were treated with trypsin and transferred to chamber slides. A peroxidase-based TACS-TdT kit (Trevigen) was used according to the manufacturer's directions. After staining for apoptosis, three to six fields were chosen at random, and the numbers of total nuclei and apoptotic nuclei were counted. The experiments were repeated four to eight times with similar results.

18. For protein immunoblot analysis, the cells were harvested 3 days after transfection, lysed in radioimmunoprecipitation assay (RIPA) buffer containing the protease inhibitors aprotinin, pepstatin, and leupeptin (100 μg/ml each, Sigma). Cell lysates (10 μg) were denatured in modified 5× loading buffer (320 mM tris, pH 6.8, 50% glycerol, 0.5% bromophenol blue, 10% SDS, 100 mM dithiothreitol, and 8M urea) to reduce aggregation that typically occurs with proteins having seven transmembrane domains. After heating at 37°C for 45 min and then 75°C for 5 min, proteins were separated on a 4 to 20% polyacrylamide-SDS gradient gel, blotted onto nitrocellulose membranes (Gelman Science), and probed with either affinity-purified anti-PS2n (*3*), anti-APP (22C11, Boehringer Mannheim), or anti–β-tubulin (Boehringer Mannheim). Immunoblots were developed with the ECL System (Amersham).
19. For immunocytochemistry, 1 day after transfection cells were treated with trypsin and plated onto cover slips in a 24-well plate. The following day the cells were fixed for 20 min in 3% paraformaldehyde at 4°C, washed once with 1.5% glycine in PBS, twice with PBS, and blocked for 1 hour at 37°C with 10% bovine serum albumin in PBS. Samples were washed three times with 0.1% saponin in PBS (Sigma), incubated for 1 hour at 4°C with anti-FLAG (M2, Eastman Kodak), diluted to 5 μg/ml in PBS–0.1% saponin, and washed three more times with PBS–0.1% saponin. Cells were then stained for 1 hour at 4°C with fluorescein isothiocyanate–conjugated donkey antibody to mouse immunoglobulin G diluted 1:200 in PBS–0.1% saponin. After three washes, the cover slips were mounted onto a slide with fluoromount-g. Cells were viewed on a confocal Bio-Rad MRC1024 microscope at a ×20 magnification.
20. TUNEL studies were performed as described (*17*). PTX (Sigma, St. Louis, MO) was added once to achieve a final concentration of 100 ng/ml on day 2 and maintained in the medium until day 4.
21. For Aβ treatments the cells were maintained in DMEM containing 10% dialyzed FBS, 5% dialyzed horse serum, and gentamycin (50 ng/ml). The cells were treated with 10 μM Aβ(1–42) (generated from a 1 mM Aβ stock solution that had been aged 7 days at 37°C) starting on day 2 after the transfection. For all studies, the cells were fixed on day 4 and analyzed by TUNEL.
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A Cyclophilin Function in Hsp90-Dependent Signal Transduction

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Cpr6 and Cpr7, the *Saccharomyces cerevisiae* homologs of cyclophilin-40 (CyP-40), were shown to form complexes with Hsp90, a protein chaperone that functions in several signal transduction pathways. Deletion of *CPR7* caused severe growth defects when combined with mutations that decrease the amount of Hsp90 or Sti1, another component of the Hsp90 chaperone machinery. The activities of two heterologous Hsp90-dependent signal transducers expressed in yeast, glucocorticoid receptor and pp60$^{v\text{-}src}$ kinase, were adversely affected by *cpr7* null mutations. These results suggest that CyP-40 cyclophilins play a general role in Hsp90-dependent signal transduction pathways under normal growth conditions.

Immunophilins are ubiquitous and highly conserved. Nevertheless, with few notable exceptions, such as the participation of *Drosophila* cyclophilin ninaA in the maturation of rhodopsin (*1*) and the FKBP12-dependent regulation of Ca^{2+} flux by the inositol 1,4,5-trisphosphate receptor (*2*), little is known about the roles these molecules play in cell biology. Two *Saccharomyces cerevisiae* cyclophilins related to human CyP-40—Cpr6 and Cpr7—have been identified through their interactions with the transcriptional regulator Rpd3, a yeast homolog of histone deacetylase (*3*, *4*). Cpr6 and Cpr7 are the only CyP-40 homologs in *S. cerevisiae* (*4*).

