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CNS1 Encodes an Essential p60/Sti1 Homolog in *Saccharomyces cerevisiae* That Suppresses Cyclophilin 40 Mutations and Interacts with Hsp90

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Cyclophilins are *cis-trans*-peptidyl-prolyl isomerases that bind to and are inhibited by the immunosuppressant cyclosporin A (CsA). The toxic effects of CsA are mediated by the 18-kDa cyclophilin A protein. A larger cyclophilin of 40 kDa, cyclophilin 40, is a component of Hsp90-steroid receptor complexes and contains two domains, an amino-terminal prolyl isomerase domain and a carboxy-terminal tetratricopeptide repeat (TPR) domain. There are two cyclophilin 40 homologs in the yeast *Saccharomyces cerevisiae*, encoded by the *CPR6* and *CPR7* genes. Yeast strains lacking the Cpr7 enzyme are viable but exhibit a slow-growth phenotype. In addition, we show here that *cpr7* mutant strains are hypersensitive to the Hsp90 inhibitor geldanamycin. When overexpressed, the TPR domain of Cpr7 alone complements both *cpr7* mutant phenotypes, while overexpression of the cyclophilin domain of Cpr7, full-length Cpr6, or human cyclophilin 40 does not. The open reading frame YBR155w, which has moderate identity to the yeast p60 homolog *STI1*, was isolated as a high-copy-number suppressor of the *cpr7* slow-growth phenotype. We show that this Sti1 homolog Cns1 (cyclophilin seven suppressor) is constitutively expressed, essential, and found in protein complexes with both yeast Hsp90 and Cpr7 but not with Cpr6. Cyclosporin A inhibited Cpr7 interactions with Cns1 but not with Hsp90. In summary, our findings identify a novel component of the Hsp90 chaperone complex that shares function with cyclophilin 40 and provide evidence that there are functional differences between two conserved sets of Hsp90 binding proteins in yeast.

Cyclophilin 40 is one of several protein components of the Hsp90 protein complex. Hsp90 has a dual function; it acts as a chaperone after heat shock to help fold denatured proteins and also maintains the activity of signalling proteins under normal conditions. Hsp90 and associated proteins function as large chaperone units that regulate several molecules involved in signal transduction, including oncogenic kinases and members of the steroid receptor family (reviewed in references 2 and 43). These Hsp90 complexes consist of several proteins, including Hsp70, p60, p48, p23, and a large immunophilin, which may be either FKBP52, FKBP54, or cyclophilin 40. Several of these proteins have recently been shown to have chaperone activity in vitro (4, 22, 48).

Interactions between the components of the Hsp90 chaperone complex and their substrates are highly ordered and very dynamic. The order of assembly of these complexes with the progesterone receptor has been determined from reconstitution experiments in cell-free lysates (53, 54). First, Hsp70 binds the progesterone receptor, forming an early complex. Next, the progesterone receptor is found in an intermediate complex containing Hsp90, Hsp70, and p60. The trimeric Hsp90-Hsp70-p60 complex is soon displaced from the progesterone receptor by a preformed Hsp90-immunophilin-p23 complex. In this mature complex, the progesterone receptor is maintained in a state competent to bind hormone. If the receptor does not bind steroid, it is released from the mature complex and starts the association-dissociation cycle again. Recently, it has been shown that if the Hsp90 substrate is locked in a complex with Hsp90 and is not released, it is targeted for degradation by the proteasome (48). This study used the Hsp90 inhibitor geldanamycin, an antiproliferative agent that may find use as a novel chemotherapy agent. It has been previously suggested that geldanamycin blocks the binding of p23 to the Hsp90-immunophilin complex (59), which may improperly stabilize interactions between this complex and target proteins, thus stimulating degradation. Two recent studies show that geldanamycin inhibits binding of a yeast p23 homolog to yeast Hsp90 (1, 19).

By Hsp90 affinity chromatography and heterologous coexpression of the steroid receptor and a reporter gene under control of a steroid response element in the yeast *Saccharomyces cerevisiae*, it was shown that the Hsp90 complex is biochemically and functionally conserved in *S. cerevisiae* (6, 8, 25, 36, 37, 42). Previous studies and the recent completion of the yeast genome sequencing project have identified genes encoding other proteins found in Hsp90 complexes. There are two *HSP90* homologs in yeast, *HSP82* and *HSC82*; *HSC82* is expressed constitutively at high level and is moderately induced by heat shock, whereas *HSP82* is expressed constitutively at a much lower level but is much more strongly induced by heat shock (3). Yeast strains require at least one copy of either *HSP82* or *HSC82* for viability. *STI1*, the yeast p60 homolog, physically and genetically interacts with *HSP90*, and mutations in *STI1* affect *HSP90* functions in vivo (9, 16, 39). In addition, several *HSP70* homologs are found in *S. cerevisiae* (38). A yeast p23 homolog, Sba1, has also recently been identified (1, 19). There are two cyclophilin 40 genes in yeast, *CPR6* and *CPR7* (8, 15–17, 57). The Cpr6 and Cpr7 cyclophilins share 47 and 35% identity with human cyclophilin 40, respectively, and 41% identity with each other. All of the cyclophilin 40 homologs have in common an amino-terminal peptidyl-prolyl isomerase

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domain and a carboxy-terminal tetratricopeptide repeat (TPR) domain. TPR domains are loosely conserved repeats of roughly 34 amino acids that are found in several proteins that interact with Hsp90; the TPR domain of cyclophilin 40 mediates its binding to Hsp90 (13, 16, 41, 44). Yeast strains lacking *cpr6* or *cpr7*, alone or in combination, are viable. *cpr7* mutant strains, however, exhibit a slow-growth phenotype, while *cpr6* mutant strains do not (15–17, 57).

Here we have further characterized the yeast cyclophilin 40 homologs. We find that *cpr7* mutant strains are hypersensitive to the Hsp90 inhibitor geldanamycin (59). Mutant forms of the cyclophilin 40 homolog Cpr7 were analyzed to determine the unique features required for function in vegetative growth and geldanamycin resistance. The TPR domain of Cpr7 alone, when overexpressed, restores normal growth rate and geldanamycin resistance in both *cpr7* and *cpr6 cpr7* null mutant strains. Neither *CPR6* nor the human cyclophilin 40 gene can functionally replace *CPR7*. In addition, the TPR domain did not have any dominant negative effect when overexpressed in a wild-type strain. We also found that transcription of the *CPR6* gene is significantly induced by heat shock, whereas expression of *CPR7* is not.

