reporter gene. Addition of hormone to wild-type cells activated GR in a concentration-dependent manner (Fig. 4A). A 20-fold reduction in GR activity at low hormone concentrations and a 3-fold reduction at high hormone concentrations were observed in *sti1* mutant cells (Fig. 4A; Fig. 4B, bars 1 and 2). GR accumulation was not affected by the *sti1* mutation (Fig. 4C). The decreased GR activity was specifically due to the loss of functional Sti1 protein: when a *STI1* expression plasmid was introduced into *sti1* mutant cells, GR activity was restored to wild-type levels (Fig. 4B, bar 3). The *sti1* mutation had no effect on the activity of a GR truncation mutant that lacks the Hsp90 binding domain and is constitutively active as a transcription factor (Fig. 4D). Thus, the effects of the *sti1* mutation on GR function correlate with the receptor's dependence upon Hsp90.

Another Hsp90 target protein, the vertebrate oncogene product v-Src, shares no obvious structural or functional similarity with GR and is not known to interact with p60 (the vertebrate homolog of Sti1). To determine if Sti1 plays a general role in the maturation of Hsp90 target proteins, we asked if *sti1* mutations would affect v-Src maturation in vivo. Because endogenous levels of tyrosine phosphorylation are very low in yeast, the promiscuous tyrosine kinase activity of v-Src is readily assessed by reacting total cellular proteins with a phosphotyrosine-specific antibody (61). Very low levels of tyrosine phosphorylation were observed when v-Src expression was induced in the *sti1* mutant (Fig. 5A). Activity was restored to high levels by the introduction of a *STI1* expression plasmid, confirming that the low levels of v-Src activity were due to the loss of Sti1 protein. Note that the loss of Sti1 had no detectable effect on v-Src accumulation (Fig. 5A).

The closely related cellular form of v-Src, c-Src, is much less dependent on Hsp90. It is also a much less promiscuous kinase; when expressed in yeast, its only readily detectable target protein is itself (61). As shown in Fig. 5B, the tyrosine kinase activity of c-Src was not reduced by the *sti1* mutation. Thus, as with GR, the effects of the *sti1* mutation on Src function correlate with the kinase's dependence upon Hsp90.

To further investigate the role of Sti1 in Hsp90-mediated events, we asked if Sti1 overexpression could rescue GR and v-Src activity in an *hsc82* disruption strain. As shown in Fig. 6A, both the accumulation and the activity of GR (lane 2) were decreased in the *hsc82* mutant. Introduction of a multicopy *STI1* expression plasmid significantly increased GR accumulation. Since overexpression of Sti1 did not increase the levels of GR mRNA (data not shown), the increased GR accumulation is most likely due to increased stability of the protein. Sti1 overexpression did not, however, restore GR activity (Fig. 6A). As described above, in a strain expressing the *G313S* Hsp90 point mutation, overexpression of Sti1 partially suppressed temperature-sensitive growth defects. In this same strain, overexpression of Sti1 only partially rescued the GR activity defect associated with the mutant (data not shown).

Deletion of *HSC82* also decreased the accumulation and activity of v-Src (Fig. 6B). Overexpression of Sti1 partially restored both v-Src activity and accumulation. Comparison of proteins from mutant cells with serially diluted proteins from wild-type cells (data not shown) indicated that the increased v-Src activity could be attributed to the increased v-Src accumulation.

Effects of the *sti1* mutation on protein complexes. Sti1 is one of several Hsp90APs in yeast (7). The *sti1* mutation did not affect the accumulation of Hsp90, Ssa (the subfamily of Hsp70 that associates with Hsp90), Cpr6 (the yeast homolog of the human cyclophilin Cyp-40), or Ydj1 (a protein that associates with Hsp90 target proteins but not with Hsp90) (Fig. 7 and 9). (We were unable to examine effects on Cpr7, another cyclophilin homolog, because we do not yet have an antibody directed against it). To determine if the *sti1* deletion affected the composition of Hsp90 complexes, we examined the complexes in several ways.