Cpr6 was also identified as an Hsp90-associated protein (*5*). To determine if Cpr7 associates with Hsp90, we mixed glutathione-S-transferase (GST)–Cpr fusion proteins expressed in *Escherichia coli* with yeast cell lysates. Hsp90 was recovered on glutathione-affinity resin in association with both GST-Cpr6 and GST-Cpr7 but not with GST alone (Fig. 1, A and B). To determine if the interaction between Cpr6 or Cpr7 and Hsp90 requires other yeast proteins, we mixed GST-Cpr proteins with lysates of bacteria expressing Hsp90. Again, Hsp90 interacted with GST-Cpr6 and GST-Cpr7 but not with GST alone (Fig. 1C). The COOH-terminal halves of Cpr6 and Cpr7, which contain tetratricopeptide repeat (TPR) motifs thought to mediate protein-protein interactions (*4*), were sufficient for interaction with Hsp90 (Fig. 1C). Bacterially expressed GST-Cpr1, a *S. cerevisiae* cyclophilin that lacks a TPR motif (*6*), did not interact with Hsp90 (Fig. 1C). Thus, like the mammalian homolog CyP-40, Cpr6 and Cpr7 can interact directly with Hsp90 through their TPR-containing COOH-termini.

A functional relation between Cpr7 and Hsp90 was suggested through tests for genetic interaction. Because *cpr7*Δ cells display a slow-growth phenotype (*4*), we tested whether a decrease in Hsp90 concentrations would enhance this phenotype. *Saccharomyces cerevisiae* produces two nearly identical Hsp90 proteins (Hsc82 and Hsp82) that have the same functions but different patterns of expression (*7*). Deletion of *HSC82* reduces the cellular concentration of Hsp90 to approximately 1/10th of that in wild-type cells but does not impair growth below 37°C (*7*). Cells deleted for *HSC82* and *CPR7* exhibited a growth defect at 30°C that was more pronounced than that observed for *cpr7*Δ cells (Fig. 2A). Similar synthetic-enhancements of the *cpr7*Δ growth phenotype were observed in cells whose only source of Hsp90 was a temperature-sensitive allele, *hsp82*^{G170D} (*8*, *9*), and in cells deleted for *STI1*, which encodes another member of the Hsp90 chaperone complex (*5*, *10*) (Fig. 2B). In contrast, deletion of *CPR6* did not confer growth defects in either *hsc82*Δ or *sti1*Δ cells.

Hsp90 can act as a general chaperone in vitro to prevent aggregation of denatured proteins (*11*), but its functions in vivo seem to be focused on the maturation of various proteins that participate in gene regulation and signal transduction (*8*, *12*). Among the best characterized of these proteins is a subgroup of the steroid receptors (*13*–*15*). Several immunophilins are present in Hsp90 complexes, including the cyclophilin CyP-40 found in Hsp90::steroid receptor complexes (*5*, *13*, *15*, *16*). The role of these immunophilins in signal transduction, however, remains unclear.

The molecular components that mediate steroid receptor signal transduction in eukaryotes are highly conserved (*5*), and mammalian glucocorticoid receptor (GR) exhibits faithful hormone-dependent transcriptional activity in *S. cerevisiae* (*14*, *17*). Hsp90 is absolutely required for GR activity (*8*, *14*), whereas Sti1 is required for full receptor activity (*18*). We compared the activity of GR in isogenic (*19*) wild-type and cyclophilin-deleted (*cpr*Δ) cells with a *lacZ* GR reporter plasmid. With saturating concentrations of hormone (20 μM deoxy-

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corticosterone), cells deleted for *CPR7* showed about one-fifth the amount of β-galactosidase expression detected in wild-type cells (Fig. 3A). The decrease in receptor activity in *cpr7Δ* cells was not the result of decreased amount of receptor (Fig. 3B). Cpr7 was also found to be required for full activity of the estrogen receptor (*20*). Deletion of *CPR6* from wild-type cells did not result in decreased hormone-dependent activity of GR, nor did it further decrease GR activity in *cpr7Δ* cells (*20*). The absence of either Cpr6 or Cpr7 did not affect basal GR activity (*20*). Deletion of *CPR7* had only a small effect on the activity of a truncated form of GR (*20*) that activates reporter gene expression in a constitutive and Hsp90-independent manner (*21*). Thus, the role of Cpr7 in steroid receptor signal transduction appears to be in events that are regulated by Hsp90.

