Hsp90 complexes contain a class of co-chaperones characterized by a tetratricopeptide repeat (TPR) domain, which mediates binding to a carboxyl-terminal EEVD region in Hsp90. Among Hsp90 TPR co-chaperones in *Saccharomyces cerevisiae*, only Cns1 is essential. The amino terminus of Cns1, which harbors the TPR domain, is sufficient for viability when overexpressed. In a screen for temperature-sensitive alleles of CNS1, we identified mutations resulting in substitutions of conserved residues in the TPR domain. Mutations in *CNS1* disrupt *in vitro* and *in vivo* interaction with Hsp90 and reduce Hsp90 function, indicating that Cns1 is a bona fide co-chaperone. Genetic interactions between CNS1 and another Hsp90 co-chaperone, CPR7, suggest that the two co-chaperones share an essential role in the cell. Although both the TPR and the isomerase domains of the cyclophilin Cpr7 are required for viability of *cns1* mutant cells, this requirement does not depend on the catalytic function of the isomerase domain. Instead, hydrophilic residues on the surface of this domain appear to be important for the common Cns1-Cpr7 function. Although both co-chaperones interact with Hsp90 primarily through the carboxyl terminus (EEVD), Cns1 and Cpr7 are mostly found in complexes distinct from Hsp90. EEVD is required for normal growth in *cns1* mutant cells, demonstrating for the first time in *vivo* requirement for this conserved region of Hsp90. Overall, our findings reveal a considerable degree of complexity in the interactions not only between Hsp90 and its co-chaperones, but also among the co-chaperones themselves.

Heat shock protein 90 (Hsp90) is an abundant molecular chaperone that is conserved from prokaryotes to humans and is essential in eukaryotes (reviewed in Refs. 1–3). The number of Hsp90 substrates identified to date is large and, although they differ greatly in structure and function, most appear to be proteins involved in signal transduction (reviewed in Ref. 4).

How Hsp90 regulates a vastly diverse group of client proteins remains an important question. In part, this versatility can be attributed to the assistance of co-chaperone proteins that modulate the role of Hsp90 in *vivo*. These co-chaperones impinge on several properties of Hsp90, including its ATPase activity, physical conformations, interaction with substrates, substrate specificity, and subcellular localization (5–11). Thus, a clear understanding of how Hsp90 function requires investigation of the roles played by its co-chaperones.

In *Saccharomyces cerevisiae*, Hsp90 is encoded by two genes, *HSP82* and *HSC82*, either of which is sufficient for viability (12). The largest subset of Hsp90 co-chaperones is characterized by the presence of tetratricopeptide repeat domains, which mediate binding to Hsp90. TPR-containing co-chaperones were first identified in Hsp90-steroid receptor complexes (reviewed in Ref. 13). One of these, Hop (Sti1 in *S. cerevisiae*) can bridge Hsp90 and another essential chaperone, Hsp70, via two sets of TPR domains (14, 15). Two classes of large TPR-containing immunophilins have also been identified in Hsp90 complexes: FK506-binding proteins FKBP51 and FKBP52, and cyclophilin CyP40, which binds cyclosporin A (CsA) (16, 17). In addition to a carboxyl-terminal TPR domain, both immunophilin groups contain peptidyl prolyl isomerase (PPIase) domains in the amino termini. Although *S. cerevisiae* does not possess orthologs of the large FKBP5, it contains two CyP40 relatives: CPR6 and CPR7 (18, 19). Another protein found in Hsp90 complexes, Cns1, is essential for viability and in this regard unique among the TPR co-chaperone family (20, 21).

Several studies have addressed the roles played by TPR co-chaperones with regard to *in vivo* function of Hsp90. Assays that measure the activity of heterologous Hsp90 substrates such as maturation of the glucocorticoid receptor (GR) revealed the requirement for CPR7 and STI1 for full Hsp90 activity (22, 23). STI1 and CPR7 are also required for normal growth; the functional relationship between these co-chaperones is revealed by the severe growth defect of cells containing null alleles of both genes (22).