First, we used a histidine-tagged Hsp82 protein to collect Hsp90 complexes by one-step affinity chromatography on a nickel resin (7). As previously described, Sti1, Ssa, and Cpr6 were recovered in association with Hsp90 in wild-type cells (Fig. 7). Contrary to expectation, the *sti1* mutation did not impair the recovery of Ssa and Cpr6 in this experiment. In fact, it increased their recovery. Ydj1 was not recovered in Hsp90 complexes in either wild-type or *sti1* mutant strains.

To examine the composition of Sti1 complexes, proteins were immunoprecipitated with a monoclonal antibody specific for Sti1. As shown in the silver-stained gel of Fig. 8, a 90-kDa protein, identified as Hsp90 by Western blotting, was the single most abundant protein in these precipitates. Although no other proteins were visible on the silver-stained gel, Western blotting revealed that Ssa (Hsp70) was slightly enriched in the Sti1 immunoprecipitates. Ydj1 was not. More importantly, Cpr6, which is abundant in Hsp90 complexes, was not recovered in Sti1 immunoprecipitates. Thus, as in vertebrate cells, multiple Hsp90 complexes with distinct subunit compositions must exist in yeast.

Finally, to examine the effects of the *sti1* mutation on target protein complexes, we used an epitope-tagged GR protein (FLAG GR) that possesses wild-type GR activity in vivo (24). FLAG GR, but not wild-type GR, was precipitated from lysates by a FLAG-specific antibody covalently attached to agarose beads (Fig. 9, lanes 1 and 2). Hsp90, Ydj1, and Ssa were coprecipitated with FLAG GR (Fig. 9), but Cpr6 and Sti1 could not be detected. In separate experiments, Sti1 antibody did not precipitate GR (data not shown). The *sti1* mutation did not affect the recovery of Hsp90 or Ssa in FLAG GR precip-

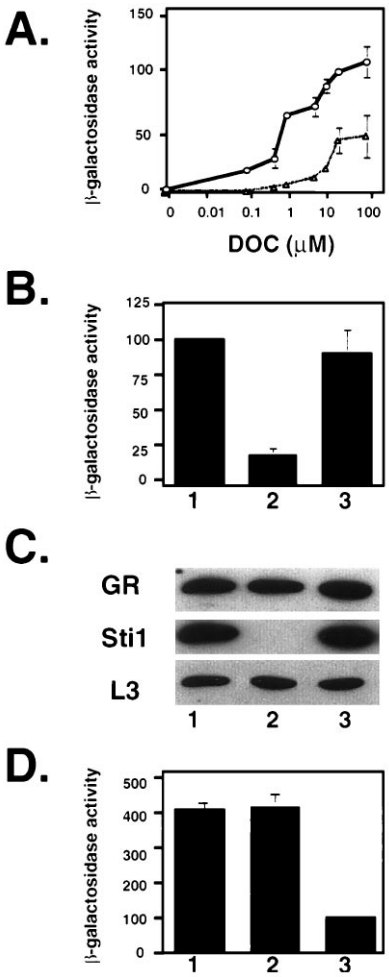

FIG. 4. Effects of the *sti1* mutation on GR activity and accumulation. (A) GR activity assessed by the activation of a *lacZ* reporter plasmid in wild-type (JC-21/GR; ○) and *sti1* mutant (JC-22/GR; △) cells treated with 0, 0.1, 0.5, 1, 5, 10, 20, or 100 μM DOC for 1 h at 25°C. Each point is the mean ± standard deviation of three to six independent experiments. (B) Hormone-dependent GR activity in wild-type cells (JC-21/GR) (bar 1), *sti1* mutant cells (JC-22/GR) (bar 2), and *sti1* mutant cells rescued by a low-copy-number *STI1* expression plasmid (JC-22/pUSti1/GR) (bar 3). Each bar is the mean ± standard deviation of six independent experiments expressed as a percentage of wild-type activity. (C) Accumulation of GR and Sti1 proteins in the strains analyzed in panel B. Total cellular proteins were separated by SDS-PAGE, transferred to Immobilon-P membranes, and reacted with antibodies specific for GR and Sti1. Equal sample loading was confirmed by Coomassie blue staining (not shown) and by reacting the blot with an antibody specific for ribosomal protein L3. (D) Hormone-independent activity of a GR truncation mutant that lacks the Hsp90 binding domain in wild-type (JC-21/GR^T) (bar 1) and *sti1* (JC-22/GR^T) (bar 2) cells. Bar 3, hormone-dependent activity of wild-type GR in wild-type cells (JC-21/GR) treated with 20 μM DOC for 1 h. Each bar is the mean ± standard deviation of three independent experiments expressed as a percentage of the activity of wild-type GR in wild-type cells.