A novel yeast gene with homology to the yeast p60 homolog *STI1* was isolated as a high-copy-number suppressor of the *cpr7* slow-growth phenotype and has been named *CNS1*, for cyclophilin seven suppressor. When overexpressed, *CNS1* complements both the slow growth and the geldanamycin sensitivity of both *cpr7* single-mutant and *cpr6 cpr7* double-mutant strains. *CNS1* is required for viability in yeast, and the lethality of a Δ*cns1* null mutant strain is not rescued by overexpression of *STI1*, *CPR6*, *CPR7*, *HSP90*, or other genes implicated in Hsp90 functions. Unlike its homolog *STI1*, *CNS1* is not transcriptionally regulated by heat shock. Finally, we show that the Cns1 protein is found in protein-protein complexes containing yeast Hsp90 and the yeast cyclophilin 40 homolog Cpr7 but not the Cpr6 cyclophilin. Taken together, our findings and previous studies reveal that the components of the Hsp90-associated chaperone machinery are duplicated in yeast and that one partner of each pair is heat inducible and nonessential (*HSC82*, *CPR6*, and *STI1*) whereas the other partner is constitutive and often more important for vegetative growth (*HSC82*, *CPR7*, and *CNS1*).

MATERIALS AND METHODS

Media and strains. Media were prepared as described in reference 49. Medium containing geldanamycin (National Cancer Institute) was prepared by adding a sterile stock of geldamycin in dimethyl sulfoxide to autoclaved medium at a final concentration of 20 μg/ml before pouring.

Strains used in this study were isogenic derivatives of JK93da (*leu2-3,112 ura3-52 rme1 trp1 his4 HML*a [27]) with the following genotypic changes: KDY46, Δ*cpr6::G418*; and KDY65, Δ*cpr7::G418* (strain construction described in reference 15). The Δ*cpr6* Δ*cpr7* double-mutant strain was constructed by crossing a *MAT*α derivative of KDY46 to KDY65. The diploid was sporulated and dissected, and G418-resistant segregants were selected from tetrads with a 2 G418-resistant:2 G418-sensitive segregation pattern. The G418-resistant segregants were confirmed to be Δ*cpr6* Δ*cpr7* double mutants phenotypically (slow growth) and by PCR analysis of genomic DNA; the resulting Δ*cpr6::G418* Δ*cpr7::G418* strain was designated KDY66.5a.

Transformations and one-step gene disruptions. Yeast transformation and one-step gene disruption were as described elsewhere (23, 45).

Cloning of *CPR6* and *CPR7*. The wild-type *CPR6* and *CPR7* genes were cloned by PCR using the following primers: for *CPR6*, 5′-GCCCGGATCCCCCACTG CATAAATGGACATCCGG-3′ and 5′-GCCCGTCGACCCCTTTATAGAAC ATAACTG-3′; for *CPR7*, 5′-GATCGGATCCGGGCGCTTCTTACCAAAGT TGCG-3′ and 5′-GGCGAATTCGGGTTGCAATTACCTGGC-3′. The resulting *CPR7* PCR product was cleaved with *Eco*RI and *Bam*HI and cloned into the corresponding sites of both the *CEN* (centromeric) *URA3* vector pRS316 (50) and the 2μm *URA3* vector YEplac195 (24) to generate plasmids pKS17 and pKS24, respectively. Similarly, to clone *CPR6*, the PCR product was cleaved with *Bam*HI and *Sal*I and cloned into the corresponding sites of pRS316 and YEp24 (5) to generate plasmids pKDw16 and pKDw10, respectively.

Construction of Cpr7 deletion mutant and Cpr6-Cpr7 fusion proteins. The ΔCYP Cpr7, ΔTPR Cpr7, and Cpr6-Cpr7 hybrid proteins were engineered and expressed with CPR7 5′ and 3′ untranslated regions by PCR overlap mutagenesis as described in reference 28 by using the following primers: for ΔCYP Cpr7, 5′-TCCAACGCGATGTGGGAAAAA-3′ and 5′-CATAGTTTTTTCCCACAT CGCGTT-3′; for ΔTPR Cpr7, 5′-ACAAGTAACTAATTACACTCCACAGTC GCTGATTCTAAC-3′ and 5′-GGAGTGTAATTAGTTACTTGTAAGGCT-3′; for Cpr6-Cpr7, 5′-TCTAGTCATCGCGTTGGATGTAGGTTG-3′, 5′-A ACGCGATGACTAGACCTAAAACTTTT-3′, 5′-TTTTTCCCACACGCCAC AGTCATCAAT-3′, and 5′-TGTGGCGTGTGGGAAAAACTATGGGT-3′. Flanking primers for these constructs were 5′-GATCGGATCCGGGCGCTTC TTACCAAAGTTGCG-3′ and 5′-GGCGAATTCGGGTTGCAATTACCTGG C-3′. The resulting PCR products were cleaved with *Eco*RI and *Bam*HI and cloned into the corresponding sites of both pRS316 (50) and YEplac195 (24).

The human cyclophilin 40 gene was fused to the 5′ and 3′ untranslated regions of *CPR7* by gap repair as described elsewhere (40). Human cyclophilin 40 cDNA was amplified (cDNA clone provided by R. Handschumacher [31]) by using primers each with 39 bases of 5′ homology to either the 5′ or 3′ untranslated region of *CPR7*: 5′-ATTCTGAAAGGTGTTCGGCAGCAACCTACATCCAA CGCGATGTCGCACCCGTCCCCCCAA-3′ and 5′-TTGGGTTATTTAATCT CAAATTTCAGCCTTACAAGTAACTAACTAAGCAAACATTTTTGCAT A-3′. The wild-type yeast strain JK93da was cotransformed with the resulting PCR product and pKS17 (wild-type *CPR7* plasmid) cleaved with *Sna*BI. The gap-repaired plasmid was rescued from yeast and sequenced, and expression of the human cyclophilin 40 clone was confirmed by Western blotting using antibodies that react specifically with human cyclophilin 40 (Affinity Bioreagents).

Northern analysis. Wild-type (JK93da) and mutant (KDY98.4a Δ*cpr1::LEU2* Δ*cpr2::TRP1 cpr3::HIS3* Δ*cpr4::URA3* Δ*cpr5::LEU2* Δ*cpr6::G418* Δ*cpr7::G418* Δ*cpr8::MET15 fpr1::ADE2* Δ*fpr2::URA3* Δ*fpr3::URA3* Δ*fpr4::G418*) yeast strains (15) were grown at 24°C to mid-log phase and heat shocked at 37°C, and samples were removed at 0, 2, 5, and 30 min. RNA was isolated as described elsewhere (47). Probes spanning the open reading frame for each gene were amplified by PCR and radiolabeled with [^{32}P]dCTP by using a random primer DNA labeling kit (Boehringer Mannheim). Northern blot analysis was done as described in reference 46; levels of induction were normalized to actin message and quantified by PhosphorImager analysis.