The tetratricopeptide repeat domains in these co-chaperones consist of a tandem array of three repeat units, each of which forms a pair of anti-parallel α-helices (24). Conserved amino acids at specific positions within the TPR units define the TPR fold, whereas other residues line an internal groove formed by the overall structure and make direct contacts with the EEVD sequence found at the end of the Hsp90 carboxyl terminus (15, 25). Surprisingly, although EEVD is conserved in all Hsp90 homologs, the deletion of this sequence does not affect growth of *S. cerevisiae* cells (26). This finding brings into question the importance of the Hsp90 carboxyl terminus *in vivo* and suggests either that there is an alternative mode of interaction.
between Hsp90 and TPR co-chaperones or that their physical association is not association.

The role of immunophilin PPIase domains in Hsp90 function also remains unclear. It is possible that the catalytic activity these proteins show in vitro (21, 28) is involved in protein folding and may even be important for the mechanism of Hsp90 action on some substrates. However, a mutant form of the yeast cyclophilin Cpr7 in which the PPIase domain has been deleted supports full Hsp90 activity and normal growth (29).

A functional relationship between Cpr7 and Cns1 was first revealed by the discovery that overexpression of CNS1 can suppress both the slow growth phenotype and decreased Hsp90 activity that result from deletion of CPR7 (20, 21). Although Cns1 contains a three-unit TPR domain related to those found in Hsp90-associated immunophilins, a direct interaction between Cns1 and Hsp90 has not been demonstrated prior to this study. Furthermore, Cns1 can associate with Cpr7 (20, 21), although whether this interaction is direct or whether it requires Hsp90 is unclear. In addition, the full scope of Hsp90 functions in which these co-chaperones participate remains unknown.

In this study, we have taken different approaches to investigate the function of Cns1 in general and its role in Hsp90 complexes in particular. We isolated temperature-sensitive alleles of CNS1 and assessed their effects on Hsp90 activity and Hsp90-Cns1 physical interactions. We have also more fully explored the functional relationship between Cns1 and Cpr7 by examining genetic interactions between cpr7 and cns1 mutants. Our studies reveal that in cells with compromised Cns1 function the PPIase domain of Cpr7 and the EEVD Hsp90 carboxy terminus are essential for viability.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Growth Conditions**—Standard genetic techniques (3) and growth media were used in the study. Yeast transformations were performed using the lithium acetate method (30), and bacterial transformations by electroporation were done using Gene Pulser (Bio-Rad). Plasmids expressing mutant forms of GST-Cns1, i.e. pgst-Cns1G90D (pMT142), pGST-Cns1 C140R (pMT140), and pGST-Cns1 3C (pMT138), were made by ligation of mutant plasmids into pESC-LEU vector (Stratagene). For the experiment shown below in the Fig. 8, 200 ng of mutagenic PCR reaction products. Conditions used for mutagenic PCR were the following: 10 ng of pJM105 template, 100 ng of each primer, 0.4 μM MnCl₂, 0.25 μM normal dNTPs, 0.05 μM lower dNTP, and buffer (10 μM Tris-HCl, pH 8.3, 50 μM KCl, 1.5 μM MgCl₂, 0.01%). Primers were designed to amplify the entire Cpr7 ORF from start to 603 bp and Cpr6 ORF from 538 bp to the end and ligation into the pESC-LEU vector (Stratagene). For the experiment shown below in the Fig. 8, pCpr7 was expressed from the pMT194 vector. The experiment was done with pMT105 and pMT175, respectively. A plasmid expressing GAL-driven FLAG-tagged Cpr7 (pMT194) was made by PCR amplification of fragments of CPR7 ORF from start to 603 bp and Cpr6 ORF from 538 bp to the end and ligation into the pESC-LEU vector (Stratagene). For the experiment shown below in the Fig. 8, a plasmid expressing Cns1 3HA-tagged at the amino terminus under control of the galactose-driven promoter, was the gift of J. Heitman (Duke University Medical Center, Durham, NC) (20). pTCA/Hsp82, used to exchange wild-type Hsp82 for Hsp82 and, for the experiment shown below in the Fig. 8, was the gift of D. Picard (Université de Genève Sciences III, Genève, Switzerland) (33). pYeF1 was the gift of L. Minville-Sebastia (Institute de Biochimie et Génétique Cellulaires, Bordeaux 2 Université, Bordeaux, France) (34).