itates. It did, however, significantly enhance the recovery of Ydj1 (Fig. 9).

DISCUSSION

The genetic interactions that we observe between Sti1 and Hsp90 demonstrate that the functions of these two proteins are closely allied in vivo. Individual mutations in Hsp90 that have no effect on growth at 25 or 35°C, severely reduce and in some cases eliminate the growth of cells that do not express Sti1. Thus, simply reducing the concentration of Hsp90 greatly increases the cellular requirement for Sti1. Furthermore, elimination of Sti1 reduces the activity of two very different proteins that depend on Hsp90 for activity, the steroid receptor GR and the oncogenic tyrosine kinase v-Src. Taken together with the strong allele-specific effects of Sti1 overexpression on Hsp90 point mutants, these results confirm that Sti1 interacts with Hsp90 in vivo and plays an important role in modulating the activity of the Hsp90 chaperone machinery.

Although they are found in the same protein complex and potentiate the activity of the same target proteins, Hsp90 and Sti1 functions are distinct. Reducing the cellular concentration of Hsp90 reduces both the activity and the accumulation of GR and v-Src. In contrast, deletion of *STI1* reduces the activities of

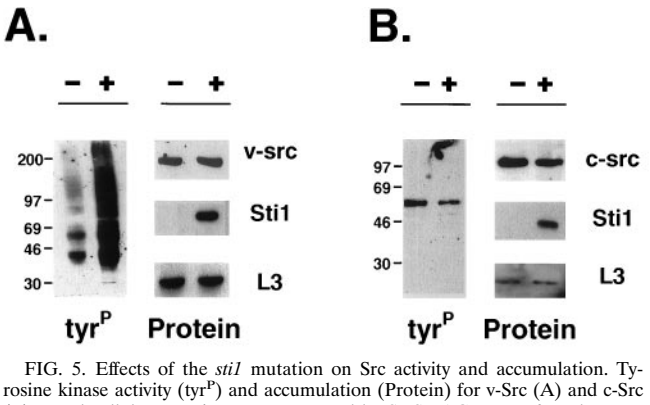

FIG. 5. Effects of the *sti1* mutation on Src activity and accumulation. Tyrosine kinase activity (tyr^P) and accumulation (Protein) for v-Src (A) and c-Src (B). Total cellular proteins were separated by SDS-PAGE, transferred to Immobilon-P membranes, and reacted with an antibody specific for phosphotyrosine. Duplicate blots were reacted with antibodies specific for Src and Sti1. Equal sample loading was confirmed by Coomassie blue staining (not shown) and by reacting blots with an antibody specific for ribosomal protein L3. −, *sti1* mutant cells (JC-22/v-src or c-src); +, *sti1* mutant cells rescued by a low-copy-number *STI1* expression plasmid (JC-22/pUSti1/v-src or c-src). Sizes are indicated in kilodaltons.