Δ*cpr7::G418* high-copy-number suppressor screen. A Δ*cpr7::G418* mutant strain was transformed with a high-copy-number *URA3* yeast genomic library (provided by C. Alarcon), and transformants were selected on medium lacking uracil. Large colonies were streak purified, and plasmids were rescued from yeast transformants as described elsewhere (30) and amplified in *Escherichia coli*. The resulting plasmid DNA was used to transform both *cpr7* and *cpr6 cpr7* mutant strains to determine which suppressors were plasmid linked. Plasmids containing suppressing clones were classified by restriction mapping and PCR with primers to the *CPR7* gene and then sequenced by Sequetech (Mountain View, Calif.). The yeast genome was then searched for homology to the cloned sequence (11), and it was determined that YBR155w was contained within the complementing clone for six isolates and the *CPR7* gene in one isolate.

Cloning of *STI1* and *CNS1*. *STI1* and *CNS1* were cloned by PCR using the following primers: for *CNS1*, 5′-CGCGGATCCCCACTTTAATTTTAAATGC TT-3′) and 5′-CGTGGATCCCTGCATTTAGTACCGACAATA-3′; for *STI1*, 5′-CGCGGATCCCCCCGTCATAAGTTCCTATAC-3′ and 5′-CGTGGATCC TATGGCAGGCACATTACTAAA-3′. The *CNS1* and *STI1* PCR products were digested with *Bam*HI and cloned into the corresponding sites of YEplac195 (24) to generate plasmids pKDw20 and pKDw19, respectively.

Construction of Δ*cns1::G418*. Disruption of the *CNS1* open reading frame was done as described previously (35). Primers used to amplify the G418 resistance gene (56) were 5′-TATGTGCCAGGGCCAGGTGATCCTGAACTTCCACCC CAACTACAGCTGAAGCTTCGTACGC-3′ and 5′-TTGCTTATCCCACTTG GAAATCCACCCTTCACTTTCTACCTTGCATAGGCCACTAGTGGATCT G-3′. The resulting PCR product containing the G418 resistance gene open reading frame flanked by sequences identical to *CNS1* was used to transform a diploid strain. G418-resistant colonies were screened by PCR to identify Δ*cns1::G418*/*CNS1* transformants.

Epitope tagging of Cns1. The *CNS1* gene was amplified by PCR with primers 5′-AAGCGATCCGCGGCCGCAATGAGCTCCGTTAACGCAAAT-3′ and 5′-AAGCTTGATGCGGCCGCACTGCATTTAGTACCGACAATA-3′. The PCR product was cleaved with *Not*I and cloned into the corresponding site of pYeF1 (12), which contains the hemagglutinin (HA) epitope under control of the *GAL* promoter, to result in fusion of the HA epitope to the amino terminus of *CNS1*. The resulting plasmid, pKDE3, expresses HA-Cns1 and restored viability in a Δ*cns1::G418* mutant strain, indicating that the HA epitope-tagged Cns1 is functional.

Antisera and immunoprecipitation experiments. Immunoprecipitation experiments were done as described in reference 46. Wild-type (JK93da) yeast was transformed with pYeF1 (empty vector) or pKDE3 (HA-tagged Cns1), transformants were grown to an optical density at 600 nm (OD_{600}) of 1 in the presence of galactose to induce Cns1 expression, and total-cell extracts were prepared as described elsewhere (7). Total-cell extracts were incubated for 12 h at 4°C with antibodies against HA coupled to Sepharose beads (Boehringer Mannheim), washed four times in lysis buffer (20 mM Tris-HCl, 100 mM KCl [pH 7.4]), and analyzed by Western blotting. Rabbit polyclonal antisera specific for Hsc82 and

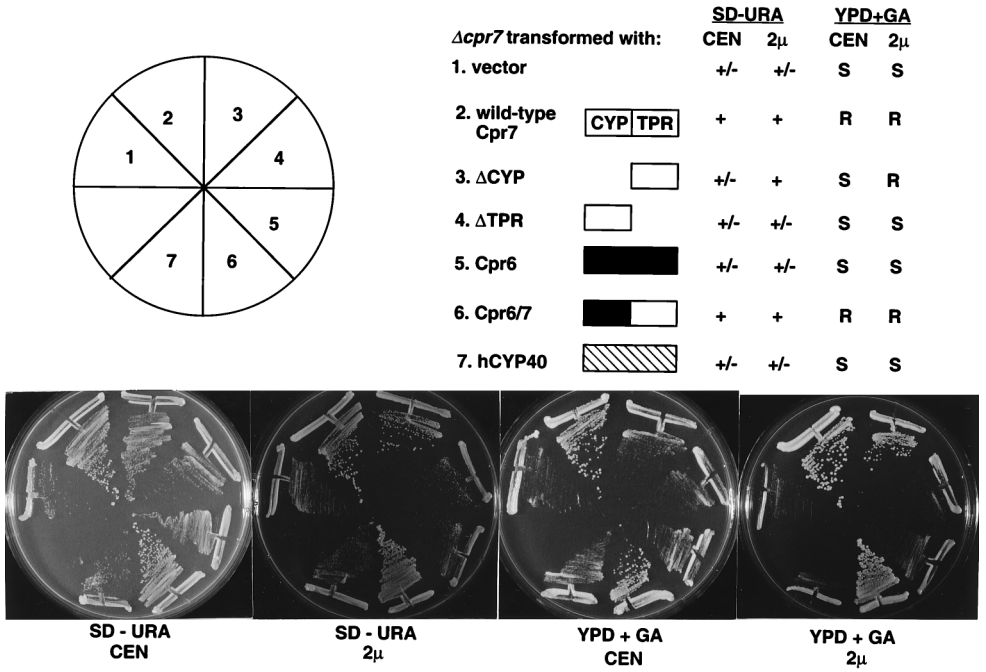

FIG. 1. The TPR domain is necessary and sufficient for Cpr7 function when overexpressed. The Δ*cpr7* mutant strain KDY65 was transformed with 2μm or *CEN URA3* plasmids expressing wild-type Cpr7, the TPR domain of Cpr7 (ΔCYP), the cyclophilin domain of Cpr7 (ΔTPR), wild-type Cpr6, a hybrid protein containing the cyclophilin domain of Cpr6 fused to the TPR domain of Cpr7 (Cpr6/7), or human cyclophilin 40 (hCYP40). Expression of Cpr6 and human cyclophilin 40 was confirmed by Western blot (data not shown). Transformants were grown on synthetic dextrose medium lacking uracil (SD − URA) or on YPD medium containing 20 μg of geldanamycin per ml (YPD + GA) for 48 h at 30°C. The criteria used to establish complementation of the slow-growth, small-colony phenotype of the Δ*cpr7* mutant strain were twofold: first and most importantly, whether the introduced plasmid restored colony size to the wild-type level, and second, the overall level of growth. Findings presented are representative of several similar experiments. Open, solid, and hatched boxes, Cpr7, Cpr6, and human cyclophilin 40 protein sequences, respectively; +, wild-type colony size; +/−, smaller colony size and poorer growth; R and S, drug resistant and drug sensitive, respectively.