The nuclear Hsp90 co-chaperones Cpr7, Cpr6, and Cns1 (pMT161), pURA3-GPD-Gal82-Hsp82::ADH1-Hsp82 (pMT179) was made by replacing codon 705 in pURA3-GPD-Gal82-Hsp82 (isolated from pGDP-HSP82FP cells) with a stop codon using site-directed mutagenesis (Stratagene). A plasmid encoding the wild-type was replaced by pMT179 in GPD: HSP82FP to generate AS5 strain.

pSC1 was used for overexpression of Cpr7 under the control of endogenous promoter in a multicopy URA3-marked plasmid. Plasmids expressing Cpr7 and Cpr7ΔTRP1 under control of the endogenous promoter have essentially been described previously (29) except that the selectable marker was replaced with LEU2, resulting in plasmids pMT160 and pMT175, respectively. A plasmid expressing GAL-driven FLAG-tagged Cpr7 (pMT194) was made by PCR amplification of fragments of CPR7 ORF from start to 603 bp and Cpr6 ORF from 538 bp to the end and ligation into the pESC-LEU vector (Stratagene). For the experiment shown below in Fig. 8, pCpr7 was expressed from pMT184, which contains a TRP1 instead of a LEU2 selectable marker. Cpr6PP-Cpr7PP chimera (pMT204) is expressed from a pRS425-based vector, which contains bp 1–600 of the CPR6 ORF and bp 603–1191 of CPR7 ORF under control GAL promoter. pCpr7PP-Cpr7ΔTRP1 (pMT200) was made by PCR amplification of fragments of CPR7 ORF from start to 603 bp and Cpr6 ORF from 538 bp to the end and ligation into the pESC-LEU vector. pCpr7PPΔ40A (pMT203) and pCpr7PPΔ40T, ΔN50 (pMT188) were made by site-directed mutagenesis of pMT160. Normal expression levels of the wild-type and mutant forms of Cpr7 were confirmed by Western blotting.

**Screen for Temperature-sensitive Alleles of CNS1—**JMY84 cells containing pm105 were co-transformed with 50–100 ng of linearized pRS314 and 100–200 ng of mutant genomic PCR reaction products. Conditions used for mutagenic PCR were the following: 10 ng of pJM105 template, 100 ng of each primer, 0.4 μM MnCl₂, 0.25 μM normal dNTPs, 0.05 μM lower dNTP, and buffer (10 μM Tris-HCl, pH 8.3, 50 μM KCl, 1.5 μM MgCl₂, 0.01%). Primers were designed to amplify the entire CPR7 ORF and pF100 400 bp of the promoter and –200 bp of vector sequences flanking the gene. Transformants were selected on the medium lacking uracil and theophylline. Colonies were grown for 3 days and replicated onto medium containing 5-FOA at 23 and 37 °C. DNA was isolated from the colonies that grew at 23 °C but not at 37 °C, transformed into Escherichia coli DH5α, and re-introduced into the original
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yeast strain. These plasmids were sequenced to determine mutations responsible for the phenotype.

Screen for Multicopy Suppressors of cns1Δ Alleles—cns1-1 cells were transformed with a S. cerevisiae genomic library prepared using multicity URA3-marked plasmids (gift of P. Hieter, University of British Columbia, Vancouver, Canada) and grown on plasmid-selective medium at permissive temperature (23 °C). After 3 days, the cells were replicated plated onto plasmid-selective medium at 23 and 37 °C and incubated for 3–5 days. Plasmid dependence of the ability to grow at the non-permissive temperature was confirmed by streaking these cells on 5-FOA. Plasmid DNA was isolated from cells expressing suppressed phenotype, transformed into E. coli DH5α, and re-introduced into cns1-1 recipients. Genomic inserts present were identified by sequencing.