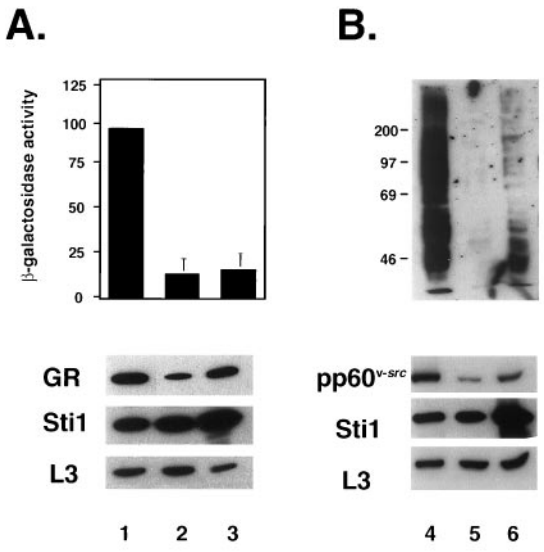

FIG. 6. Effects of Sti1 overexpression on target-protein function in *hsc82* mutants. (A) GR activity (top) and accumulation (bottom) were assessed as in Fig. 4B and C. Lane 1, wild-type cells (JC-21/GR); lane 2, *hsc82* mutant cells (JC-25/GR); lane 3, *hsc82* mutant cells carrying a multicopy *STI1* expression plasmid (JC-25/YEpSti1/GR). (B) v-Src activity (top) and accumulation (bottom) were assessed as in Fig. 5. Lane 4, wild-type cells (JC-21/v-src); lane 5, *hsc82* mutant cells (JC-25/v-src); lane 6, *hsc82* mutant cells carrying a multicopy*STI1* expression plasmid (JC-25/YEpSti1/v-src).

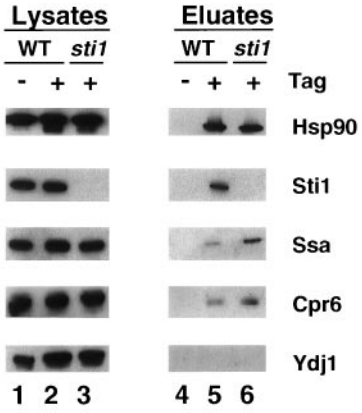

FIG. 7. Effects of the *sti1* mutation on the composition of Hsp90 complexes. Total cellular proteins (lanes 1 to 3) and proteins bound to a nickel affinity matrix and eluted with 150 mM imidazole (lanes 4 to 6) were separated by SDS-PAGE, transferred to Immobilon-P membranes, and reacted with antibodies specific for Hsp90, Sti1, Ssa, Cpr6, and Ydj1. Lanes 1 and 4, wild-type (WT) cells (JC-21); lanes 2 and 5, *sti1 hsp82 hsc82* (JC-S2) cells transformed with a low-copy-number histidine-tagged *HSP82* expression plasmid and a low-copy-number *STI1* expression plasmid; lanes 3 and 6, *sti1 hsp82 hsc82* (JC-S1) cells transformed with a low-copy-number histidine-tagged *HSP82* expression plasmid.

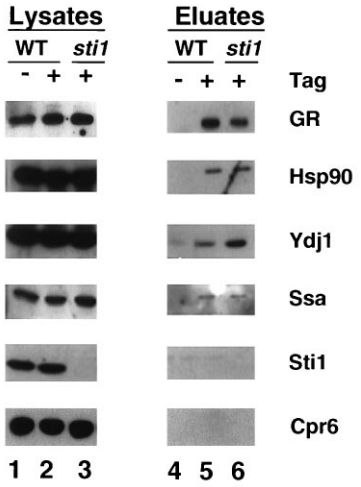

FIG. 9. Effects of the *sti1* mutation on GR complexes. Total cellular proteins (lanes 1 to 3) and proteins bound to an anti-FLAG affinity gel and eluted with sample buffer (lanes 4 to 6) were separated by SDS-PAGE, transferred to Immobilon-P membranes, and reacted with antibodies specific for GR, Hsp90, Ydj1, Ssa, Sti1, and Cpr6. Lanes 1 and 4, wild-type (WT) cells expressing GR (JC-21/GR); lanes 2 and 5, wild-type cells expressing FLAG GR (JC-21/GR^{FLAG}); lanes 3 and 6, *sti1* mutant cells expressing FLAG GR (JC-22/GR^{FLAG}).

both proteins but does not reduce their accumulation. Although Sti1 is not required for target protein accumulation, it can increase accumulation when it is overexpressed in an *hsp90* mutant background. Even so, overexpression of Sti1 cannot compensate for reduced concentrations of Hsp90 in target protein activation. (This was most apparent for GR.) The more restricted biochemical role of Sti1 is in keeping with genetic data: *hsp90* null mutations have a much stronger phenotype (lethal at all temperatures) than *sti1* null mutations (slow growth only at high and low temperatures).