Cpr6 were generously provided by Susan Lindquist and Didier Picard, respectively. Mouse polyclonal antisera to yeast Hsc82 was generously provided by Avrom Caplan. Antisera against human cyclophilin 40 was purchased from Affinity Bioreagents.

GST-Cpr7. The open reading frame of *CPR7* was PCR amplified by using primers 5′-CGAGGATCCATGATTCAAGATCCCCTTGTA-3′ and 5′-CGAG GATCCTACTGCTAGGATGAGGCCCAG-3′. The resulting PCR product was cleaved with *Bam*HI and cloned in the corresponding site of plasmid pGEX-2TK (52). Purification of the glutathione *S*-transferase (GST)–Cpr7 protein was performed as described previously (21). Yeast strains transformed with pYeF1 or pKDE3 were grown to an OD_{600} of 1. Protein extracts were made as described above and then incubated for 12 h at 4°C with either purified GST-Cpr7 or GST alone. In some cases, reaction mixtures contained 20 μM cyclosporin A (CsA) or 50 μM geldanamycin. Reaction products were then washed four times in lysis buffer and analyzed by Western blotting with mouse monoclonal antibodies against HA (Boehringer Mannheim) or mouse polyclonal antisera against yeast Hsc82 (provided by Avrom Caplan).

RESULTS

The TPR domain of the yeast cyclophilin 40 homolog Cpr7 is critical for function. To determine which domains of the yeast cyclophilin 40 homolog Cpr7 are important for function, we engineered a series of deletion and fusion proteins and tested whether these restore normal growth in a *cpr7* null mutant strain when expressed from either a low-copy-number *CEN* plasmid or a high-copy-number 2μm plasmid. As expected, expression of the wild-type *CPR7* gene from either a *CEN* or 2μm plasmid complemented the slow-growth defect of the *cpr7* mutation, restoring colony size to the wild-type level (Fig. 1). Interestingly, overexpression of the Cpr7 TPR domain alone from a 2μm plasmid (but not from a *CEN* plasmid) was sufficient to complement the *cpr7* slow-growth mutant phenotype and restore colony size to wild-type (Fig. 1). In contrast, the Cpr7 cyclophilin domain failed to complement the *cpr7* mutation, even when overexpressed (Fig. 1). These findings are in accord with a recent report by others (18). Expression of the yeast Cpr6 cyclophilin homolog, or human cyclophilin 40, also failed to complement the *cpr7* mutation (Fig. 1). Western blot analysis with specific antisera confirmed that both Cpr6 and human cyclophilin 40 were expressed (data not shown). Finally, we note that when the Cpr7 TPR domain was fused to the cyclophilin domain of either Cpr7 (wild-type protein) or Cpr6 (Cpr6-7 hybrid protein), complementation was observed even with expression from a *CEN* plasmid. When overexpressed in a wild-type background, none of the cyclophilin 40 deletion or fusion proteins had any dominant negative effects on growth rate (data not shown).

To test whether the TPR domain of Cpr7 required the presence of the Cpr6 protein to function, we repeated the preceding experiments in a *cpr6 cpr7* double-mutant strain. When overexpressed, the TPR domain of Cpr7 still complemented the growth defect of the *cpr6 cpr7* mutant strain (Fig. 2). In addition, the Cpr7 TPR domain alone restored normal growth in a *cpr1 cpr6 cpr7* triple-mutant strain (*CPR1* encodes the yeast cytoplasmic cyclophilin A); thus, no cytoplasmic cyclophilin domain is required for the TPR domain to complement the slow-growth defect of *cpr7* (data not shown). Taken together, these findings indicate that it is the TPR domain of Cpr7 that is most critical for its in vivo function.

***cpr7* mutants are sensitive to the Hsp90 inhibitor geldanamycin.** Geldanamycin is a potent antitumor drug whose target is Hsp90 (59). We found that *cpr7* mutant strains, and also *cpr6* and *cpr6 cpr7* mutant strains, are hypersensitive to geldanamycin, indicating that in the absence of the yeast cyclophilin 40 homologs the cell is sensitive to perturbations in Hsp90 func-

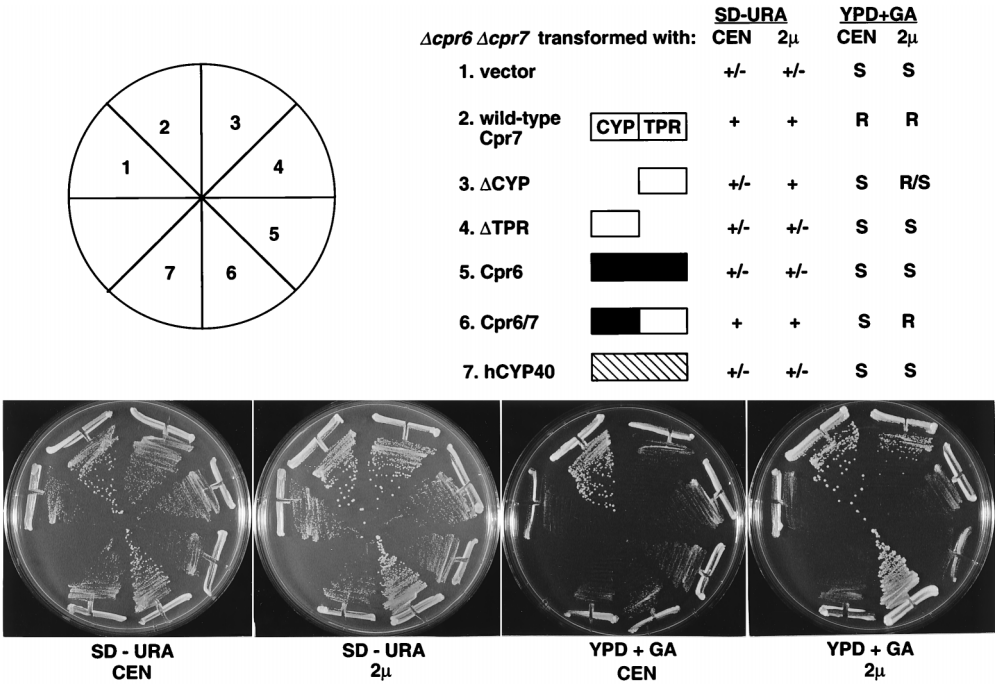

FIG. 2. The TPR domain does not require the presence of Cpr6 to functionally replace Cpr7. The Δ*cpr6* Δ*cpr7* mutant strain KDY66.5a was transformed with 2μm or *CEN URA3* plasmids expressing wild-type Cpr7, the TPR domain of Cpr7 (ΔCYP), the cyclophilin domain of Cpr7 (ΔTPR), wild-type Cpr6, a hybrid protein containing the cyclophilin domain of Cpr6 fused to the TPR domain of Cpr7 (Cpr6/7), or human cyclophilin 40 (hCYP40). Transformants were grown on synthetic dextrose media lacking uracil (SD − URA) or on YPD medium containing 20 μg of geldanamycin per ml (YPD + GA) for 48 h at 30°C. Criteria used to establish complementation of the Δ*cpr6* Δ*cpr7* mutant strain were as described in the legend to Fig. 1. Findings presented are representative of several similar experiments. Open, solid, and hatched boxes, Cpr7, Cpr6, and human cyclophilin 40 protein sequences, respectively; +, wild-type colony size; +/−, smaller colony size and poorer growth; R, R/S, and S, drug resistant, partially drug resistant, and drug sensitive, respectively.

tion (Fig. 3). These findings suggest that Cpr7 and Hsp90 normally interact, either physically, functionally, or both, in accord with previous genetic analyses that revealed a synthetic lethal interaction between yeast *hsp90* and *cpr7* mutations (16).