To determine the extent of cyclophilin involvement in Hsp90-dependent functions, we examined the activity of a different Hsp90 substrate, the oncogenic tyrosine kinase $pp60^{v\text{-}src}$ (*22*, *23*). Because the endogenous amount of tyrosine phosphorylation in *S. cerevisiae* is very low, the in vivo activity of exogenous $pp60^{v\text{-}src}$ is readily monitored by the appearance of ectopic phosphorylation of yeast proteins (*23*). The activity of this kinase is absolutely dependent on Hsp90 (*8*) and is reduced by a *STI1* deletion (*18*). Some mutations in Hsp90 that decrease $pp60^{v\text{-}src}$-mediated tyrosine kinase activity also decrease kinase accumulation (*8*), whereas deletion of *STI1* reduces the activity of $pp60^{v\text{-}src}$ without reducing its accumulation (*18*).

When $pp60^{v\text{-}src}$ was expressed in *cpr7Δ* cells, both its activity and accumulation were reduced relative to those in wild-type cells (Fig. 4A). In *cpr6Δ* cells, $pp60^{v\text{-}src}$ activity decreased by ~50%, whereas accumulation was unaffected. In contrast, the accumulation and activity of the cellular, nononcogenic form of the kinase, $pp60^{c\text{-}src}$, were similar in wild-type, *cpr6Δ*, and *cpr7Δ* cells (*24*) (Fig. 4B). Because $pp60^{c\text{-}src}$ is much less sensitive to Hsp90 perturbations than $pp60^{v\text{-}src}$ (*23*), these results reinforce the hypothesis that the cyclophilins are specifically involved in Hsp90-mediated events.

Studies with drugs that inhibit the prolyl-isomerase activity of CyP-40 have yielded conflicting results regarding the role of this cyclophilin in vivo (*25*). Using genetic methods, we show that cyclophilins of the CyP-40 class are required for full activity of several Hsp90-dependent signal transducers. Although the experiments were performed in *S. cerevisiae* (*26*) with heterologous Hsp-90 substrates, the structural (*5*) and functional (*14*, *17*) conservation of the Hsp90 machinery in yeast and mammalian cells suggest that our results reveal an authentic role of CyP-40 cyclophilins. CyP-40 exhibits chaperone activity in vitro (*27*), and the functions of Cpr6 and Cpr7 are closely allied with those of Hsp90 in vivo. Thus, this class of cyclophilin may enhance the ability of the Hsp90 machinery to maintain signal trans-

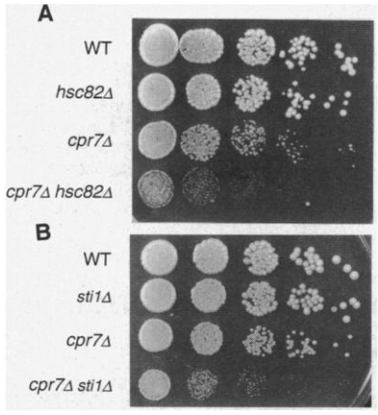

Fig. 2. Genetic interactions between Cpr7 and Hsp90 components. Serial dilutions of logarithmically growing cells (*19*) (1.4×10^6 per milliliter) were spotted onto rich medium and photographed after incubation (2 days) at 30°C. Enhancement of the *cpr7Δ* slow-growth phenotype by (**A**) *hsc82Δ* or (**B**) *sti1Δ* mutations was tested separately. The enhanced growth defect cosegregated with the *cpr7Δ hsc82Δ* and *cpr7Δ sti1Δ* genotypes among tetrads derived from crosses between congenic parent strains (*9*, *20*).

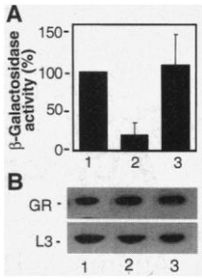

Fig. 3. Potentiation of hormone-dependent activation of GR by Cpr7. (**A**) Expression of β-galactosidase from a GR-dependent reporter gene in wild-type (lane 1), *cpr7Δ* (lane 2), or *cpr7Δ* cells expressing *CPR7* from a plasmid (lane 3) (*30*). Indicated activities are relative to that obtained from wild-type cells. All data are means ± SD ($n = 4$). (**B**) Abundance of GR and ribosomal protein L3 (loading control) visualized by immunostaining.