Assays of β-Galactosidase Activity (GRE, HSE, and FUS1)—MTY189 or JMY484 cells containing wild-type and mutant CNS1 on TRP1-marked plasmids were transformed with the appropriate reporter plasmids. β-Galactosidase activity was measured as described previously (22, 35) at 23 and 37 °C.

Protein Interaction Assays—Lysis of yeast cells was performed essentially as described previously (21), except that 1 mM dithiothreitol was omitted in preparing lysates that were used for purification on Ni-NTA beads (Qiagen).

Protein Concentrations Were Determined using the Bio-Rad Protein Assay—Precipitations using GST fusion proteins were performed as described previously (21, 22). BL21-CodonPlus®(DE3)-RIL pLexS E. coli cells (Stratagene) were used in all experiments except for the one shown in Fig. 1a.

GPD:HSP82FP or AS5 cells were used to analyze endogenous yeast Hsp90 complexes. These experiments were performed as described previously (21), except that additional washes were performed with a buffer containing 20 mM imidazole and that 200 mM imidazole was used for elution of Hsp90 complexes. Laemmli sample buffer (36) was added to the eluted fractions without precipitation with trichloroacetic acid, and samples were frozen prior to analysis by SDS-PAGE.

Recombinant His6-Hsp90 was used to test the interactions between Hsp90 and truncated forms of Cns1. In this experiment, E. coli lysate prepared as above was bound to Ni-NTA-agarose (Qiagen) for 30 min, washed three times with phosphate-buffered saline, and subsequently bound to E. coli lysates expressing GST fusions of truncated and full-length Cns1 for 1 h at 4 °C. The agarose was washed with phosphate-buffered saline five times, and bound proteins were eluted from Ni-NTA using 150 mM imidazole as described above.

Western Blot Analysis—Protein samples were separated on 10% SDS-PAGE gels, which were either stained with Coomassie Blue or transferred onto Protran® nitrocellulose membranes (Schleicher & Schuell). Equal amounts of loaded sample and efficient transfer were confirmed by staining with Ponceau S (Sigma). ECL (Amerham Biosciences) was used according to the manufacturer’s protocol. The following antibodies were used for immunoblotting: GST (Amerham Biosciences), HA (12CA5, BAbCO, Inc.), FLAG (M2, Kodak), Hsp90 (gift of D. Toft, Mayo Graduate School, Rochester, MN), and Cns1 (this study). Cns1 antibody was raised in rabbits according to the following procedure. 3 liters of logarithmically growing E. coli cells, centrifuged at 14,000 and 20,000 rpm in an An-60 Ti rotor, and allowed to reach equilibrium. Data sets were fitted individually and simultaneously using software packages that employ a non-linear least squares method: WinNonLin 1.050, Sednterp 1.01, and Nonlin 1.01. The specific partial volume was 0.7362 ml/mg, and the viscosity of the buffer (50 mM sodium phosphate, 150 mM NaCl, pH 7.0) was 1.1090 mg/ml. Two models for fitting were used: the first one assumed an ideal solution of a single species, and the second one used monomer-dimer equilibria with a fixed monomer molecular weight of Cns1 of 44,107 (determined from the amino acid sequence).

GeI Filtration—200 ml of logarithmically growing cells expressing His10-Hsp92 or His6-Hsp82 were harvested and lysed in the equilibration buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol) as described above. Lysates were centrifuged twice in the TL-100 Ultracentrifuge (Beckman) at 100,000 × g, 4 °C for 30 min. A Superdex-200 column (Amersham Biosciences) was washed in equilibration buffer. 1.5 mg of total protein was resolved on the column, and 0.5-ml fractions were collected using BioTec Liquid Chromatography Collector LCC-500 (Amersham Biosciences). Collected fractions were analyzed by SDS-PAGE and Western blotting as described above. The following molecular mass standards were used (Sigma): thyroglobulin (669 kDa), apoferritin (443 kDa), ovalbumin (45 kDa), and carbonic anhydride (29 kDa).

Requirement for the Amino Terminus of Cns1—The TPR domain of Cns1 is located in the amino terminus and possesses characteristics of other Hsp90-interacting TPR domains (15).