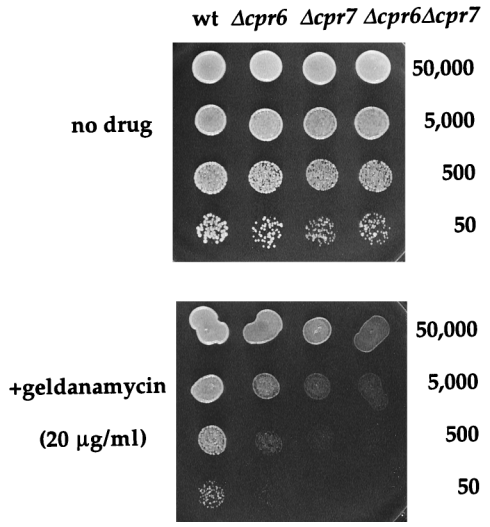

FIG. 3. Yeast mutants lacking cyclophilin 40 homologs are hypersensitive to the Hsp90 inhibitor geldanamycin. Isogenic wild-type (JK93da), Δ*cpr6* (KDY46), Δ*cpr7* (KDY65), and Δ*cpr6* Δ*cpr7* (KDY66.5a) mutant yeast strains were grown overnight in YPD medium, diluted to equal OD, and 10-fold serially diluted; 5-μl portions were plated on YPD medium containing no drug or 20 μg of geldanamycin per ml and incubated for 48 h at 30°C. Approximate numbers of cells plated are indicated to the right.

We tested whether Cpr6, human cyclophilin 40, or any of the Cpr7 deletion proteins could restore growth to a *cpr7* (or *cpr6 cpr7*) mutant strain on medium containing 20 μg of geldanamycin per ml. As in the growth rate studies, the Cpr6-Cpr7 fusion protein and the overexpressed Cpr7 TPR domain alone (but not Cpr6 or human cyclophilin 40) complemented the geldanamycin-sensitive phenotype of a *cpr7* mutant strain (Fig. 1 and 2). This result provides further evidence that the slow-growth phenotype of *cpr7* mutant strains is linked to defects in Hsp90 function.

The overexpressed Cpr7 TPR domain only partially restored normal growth on medium containing geldanamycin in a *cpr6 cpr7* double-mutant strain (Fig. 2). In addition, the Cpr6-Cpr7 fusion protein must be overexpressed to complement the geldanamycin sensitivity in the *cpr6 cpr7* double-mutant strain, while in the *cpr7* single mutant the fusion protein in low copy number was sufficient for full function. These results suggest that Cpr6 and Cpr7, though not functionally redundant, may to some extent overlap in function.

***CPR6* expression is heat induced, whereas *CPR7* expression is not.** Because several other proteins found in Hsp90 complexes are inducible by heat shock, we tested whether Cpr6 or Cpr7 expression is regulated by heat shock via Northern blot analysis. *CPR6* was induced 3.3-fold after 5 min at 37°C (Fig. 4A), in accord with previous studies that have shown that Cpr6 protein levels are induced fourfold after heat shock at 39°C (57). In contrast, expression of the *CPR7* gene was not induced by heat shock (Fig. 4B). In accord with these findings, the *CPR6* gene promoter contains consensus heat shock response elements (57), whereas the *CPR7* gene promoter does not. *cpr6* and *cpr7* single-mutant and *cpr6 cpr7* double-mutant strains

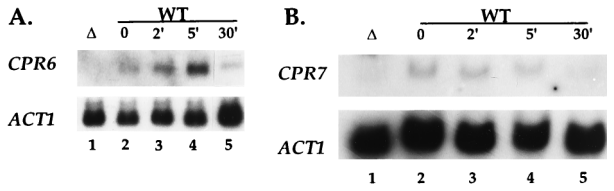

FIG. 4. Cpr6 transcription is regulated by heat shock; Cpr7 expression is not. Total RNA was purified from wild-type (WT) and mutant (Δ; Δ*cpr1* Δ*cpr2 cpr3* Δ*cpr4* Δ*cpr5* Δ*cpr6* Δ*cpr7* Δ*cpr8 fpr1* Δ*fpr2* Δ*fpr3* Δ*fpr4* [15]) immunophilin strains and analyzed by Northern blot with probes specific to the gene indicated on the left. Lanes: 1, total RNA from the Δ*cpr1-8* Δ*fpr1-4* strain lacking *CPR6* and *CPR7* as a control; 2 through 5, total RNA from a wild-type yeast strain was isolated after 37°C heat shock for 0, 2, 5, or 30 min. Fold induction was determined by normalizing the amounts of immunophilin RNA to actin RNA by phosphorimaging.

were not more sensitive to heat shock at 45 or 48°C than the isogenic wild-type strain (data not shown).

***cpr7* mutants are not suppressed by overexpression of known Hsp90-interacting proteins Ppt1, Cdc37, Cdc23, Ubc4, and Sun2.** We tested whether the yeast Hsp90 homologs can function as high-copy-number suppressors of the *cpr7* slow-growth phenotype. Overexpression of Hsc82 or Hsp82 did not complement the slow-growth phenotype of a *cpr7* mutant strain (data not shown). We also tested the following proteins that interact with Hsp90 or may be involved in Hsp90 functions: Ppt1, a serine/threonine phosphatase with four TPR domains that copurifies with the glucocorticoid receptor (10); Cdc37, the p50 component found in several Hsp90-kinase complexes (20, 32); Cdc23, which contains several TPR domains and is involved in ubiquitination and degradation of B-type mitotic cyclins (14, 51); Ubc4, a ubiquitin-conjugating enzyme with TPR domains which, when mutated, is synthetically lethal with *cdc23* mutations (29); and Sun2, a component of the 26S proteasome (33). None of these proteins restored normal growth to a *cpr7* mutant strain when overexpressed from a 2μm high-copy-number plasmid (data not shown).