A coherent biochemical model for Hsp90 target protein maturation has recently been derived from investigations of Hsp90's ability to reactivate salt-disrupted steroid receptor complexes in vitro (51). Receptor maturation appears to be a highly ordered, dynamic process involving Hsp90 at multiple steps and Sti1 transiently at a specific intermediate step (51). The cochaperones Hsp70 and p48 are thought to participate in earlier stages (36, 51); p23 and the immunophilins are thought to participate in later stages (51, 57). Our findings provide support for this pathway but also extend and modify it.

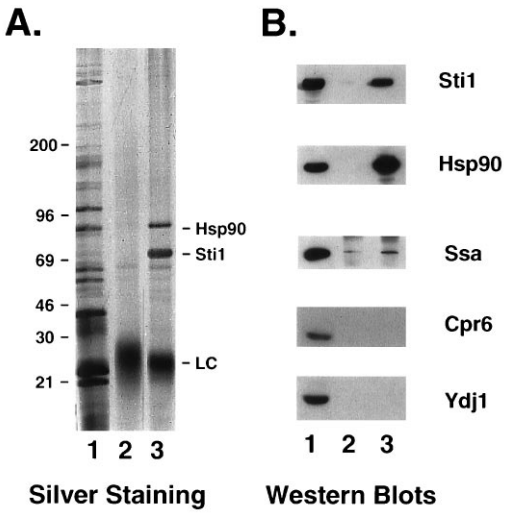

FIG. 8. Composition of Sti1 complexes. Total cellular proteins (lane 1) from wild-type cells (JC-21) were immunoprecipitated with nonimmune serum (lane 2) or with a Sti1-specific antibody (lane 3). Proteins were separated by SDS-PAGE and stained with silver (A) or transferred to Immobilon-P membranes and reacted with antibodies specific for Sti1, Hsp90, Ssa, Cpr6, and Ydj1 (B). Sizes are indicated in kilodaltons.

First, overexpression of Sti1 exerts strong allele-specific effects on Hsp90 point mutations, all of which reduce the capacity of the chaperone to mature GR and v-Src (32). Since these point mutations are scattered throughout the Hsp82 coding region and were isolated in a general temperature-sensitive screen, it seems unlikely that all of them directly affect Hsp90-Sti1 interactions. Yet, Sti1 enhances the growth defects of some mutants, suppresses the growth defects of others, and has no effect on others. These finding support the notion that Hsp90 functions at several steps in target protein maturation, with the point mutations affecting different steps—before, during, or after interaction with Sti1.

Second, although Hsp90 associates with several putative cochaperones, including Cpr6 and Cpr7 (the yeast homologs of the human cyclophilin Cyp-40) (13) and Hsp70 and Sti1 (7), no Cpr6 and very little Hsp70 coprecipitate with Sti1. This finding coincides with a recent report that the p60 protein (the vertebrate Sti1 homolog) does not coprecipitate with the Hsp90-associated immunophilins FKBP59 and Cyp-40 (34). Again, these results support the notion that target protein maturation occurs through a discrete series of Hsp90 complexes, only a subset of which includes Sti1.

Third, we do not detect significant quantities of Sti1 in GR complexes in yeast. Sti1's homolog p60 is also not found in mature GR and progesterone receptor complexes of vertebrate cells (38, 51–53). These data indicate that, as is the case in vitro, any association between target proteins and Sti1 in vivo must be transient.