To determine if the interaction between Cns1 and Hsp90 is direct, recombinant yeast proteins were used. His8-Hsp82 was purified using the nickel affinity matrix and incubated with bacterial lysates containing full-length or truncated Cns1 proteins fused to the GST moiety. Both the full-length Cns1 and the amino-terminal domain (amino acids 1–212) interacted with Hsp90, whereas the carboxyl terminus (amino acids 213–385) did not (Fig. 1a). A further truncation of 80 amino acids from the amino terminus, leaving just the TPR domain (amino acids 81–212), disrupted binding to Hsp90. In other Hsp90 binding partners, including Cyp40, FKBP51, and FKBP52, regions adjacent to the TPR domains are required for interaction with Hsp90 (37, 38). Thus, Cns1 interacts with Hsp90 directly, and in a manner similar to other TPR-containing co-chaperones.

Overexpression of the Cns1 amino terminus (amino acids 1–212) was sufficient to suppress cns1Δ lethality (Fig. 1b).

Generation of Temperature-sensitive Alleles of CNS1—To investigate its in vivo functions, we performed a screen for conditional alleles of CNS1. DNA encompassing the CNS1 open reading frame was randomly mutagenized by PCR and co-transformed with a linearized vector into a cns1Δ recipient (see “Experimental Procedures” for details). Several temperature-sensitive alleles of CNS1 were obtained; this study focuses on those presented in Fig. 2a. Although growth at elevated temperature (37 °C) was weaker than at lower temperatures, the portion of Cns1 that interacts with Hsp90 is sufficient for viability under normal growth conditions.

Sedimentation equilibrium experiments were conducted at 16 °C in a Beckman Optima XL-I analytical ultracentrifuge. Six samples of purified Cns1 were prepared, resulting in A230 values of 0.15, 0.2, 0.3, 0.5, 0.8, and 1.0. Approximately 100 μl of each sample was loaded into 1.2-cm path length cells, centrifuged at 14,000 and 20,000 rpm in an
tions in tetratricopeptide repeats have been reported to impact functions of other TPR proteins (39–41).

Effect of CNS1 Mutations on Hsp90 Function—Cells in which CPR7 is deleted exhibit decreased Hsp90 function (22, 35), and overexpression of Cns1 in these cells largely restores wild-type Hsp90 activity (21). To determine if Cns1 plays a basic role in Hsp90 function, the effect of geldanamycin on the growth of cns1 cells was tested. GA is a specific inhibitor of Hsp90 (42); cells lacking certain Hsp90 co-chaperones exhibit hypersensitivity to GA (20, 43, 44). In the presence of 35 μM GA, growth of cns1 cells was severely impaired, whereas wild-type cells were unaffected (Fig. 3a).

Maturation of glucocorticoid receptor is a commonly used assay for the ability of Hsp90 complexes to function properly in yeast cells (45). To examine the role of Cns1 in the maturation of GR, a reporter plasmid was used that expresses mammalian GR under the control of a constitutive promoter and in which glucocorticoid response elements were fused to lacZ. The activity of the reporter was measured after treatment with deoxycorticosterone at the permissive temperature (23 °C); β-galactosidase activity was quantified as described under “Experimental Procedures.” Each bar represents the mean ± S.D. from a minimum of five independent measurements. Indicated values were calculated relative to those of wild-type cells (column on the left) and represent percent of wild-type activity.

FIG. 1. **The amino terminus of Cns1 binds directly to Hsp90 and is sufficient for viability.** a, bacterial lysates from cells expressing His6-Hsp82 were purified on the Ni-NTA resin and incubated with E. coli lysates expressing GST fusion proteins indicated. Proteins eluted from Ni-NTA were analyzed by SDS-PAGE and visualized by Coomassie Blue staining (top panel) or by Western blotting using an antibody against GST (bottom panel). Binding of GST-Cpr7 is presented for comparison. b, serial dilutions of cns1 cells (JMY84h) harboring URA3-marked plasmids expressing galactose-inducible full-length (pGAL-Cns1) or truncated (pGAL-Cns1AC) forms of Cns1 were spotted on medium containing galactose at 23 and 37 °C to test the ability of these plasmids to suppress the lethality of the cns1Δ cells.