Identification of a multicopy suppressor of *cpr7* mutations as the p60/Sti1 homolog *CNS1*. To identify the target(s) or novel components of the Cpr7-Hsp90 complex, a 2μm *URA3* yeast genomic library was screened for genes that, when overexpressed, suppress the Δ*cpr7* slow-growth phenotype. We screened ~55,000 Ura^+ transformants on synthetic medium lacking uracil and identified eight potential suppressors. The plasmids containing the putative suppressor clones were rescued from the Δ*cpr7* mutant strain, amplified in *E. coli*, and retransformed into both a *cpr7* single-mutant and a *cpr6 cpr7* double-mutant strain. Seven of the eight rescued plasmids complemented the slow growth of both the *cpr7* single and *cpr6 cpr7* double-mutant strains and were further analyzed. By subcloning and sequencing, we determined that one of these clones contained the *CPR7* gene, as expected. The remaining six clones were overlapping genomic sequences; all contained YBR155w, a previously uncharacterized open reading frame with moderate homology to *STI1* (20% identity and 39% similarity [Fig. 5]). Another group has also independently identified YBR155w in a similar Δ*cpr7* suppressor screen (25a), and this open reading frame has been named *CNS1*, for cyclophilin seven suppressor.

Cns1 is an essential Sti1/p60 homolog that is not induced by heat shock. The *CNS1* gene was amplified by PCR and cloned in YEplac195, a 2μm *URA3* vector; the resulting plasmid carrying the *CNS1* gene alone was able to complement the slow-growth and geldanamycin-sensitive phenotypes of the Δ*cpr7* single-mutant and Δ*cpr6* Δ*cpr7* double-mutant strains. The

```
Sti1      1 MSLTADEYKQQGNAAFTAKDYDKAIELFTKAIEVSETPNHVLYSNRSACY  50
Cns1
Sti1     51 TSLKKFSDALNDANECVKINPSWSKGYNRLGAAHLGLGDLDEAESNYKKA 100
Cns1      1              MSSVNANGGYTK-------------------------  12
Sti1    101 LELDASNKAAKEGLDQVHRTQQARQAQPDLGLTQLFADPNLIENLKKNPK 150
Cns1     13 ---------------------------P--------------QKYVPGP-  20
Sti1    151 TSEMMKDPQLVAKLIGYKQNPQAIGQDLFTDPRLMTIMATLMGVDLNMDD 200
Cns1     21 -----GDPELPPQLSEFK-------------------------DKTSDE  39
Sti1    201 INQSNSMPKEPETSKSTEQKKDAEPQSDSTTSKENSSKAPQKEESKESEP 250
Cns1     40 ILK--EMNRMPFFMTKLDETDGAGG--------ENVELEALKALAYEGEP  79
Sti1    251 MEVDEDDSKIEADKEKAEGNKFYKARQFDEAIEHYNKAWELHKD-----I 295
Cns1     80 HEIAEN--------FKKQGNELYKAKRFKDARELYSKGLAVECEDKSINE 121
Sti1    296 TYLNNRAAAEYEKGEYETAISTLNDAVEQGREMRADYKVISKSFARIGNA 345
Cns1    122 SLYANRAACELELKNYRRCIEDCSKALTINPKNVKCYYRTSKAFFQLN-- 169
Sti1    346 YHKLGDLKKTIEYYQKSLTEHR--TADILTKLRNAEKELKKAEAEAYVNP 393
Cns1    170 --KLEEAKSAATFANQRIDPENKSILNMLSVIDRKEQELKAKEEKQQR-- 215
Sti1    394 EKAEEARLEGKEYFTKSDWPNAVKAYTEMIKRAPEDARGYSNRAAALAKL 443
Cns1    216 --EAQERE-NKKIMLESAMT--LRNITNIKTHSPVELLNEG--KIRLEDP 258
Sti1    444 MSFPEAIADCNKAIEKDPNFVRAYIRKATAQIAVKEYASALETLDAARTK 493
Cns1    259 MDFESQLIYP-ALIMYPTQDEFDFVGEVSELTTVQELVDLVLEGPQERFK 307
Sti1    494 DAEVNNGSSAREIDQLYYKASQQRFQPGTSNETPEETYQRAMKDPEVAAI 543
Cns1    308 KEGKENFTPKKVLVFMETKAG------GLIKAGKKLTFHDILKKESPDVP 351
Sti1    544 MQDPVMQSILQQAQQNPAALQEHMKNPEVFKKIQTLIAAGIIRTGR 589
Cns1    352 LFDNALKIYIVPKVESEGWISKWDKQ-KALERRSV             385
```

FIG. 5. Alignment of Sti1 and Cns1 protein sequences by the Clustal method (MacVector software). Identical residues are marked with asterisks; similar residues are marked with periods.

CNS1 open reading frame was replaced by the G418 resistance gene in a wild-type diploid strain. The resulting heterozygous *CNS1*/Δ*cns1::G418* strain was sporulated and dissected, and 18 of 19 tetrads yielded two viable and two inviable segregants (a representative sample is shown in Fig. 6). All of the viable segregants were found to be G418 sensitive, consistent with the cosegregation of lethality and the Δ*cns1::G418* allele (data not shown).

The *CNS1*/Δ*cns1::G418* heterozygous diploid was trans-

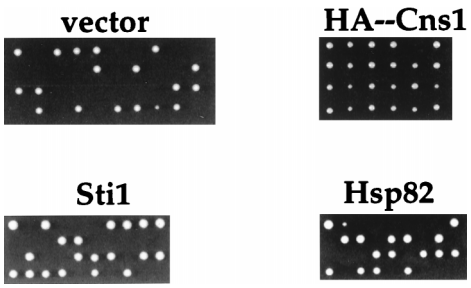

FIG. 6. *CNS1* is an essential gene. A *CNS1*/Δ*cns1::G418* diploid strain was transformed with high-copy-number 2μm plasmids alone (vector) or containing the HA epitope-tagged *CNS1*, wild-type *STI1*, or wild-type *HSP82* gene. The transformed diploid strains were then sporulated and dissected; representative samples of the segregants are illustrated.

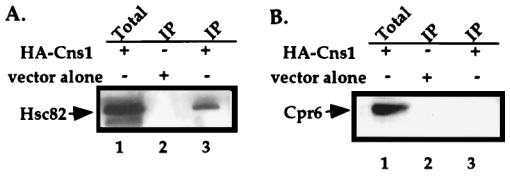

FIG. 7. The yeast Hsp90 protein Hsc82 and the p60/Sti1 homolog Cns1 are present in protein-protein complexes. Cellular lysates were incubated with anti-HA antibodies coupled to Sepharose beads, washed four times in lysis buffer, and then analyzed by Western blotting with antibodies to Hsc82 (A) or Cpr6 (B). Lanes: 1, total-cell extract control; 2, wild-type extract (immunoprecipitate [IP]) containing the empty plasmid pYeF1; 3, wild-type extract (IP) expressing the HA-tagged Cns1 from plasmid pYeF1.

formed with a plasmid containing the wild-type *CNS1* and *URA3* genes, sporulated, and dissected. The majority of the tetrads showed four viable and no inviable segregants (Fig. 6), which consisted of two G418-sensitive, 5-fluoro-orotic acid-resistant and two G418-resistant, 5-fluoro-orotic acid-sensitive segregants (data not shown). These findings indicate that *CNS1* is an essential gene and that reintroduction of the wild-type *CNS1* gene restores viability in the Δ*cns1::G418* mutant strain. Overexpression of *CPR7*, *STI1*, *HSP82*, *HSC82*, *CDC23*, *UBC4*, *PPT1*, or *SUN2* did not restore viability of the Δ*cns1::G418* mutant (Fig. 6 and data not shown). In addition, overexpression of *CNS1* did not suppress the conditional synthetic lethality exhibited by a Δ*sti1* mutation in combination with an *hsp82* mutation (data not shown).