Two of our findings were unexpected from models derived from reticulocyte reconstitution experiments. First, deletion of *STI1* does not reduce the concentrations of Hsp90 and Hsp70

(Ssa) in GR complexes. Clearly, in yeast cells, Sti1 is not required to mediate associations between Hsp70, Hsp90, and target proteins, as previously suggested for mammalian proteins (8). It may be that subtle differences exist in the Hsp90 chaperone machineries of yeast and mammalian cells. Alternatively, the dynamic nature of chaperone complexes might provide multiple pathways for Hsp90-target protein interaction in vivo that do not operate efficiently in vitro. Second, the *sti1* mutation increases the concentration of Ydj1 in GR complexes. Ydj1 binds unfolded proteins (16). If the *sti1* deletion increases the concentration of unfolded GR, Ydj1 might simply be serving to scavenge GR. Other observations, however, lead to a different interpretation. The stability of GR is not affected in the *sti1* mutant, as it is in *hsp90* mutants, suggesting that the protein is not unfolded. In addition, Ydj1 associates with many nascent polypeptides and appears to be a general chaperone for the maturation of cellular proteins (16). Finally, a particular point mutation in Ydj1 (*G315D*) exhibits strong genetic interactions with Hsp90 and exerts strong effects on the activities of three different Hsp90 target proteins. Surprisingly, it reduces the activity of v-Src but increases the constitutive activity of GR and estrogen receptor (24). Thus, the increase in GR-Ydj1 complexes in the *sti1* mutant supports a previous proposal that Ydj1 plays an early, pivotal role in Hsp90 target protein maturation (24, 36), with Sti1 acting at a downstream step (36). We suggest that steroid receptor reconstitution experiments did not reveal interactions with Ydj1 because salt-disrupted receptors retain a level of folding that places them downstream of Ydj1.

While the regulatory function of Ydj1 in the maturation of Hsp90 target proteins derives from its general role in protein folding, this is not likely to be the case with Sti1. Virtually all of the Sti1 protein in yeast cells is complexed with Hsp90 (7). Furthermore, the effects of the *sti1* mutation on receptor activity are eliminated when the Hsp90 interaction domain is removed from GR. Similarly, the *sti1* deletion has no effect on the activity of c-Src, a protein that is closely related to v-Src but is much less dependent on Hsp90. We suggest that the functions of Sti1 are likely to be restricted to Hsp90 target proteins.

Perhaps the most important implication of our work concerns the general mechanism of Hsp90 target protein maturation. Hsp90 associates with a bewildering variety of cellular proteins (1, 37). Some, e.g., Sti1 (p60) and Cpr7 (Cyp-40), are now being defined as cochaperones; others are being defined as target proteins that depend on Hsp90 for function. The variety of these target proteins is remarkable. Included are steroid receptors (1), helix-loop-helix transcription factors (49), oncogenic tyrosine kinases (5), and normal cellular serine/threonine kinases (30, 42, 58, 59). Some appear to require Hsp90 function continuously (32); others require it only before maturation is complete (5, 61). It has been postulated that the interactions between Hsp90 and different target proteins are governed by different cochaperones (1, 38). Indeed, p50 (cdc37) is emerging as an Hsp90 cochaperone dedicated to the maturation of particular target proteins (23, 31). In contrast, we suggest that Sti1 is a general factor acting to promote the maturation of Hsp90 target proteins. Although the vertebrate homolog of Sti1, p60, was known to interact with GR in vitro, there was no previous indication of an interaction with v-Src. GR and v-Src share no known structural or functional characteristics. Yet we find that deletion of *STI1* affects GR and v-Src similarly, reducing their activities without reducing their accumulation.

Similarly, we have found that deletion of *CPR7*, encoding an Hsp90-associated yeast homolog of human Cyp-40, reduces the ability of Hsp90 to mature both GR and v-Src (13). Previously, we reported that eight of eight Hsp90 point mutations, scattered throughout the protein and isolated solely on the basis of reduced growth rates in yeast, compromise the ability of Hsp90 to mature both GR and v-Src. Moreover, recent work by Nair et al. indicates that very different Hsp90 target proteins cycle through a similar series of Hsp90-cochaperone complexes in reticulocyte lysates (31). No doubt the maturation of different target proteins will exhibit different nuances. Nevertheless, the emerging picture is that Hsp90 matures even very different target proteins by similar molecular mechanisms, mechanisms that are conserved from lower eukaryotic to vertebrate cells.

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