FIG. 2. **Temperature-sensitive mutants of Cns1.** Temperature-sensitive cns1Δ mutants were obtained from a genetic screen described under “Experimental Procedures.” a, representation of the amino acid changes encoded by the cns1 mutant alleles discussed in this work. b, serial dilutions of cns1Δ cells expressing either wild-type or temperature-sensitive alleles of CNS1 on centromeric plasmids were incubated at permissive (23 °C) and non-permissive (37 °C) temperatures on synthetic medium for the length of time indicated.

FIG. 3. **Mutations in CNS1 impair Hsp90-dependent function.** a, effect of cns1Δ mutations on the sensitivity to the Hsp90 inhibitor, geldanamycin. Dilutions of cells expressing wild-type or temperature-sensitive alleles of CNS1 were incubated on YPD medium containing either Me2SO alone (DMSO), or Me2SO and GA at the concentration of 35 μM. Cultures were incubated at 23 °C for 72 h. b, effect of cns1 mutations on Hsp90-dependent maturation of the GR. Wild-type and temperature-sensitive cns1 cells containing a plasmid encoding mammalian GR and a GRE-lacZ reporter gene were treated with deoxycorticosterone at the permissive temperature (23 °C); β-galactosidase activity was quantified as described under “Experimental Procedures.” Each bar represents the mean ± S.D. from a minimum of five independent measurements. Indicated values were calculated relative to those of wild-type cells (column on the left) and represent percent of wild-type activity.
maturation of the steroid receptor was significantly decreased in cns1<sup>ts</sup> cells (Fig. 3b). Combined with our finding that Cns1 interacts directly with Hsp90, these results reveal that Cns1 is a bona fide co-chaperone of the Hsp90 machinery.

Hsp90 plays an important role in several signal transduction pathways in <i>S. cerevisiae</i>, including the pheromone response (33) and negative regulation of the heat shock factor (35). The potential involvement of Cns1 in these two processes was investigated by measuring the activity of β-galactosidase expressed from reporter plasmids containing heat shock response elements or a <i>FUS1</i>-pheromone-responsive promoter. cns1<sup>ts</sup> mutants exhibited normal regulation of both pathways (data not shown), suggesting that Cns1 plays a significant role in only a subset of Hsp90 functions.

**Physical Interactions between Hsp90 and Cns1**—We first determined whether <i>cns1<sup>ts</sup></i> mutations have an effect on the direct interaction between Hsp90 and Cns1. GST fusions with wild-type or mutant Cns1 proteins were expressed in <i>E. coli</i>, purified on glutathione-Sepharose, and incubated with recombinant yeast Hsp82. Whereas wild-type Cns1 exhibited a robust interaction with Hsp90, the interactions of Cns1<sup>G90D</sup> and Cns1<sup>C140R</sup> with Hsp90 were undetectable (Fig. 4a). Cns1<sup>3C</sup> retained the ability to interact with the chaperone. Thus, mutations in the TPR domain disrupt the interaction with Hsp90, in contrast with the mutations in the carboxyl terminus of Cns1. Based on the model of TPR-Hsp90 interactions (15), the amino acids substituted in Cns1<sup>G90D</sup> and Cns1<sup>C140R</sup> are unlikely to make direct contacts with the EEVD carboxyl terminus of Hsp90. Therefore, residues previously not implicated in the binding to EEVD may also be important for the TPR-Hsp90 interaction.

The effect of <i>cns1<sup>ts</sup></i> mutations on the interaction with Hsp90 in vivo was also investigated. The presence of Cns1–3HA in Hsp90 complexes was tested using cells in which His<sub>4</sub>-Hsp82 was the sole source of Hsp90. Cells were grown at permissive temperature and either maintained at 30 °C or shifted to 37 °C and lysed, and the lysates were bound to Ni-NTA resin. Lysates prior to and after purification of Hsp90 complexes are shown in Fig. 4b. Steady-state levels of Cns1<sup>3C</sup> were lower than wild-type levels at both temperatures, but the interaction of this mutant with Hsp90 was maintained to the extent proportional to the interaction of the wild-type Cns1 with Hsp90. This is consistent with our previous finding (Fig. 4a) that Cns1<sup>3C</sup> interacts well with Hsp90 in vitro.