It was previously shown that *STI1* is induced by heat shock (39). We examined *CNS1* gene expression during heat shock by Northern analysis and found that transcription of the *CNS1* gene was not induced at elevated temperatures (data not shown).

Cns1 is in protein complexes containing Hsc82 and Cpr7 but not Cpr6. To examine physical interactions between Hsp90, Cns1, and cyclophilin 40, Cns1 was tagged with the HA epitope at its amino terminus (see Materials and Methods). The HA-tagged form of Cns1 complemented the lethality of a Δ*cns1::G418* null mutant (Fig. 6). Total-cell lysate was prepared from a wild-type yeast strain containing the HA-tagged Cns1. Cns1 was then immunoprecipitated with anti-HA antibodies coupled to Sepharose beads, and immunoprecipitates were analyzed by Western blotting. Hsc82 coimmunoprecipitated with the HA-Cns1 protein (Fig. 7A), indicating that Cns1 and Hsc82 are present in protein-protein complexes and may directly interact. This observation and interpretation would be in accord with previous findings that Hsp90 is directly physically associated with the p60/Sti1 protein that shares sequence identity with Cns1. In contrast to Hsc82, the Cpr6 protein was not present in Cns1 immunoprecipitates (Fig. 7B). This observation would again be in accord with previous observations that Hsp90 can exist in distinct complexes with p60/Sti1 and Cpr6 in yeast (9) and mammalian cells (41, 44).

To examine interactions between cyclophilin 40, Cns1, and Cpr7 by a different approach, Cpr7 was fused to GST and expressed in bacteria, and GST-Cpr7 was adsorbed to glutathione-Sepharose beads. The resulting Cpr7 affinity matrix was then incubated with yeast total-cell extracts containing HA-Cns1. Interestingly, the HA-Cns1 protein interacted with the GST-Cpr7 fusion protein (Fig. 8A and B). Because Cpr6 was not detected in the Cns1 immunoprecipitate, this finding suggests that Cpr7 is distinguished from Cpr6 by its ability to interact, directly or indirectly, with the Cns1 protein. Western blot analysis revealed that Hsc82 was also specifically bound to the GST-Cpr7 affinity matrix (Fig. 8C), in accord with previous

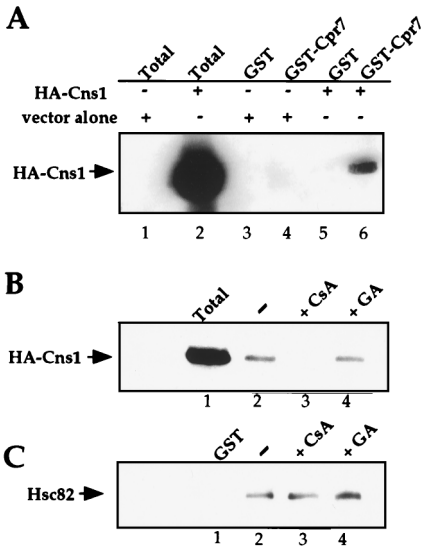

FIG. 8. The yeast p60/Sti1 homolog Cns1 and the cyclophilin 40 homolog Cpr7 are present in protein-protein complexes. (A) Cellular lysates containing HA-Cns1 protein were incubated with GST-Cpr7 Sepharose beads (lanes 4 and 6) or GST beads alone (lanes 3 and 5), washed four times in lysis buffer, and analyzed by Western blotting with anti-HA antibodies to detect the HA-Cns1 protein. Lanes: 1 and 2, total cell extracts; 3 and 4, wild-type extracts containing the control plasmid pYeF1; 5 and 6, wild-type extract expressing the HA-tagged Cns1 protein from plasmid pYeF1. (B) Cellular lysates were incubated with GST-Cpr7 beads in the absence of drug (lane 2) or in the presence of 20 μM CsA (lane 3) or 50 μM geldanamycin (GA; lane 4), washed four times, and analyzed by Western blotting to detect HA-Cns1. Lane 1 contains total cell extract. (C) Cellular lysates were incubated with GST beads alone (lane 1) or with GST-Cpr7 beads in the absence of drug (lane 2) or the presence of 20 μM CsA (lane 3) or 50 μM geldanamycin (GA; lane 4), washed four times, and analyzed by Western blotting with mouse polyclonal antisera directed against the yeast Hsc82 protein.

findings (16). Interestingly, CsA disrupted the Cpr7-Cns1 interaction (Fig. 8B), suggesting that the cyclophilin domain of Cpr7 may participate in binding of Cns1 to Cpr7. CsA did not inhibit Hsc82 binding to Cpr7 (Fig. 8C), in accord with previous findings that the TPR domain of Cpr7 is sufficient for binding to Hsc82 (16). Finally, the Hsp90 inhibitor geldanamycin had no effect on binding of either HA-Cns1 or Hsc82 to the GST-Cpr7 affinity matrix (Fig. 8B and C).

DISCUSSION

In this study, we have further analyzed the structures and functions of components of the Hsp90-associated chaperone machinery. We performed a structure-function analysis of the yeast cyclophilin 40 homologs Cpr6 and Cpr7 that revealed the conserved TPR domain of Cpr7 is critical for function, demonstrated that yeast mutants lacking Cpr7 are hypersensitive to the Hsp90 inhibitor geldanamycin, and identified Cns1, a novel essential p60/Sti1 homolog that associates with Hsp90 and Cpr7.

The cyclophilin 40 proteins of yeast and mammals contain two conserved domains, an amino-terminal cyclophilin prolyl isomerase domain and a carboxy-terminal TPR domain (15–17, 57). We have found that the TPR domain of Cpr7 is critical for in vivo function, whereas the cyclophilin domain is largely dispensable. The TPR domain is known to be the critical domain for cyclophilin 40-Hsp90 interactions in both yeast and mammals (16, 41, 44). Because the TPR domain can complement in vivo and is the critical domain for Hsp90 interactions, Cpr7 and Hsp90 likely interact under normal physiological conditions via the Cpr7 TPR domain. While this report was in

preparation, another group reported similar findings that the Cpr7 TPR domain is important for function (18).