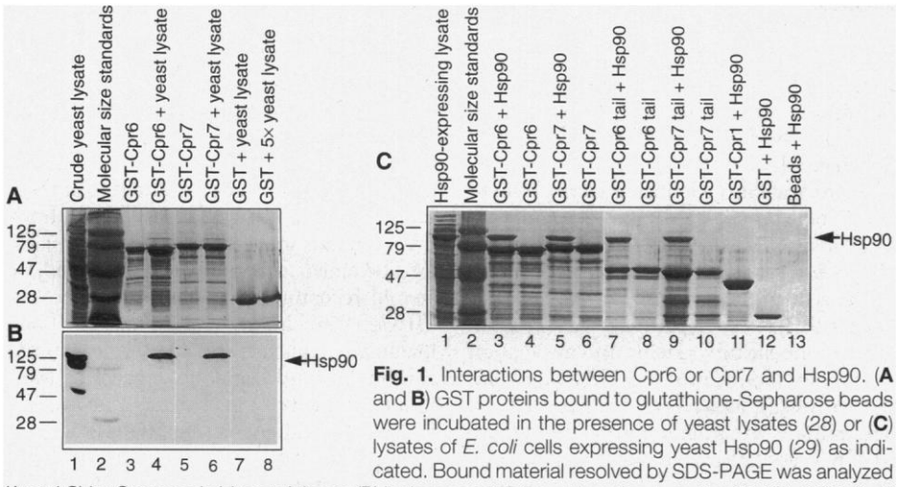

Fig. 1. Interactions between Cpr6 or Cpr7 and Hsp90. (**A** and **B**) GST proteins bound to glutathione-Sepharose beads were incubated in the presence of yeast lysates (*28*) or (**C**) lysates of *E. coli* cells expressing yeast Hsp90 (*29*) as indicated. Bound material resolved by SDS-PAGE was analyzed (A and C) by Coomassie blue staining or (B) by immunostaining with antibody to Hsp90 (*21*). GST-Cpr6 and GST-Cpr7 are fusions between GST and the entire coding sequence of Cpr6 or Cpr7; GST-Cpr6 tail and GST-Cpr7 tail are fusions between GST and the TPR-containing COOH-terminal region of Cpr6 or Cpr7 (*28*, *29*); and GST-Cpr1 is a fusion between GST and Cpr1 (*29*). (C) Lane 13, material bound in the absence of GST proteins.

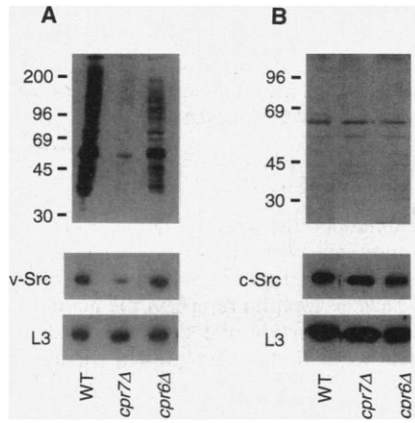

Fig. 4. Effects of *cpr6Δ* and *cpr7Δ* mutations on $pp60^{v\text{-}src}$ activity and accumulation. Wild-type, *cpr6Δ*, and *cpr7Δ* cells were grown to 2×10^6 per milliliter in raffinose medium, then transferred to galactose medium for 7 hours to induce expression of (**A**) $pp60^{v\text{-}src}$ or (**B**) $pp60^{c\text{-}src}$. Cellular proteins were separated on an SDS-polyacrylamide gel and transferred to immobilon-P membranes. Equal sample loading was confirmed by Coomassie blue staining. Blots were incubated with antibody (*8*) specific for phosphotyrosine (upper panel), Src (middle panel), or ribosomal protein L3 (lower panel).

ducers in an intermediate state from which they can be readily activated.