Steady-state protein levels of Cns1<sup>G90D</sup> and Cns1<sup>C140R</sup> are indistinguishable from wild-type Cns1 at both permissive and non-permissive temperatures (Fig. 4b, top two panels). In contrast to the in vitro experiments using recombinant proteins, Cns1<sup>G90D</sup> and Cns1<sup>C140R</sup> exhibited a decreased, but detectable interaction with Hsp90 at the permissive temperature. At the non-permissive temperature, the Cns1-Hsp90 association was further impaired (Fig. 4b, bottom panel). In these cells, a genomic copy of <i>CNS1</i> was present, in addition to the plasmid-expressed 3HA-tagged version. To address possible competition or cooperation between epitope-tagged and endogenous Cns1, the experiment was repeated using an integrated cns1-1 allele as the sole source of Cns1 protein. The strength of interaction between Cns1<sup>G90D</sup> with Hsp90 was not affected by the presence or absence of the wild-type CNS1 allele (not shown).

**Genetic Interactions between CNS1 and CPR7**—Two genetic approaches were taken to further explore the role of Cns1 in vivo. In the first, a screen for multicity suppressors of the temperature-sensitive phenotype of <i>cns1-1</i> was performed. Two genes were identified in the screen: CNS1 and CPR7. Overexpression of the cyclophilin CPR7 from a multicopy vector strongly suppressed the temperature sensitivity of Cns1<sup>G90D</sup> (Fig. 5a). Overexpression of CPR7 also suppressed the temperature sensitivity of Cns1<sup>C140R</sup> and Cns1<sup>3C</sup>, albeit to a lesser extent (Fig. 5a). Because Cns1 was originally identified by its ability to suppress the slow growth phenotype of <i>cpr7Δ</i> cells (20, 21), the discovery that overexpression of CPR7 can suppress temperature sensitivity of Cns1 mutants provides complementary evidence for the functional relationship between these two Hsp90 co-chaperones.

In the second approach, genetic interaction between <i>CNS1</i> and <i>CPR7</i> was explored by introducing <i>cns1</i> mutants into <i>cpr7Δ cns1Δ</i> cells. This experiment was performed using the plasmid shuffle method: a wild-type copy of <i>CNS1</i> was maintained on a <i>URA3</i>-marked plasmid in <i>cns1<sup>ts</sup> cpr7Δ</i> cells. The inability of these cells to grow on medium containing 5-FOA revealed a synthetic lethal relationship between the <i>cns1-1</i> and <i>cpr7Δ</i> (Fig. 5b, top row). A plasmid expressing CPR7, but not vector alone, was able to suppress this synthetic lethality (Fig. 5b, second row from top). cns1-2 and cns1-3 also exhibited synthetic interactions with <i>cpr7Δ</i> (data not shown). The syn-
Cpr6 and Cpr7 were tested for the ability to confer viability to reciprocal chimeras of the PPIase and TPR-containing regions of presence of another CyP40 homolog in yeast, Cpr6 (22). Reciprocal regions of the two Cpr7 domains, we took advantage of the isomerase domain of Cpr7 is not needed for Hsp90-related functions (29). Expression of the Cpr7 amino terminus, which harbors the PPIase domain, also proved insufficient for growth in vivo.