We also find that yeast mutants lacking the cyclophilin 40 homolog Cpr7 are uniquely hypersensitive to the antitumor agent geldanamycin. Given previous studies that *cpr7* and the yeast *hsp90* homologs genetically interact and that geldanamycin binds to and perturbs Hsp90 function in mammalian cells and in yeast (1, 19, 26, 48, 55, 59), our findings provide additional evidence that Hsp90 function is compromised in *cpr7* mutant strains. In addition, our findings open the door to a genetic dissection of geldanamycin action in yeast. Previous studies have revealed that Hsp90 and its associated partner proteins have been conserved, both in structure and in function, from yeast to mammals (3, 8, 9, 25, 39, 42); thus, our findings should be generally applicable to understanding Hsp90, cyclophilin 40, and p60/Sti1 functions in mammalian systems.

Our studies have also identified a previously uncharacterized open reading frame as a multicopy suppressor of the Δ*cpr7* mutation. The product of this suppressor gene, Cns1, shares limited sequence identity with Sti1, the yeast homolog of the mammalian p60 protein, which genetically and physically interacts with the yeast Hsp90 homologs. We have shown that Cns1 is found in protein complexes that contain Hsc82 and Cpr7. Others have shown that the Cns1 homologs, Sti1 in yeast and p60 in mammals, are also components of Hsp90 complexes (9, 53). It has also been shown that p60 and cyclophilin 40 are present in distinct complexes with Hsp90. p60 and cyclophilin compete, via their TPR domains, for Hsp90 binding and do not bind to each other (16, 41, 44). We have found, however, that the yeast Cns1 protein is present in complexes that contain the Cpr7 cyclophilin 40 homolog but not the Cpr6 cyclophilin. There are several different interpretations and implications of this result. First, there may subtle differences between the constitution of yeast and mammalian Hsp90 complexes. Studies that support a similarity between the protein content of yeast and mammalian Hsp90 complexes examined the presence of only Cpr6 and Sti1 (9). Second, because our experiments involved incubation of yeast extracts containing HA-Cns1 with bacterially expressed Cpr7 protein, Cns1 and Cpr7 need not directly interact and could, for example, be present in a ternary Cns1-Hsc82-Cpr7 complex in which Cns1 and Cpr7 are not in direct contact. However, our finding that cyclosporin A inhibits formation of Cpr7-Cns1 complexes, but not of Cpr7-Hsc82 complexes, suggests that Cpr7 directly interacts with both Cns1 and Hsc82. Finally, although the p60 homologs Sti1 and Cns1 are related, the level of sequence identity is low, the two genes are differentially regulated, and Cns1 is essential whereas Sti1 is not. Thus, Cns1 may have functions quite distinct from those of Sti1 that could involve direct protein-protein interactions with both Hsp90 and the cyclophilin 40 homolog Cpr7, whereas p60 and Sti1 have evolved to compete with cyclophilin 40 homologs for Hsp90 binding. Further study will be required to address these issues in detail.

Several of the proteins in Hsp90 complexes are encoded by two differentially regulated genes. For instance, the yeast homologs of Hsp90 (*HSC82* and *HSP82*), Hsp70 (*SSA1*, *SSA2*, *SSA3*, and *SSA4*), cyclophilin 40 (*CPR6* and *CPR7*), and p60 (*STI1* and *CNS1*) are each encoded by at least two genes that are regulated differently at the transcriptional level, with one partner constitutively expressed and the other induced by heat shock (3, 38, 39, 57). Perhaps under normal conditions, expression of the constitutively expressed homolog is sufficient for physiological functions but growth at elevated temperatures requires higher levels of protein. Thus, it is more efficient to induce transcription of just one homolog. This is likely to be the case for the Hsp82-Hsc82 pair, which share 97% identity at the amino acid level and have seemingly overlapping functions. For more divergent sets such as Cpr6-Cpr7 (41% identity) and Sti1-Cns1 (20% identity and 39% similarity), the homologs may have partially overlapping but also unique functions. It is interesting that for the cyclophilin 40 and p60 homologs, the constitutively expressed gene has the more dramatic phenotype when mutated compared to the effects of mutating the heat-regulated homolog. One hypothesis consistent with these observations is that the stress-regulated homolog is less important during normal growth conditions. For instance, normally the constitutively expressed protein may have very transient interactions but under stressed conditions, chaperone-like interactions may persist, requiring a larger pool of protein; thus, the stress-regulated protein is induced. Alternatively, perhaps the range of substrates is broadened under stress conditions, which would also require an increase in chaperone protein levels. Examining Hsp90 complexes under stressed conditions by using reagents that detect specific homologs would help to address these alternative hypotheses.

Although our multicopy suppressor screen was exhaustive, just one suppressor of the Δ*cpr7* mutant phenotype was identified. Potential targets of the Hsp90 complex, however, were not identified in this high-copy-number suppressor screen. Perhaps targets were not isolated because there may be several, critical substrates for the Hsp90 complex, and overexpression of any one is not sufficient to restore a normal level of growth in the Δ*cpr7* mutant strain.

Possible functions and targets of cyclophilin 40 homologs have recently been identified by other studies. First, the *Schizosaccharomyces pombe* cyclophilin 40 homolog Wis2 was identified as a multicopy suppressor of a *cdc25 wee1 win1* triple mutant, suggesting that the Wis2 cyclophilin may be involved in progression from the G_2 phase to mitosis (58). Second, mammalian cyclophilin 40 has been shown to bind to and negatively regulate DNA binding by the c-Myb transcription factor (34). While the in vivo significance of this observation remains to be explored, an interesting finding was that the cyclophilin domain was required for inhibition of c-*myb* DNA binding activity; this is in contrast to our finding that the Cpr7 cyclophilin domain is not critical for in vivo function in yeast but may be in accord with our observation that the cyclophilin domain may be involved in high-affinity binding of Cpr7 to Cns1. Finally, in the cases of both Wis2 and c-Myb, a role for Hsp90 or other Hsp90-associated proteins remains to be elucidated.

Why does overexpression of the *CNS1* gene suppress the *cpr7* mutation? One model is that *CNS1* overexpression makes formation of an initial Hsp90 complex (Hsp70-Hsp90-Cns1) more efficient. Alternatively, when overexpressed, Cns1 may substitute for Cpr7 in the mature Hsp90 complex (Hsp90-p23-immunophilin). Finally, our findings suggest that Cpr7 and Cns1 may be present simultaneously in the same Hsp90 complexes, and thus overexpression of one component might compensate for the loss of a different component of the complex. It is especially intriguing that while the components of the Hsp90 complex are duplicated, some components are significantly divergent. In this regard, Cns1 is quite divergent from its homolog Sti1, and further studies will be required to further address the unique or shared features of these distinct Hsp90-associated components.

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