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26. Because the growth rate of cells originating from a different strain background (S288C) was less affected by deletion of *CPR7*, the magnitude of the effect of a *cpr7*Δ mutation on Hsp90-dependent events may vary from strain to strain.
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28. Samples of bacterial extracts containing GST-Cpr6 (*4*), GST-Cpr7 (*4*), or nonchimeric GST were incubated in the presence of glutathione–Sepharose 4B beads (Pharmacia) for 1 hour at 4°C. The beads were then washed twice with phosphate-buffered saline, and equal amounts of beads and bound proteins (~5 μg) were transferred to new tubes. The binding reactions were completed by addition to each tube of 2× binding buffer (100 μl) (*4*), yeast cell crude lysate [16 μl (80 μl in lane 8) extracted from strain W303 as described (*5*)], and doubly distilled H_2O to a final volume of 200 μl. After incubation at 4°C for 1 hour, the beads were washed four times with 1× binding buffer, and bound proteins were eluted with 0.5× final concentration of glutathione elution buffer (*4*). Material from 10 μl of each eluate (from a total of 50 μl) was resolved by SDS–polyacrylamide gel electrophoresis (PAGE) and analyzed as described in the figure legend.
29. A plasmid expressing yeast Hsp90 (pETy90) was transformed into λDE3 lysogens of *E. coli* strain BL21 and induced with isopropyl-β-D-thiogalactopyranoside. Lysate (40 μl) derived from these cells was added to fusion protein–bound glutathione beads prepared as described (*28*). The three additional *GST::CPR* fusions generated for these experiments included GST fused at the NH_4-terminus of Cpr1, a hybrid between GST and the TPR-containing COOH-terminal region of Cpr6 (amino acids 171 to 371, Cpr6 tail), and a fusion between GST and the analogous region of Cpr7 (amino acids 193 to 393, Cpr7 tail). The binding reactions were treated as described (*28*), and eluates were analyzed by SDS-PAGE. Immunoblot experiments verified the identity of Hsp90 found in association with the fusion proteins.
30. Cells were transformed with a GR expression plasmid, p2A/GRGZ, that also contains the *lacZ* gene under the control of GR elements (*8*). In the absence of the receptor, the *lacZ* reporter gene is expressed at very low levels (*14*). Mid-logarithmic phase cells grown in galactose-containing medium were treated with saturating amounts (20 μM) of deoxycorticosterone (DOC) for 1 hour at 25°C, and β-galactosidase activity was assessed as described (*8*). Cellular proteins from mid-logarithmic phase cells were separated on an SDS-polyacrylamide gel and transferred to an Immobilion-P membrane. Equal sample loading was confirmed by Coomassie blue staining before incubation with antibody specific for GR (*8*) or L3 proteins. Constitutive GR activity was measured without DOC (*8*).

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Chaperone Function of Hsp90-Associated Proteins

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The Hsp90 heat shock protein of eukaryotic cells regulates the activity of proteins involved in signal transduction pathways and may direct intracellular protein folding in general. Hsp90 performs at least part of its function in a complex with a specific set of partner proteins that include members of the prolyl isomerase family. The properties of the major components of the Hsp90 complex were examined through the use of in vitro protein folding assays. Two of the components, FKBP52 and p23, functioned as mechanistically distinct molecular chaperones. These results suggest the existence of a super-chaperone complex in the cytosol of eukaryotic cells.

The heat shock protein Hsp90 is expressed at high levels in the cytosol of eukaryotic cells (*1*) and functions as a general cytosolic chaperone under physiological (*2*, *3*) and heat shock conditions (*4*) in vitro. Unlike other heat shock proteins, it seems to perform at least part of its activity in complex with proteins of unknown function (*5*, *6*). Nine different components of the complex have been identified, including Hsp90, Hsp70, p60/Hop (Hsp90/Hsp70 organizing protein), p48 (Hip), Hdj1, p23, and any one of the three large immunophilins: FKBP52 (FK506 binding protein, p59, Hsp56, HBI), FKBP54, or cyclophilin 40 (Cyp40) (*6*–*8*). Most of these proteins were initially identified in mammals in complexes with steroid receptors. They are required to keep the receptors in a state activatable by steroids (*6*, *9*). However, it seems that these complexes have more general functions because they are conserved throughout eukaryotes (*10*).

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To analyze the functional properties of the Hsp90 partner proteins, we studied their action in in vitro protein folding and unfolding reactions. As an assay system, the thermal unfolding and aggregation of citrate synthase (CS) was used (*4*). The characterization of the unfolding pathway of CS allows the quantitative analysis of the number of intermediates formed in the unfolding process and the distribution between native and aggregated forms of CS (*4*).

One of the characteristic features of molecular chaperones is their ability to suppress the aggregation of proteins under stress conditions (*1*, *11*, *12*). Thus, we examined the ability of Hsp90 partner proteins and a number of unrelated proteins to suppress the thermal aggregation process of CS. Immunoglobulin G (IgG), lysozyme, or bovine serum albumin (BSA) (Fig. 1A) (*13*, *14*), as well as an isolated domain of Hsp90 (*14*), did not affect the aggregation process of CS. However, two components of the mature Hsp90 complex, FKBP52 and p23, specifically suppressed the aggregation of CS in a concentration-dependent manner (Fig. 1, A and B), similar to that for Hsp90 (*4*) and small Hsps (sHsps) (*13*, *15*).