To identify regions of Cpr7 that are necessary for viability in a background compromised for Cns1 function, several mutant alleles of the cyclophilin were introduced into cpr7Δ cns1-1 cells. Expression of the TPR-containing carboxyl terminus of Cpr7 was insufficient to confer viability in these cells (Fig. 5b). This was unexpected because of our earlier finding that the isomerase domain of Cpr7 is not needed for Hsp90-related functions (29). Expression of the Cpr7 amino terminus, which harbors the PPIase domain, also proved insufficient for growth of cpr7Δ cns1-1 cells (Fig. 5b). To assess specific requirements regarding the two Cpr7 domains, we took advantage of the presence of another CyP40 homolog in yeast, Cpr6 (22). Reciprocal chimeras of the PPIase and TPR-containing regions of Cpr6 and Cpr7 were tested for the ability to confer viability to cpr7Δ cns1-1 cells. Neither the Cpr7ΔΔ-Cpr7TPR chimera nor the Cpr7TPR-Cpr6TPR chimera was able to confer viability in these cells (data not shown), suggesting a specific requirement for the TPR and isomerase domains of Cpr7 that cannot be fulfilled by analogous domains of a closely related cyclophilin. Overall, our data indicate that both the isomerase and TPR domains of Cpr7 are required for viability when Cns1 function is impaired.

Role of the Isomerase Domain of Cpr7 in the Cns1-Cpr7 Genetic Interaction—We used two approaches to test whether the catalytic activity of the PPIase domain of Cpr7 is required for the essential function it shares with Cns1. In the first, cells were grown on medium containing cyclosporin A, an immunosuppressant and a potent inhibitor of the isomerase activity of cyclophilins (reviewed in Ref. 46). We reasoned that, if the isomerase activity of Cpr7 were required for viability in cns1-1 cells, then these cells should be hypersensitive to CsA. Additionally, if suppression of the temperature sensitivity of cns1-1 cells depended on the catalytic activity of Cpr7, the suppression would be sensitive to CsA. However, in the presence of CsA both strains grew normally (data not shown), suggesting that the isomerase activity of Cpr7 does not play a role in the essential function that this protein shares with Cns1.

In a complementary approach, a cpr7 allele in which a conserved amino acid within the catalytic site is altered (R64A) was tested for the ability to confer viability to cpr7Δ cns1-1 cells. The corresponding substitution in human CyP18 decreased catalytic activity to 0.1% of wild-type (47), and an equivalent mutation in the mammalian cyclophilin RanBP2 led to loss of function (48). cns1-1 cells expressing Cpr7R64A grow as well as those containing wild-type Cpr7 (Fig. 6a), providing more evidence that the Cpr7 isomerase domain plays a role independent of its catalytic activity.

Among the Cpr7 mutants that were tested for the ability to confer viability in cpr7Δ cns1-1 cells, a Cpr7K67P,N68G double mutant was unable to suppress lethality of these cells (Fig. 6b). Cpr7K67P,N68G contains amino acid substitutions at sites predicted by the crystal structure of bovine CyP40 to reside on the surface of the isomerase domain, removed from the PPIase catalytic site (49). As such, these residues may play a role in protein-protein interactions, although they do not affect the catalytic activity of Cpr7.

FIG. 5. CNS1 and CPR7 exhibit genetic interactions. a, suppression of the mutant phenotype of cns1Δ cells by overexpression of CPR7. Serial dilutions of cns1Δ mutants harboring either vector alone or a multicopy (2μ) plasmid expressing CPR7 were incubated on synthetic medium at the indicated temperatures for 72 h. Cells expressing the wild-type allele of CNS1 are included on the right for comparison. b, the requirement for both domains of Cpr7 to suppress the lethality of cpr7Δ cns1-1 cells. Serial dilutions of cns1Δ cpr7Δ cells harboring a URA3-marked plasmid expressing CNS1, a TRP1-marked plasmid expressing cns1-1, and a LEU2-marked vector expressing either wild-type CPR7 or truncated forms of CPR7 (Cpr7TPR and Cpr7ΔΔ) were spotted on YNB or 5-FOA media as indicated.

FIG. 6. The isomerase domain of Cpr7, but not the catalytic activity, is required for viability in cns1Δ cells. a, suppression of cpr7Δ cns1-1 synthetic lethality by the catalytically inactive Cpr7. Serial dilutions of cpr7Δ cns1-1 cells harboring a URA3-marked plasmid expressing CNS1- and LEU2-marked plasmids expressing either vector alone, wild-type Cpr7, or the catalytic site mutant Cpr7R64A. Cells were grown for 72 h at 30 °C on YNB as control and on 5-FOA to test for the ability to grow upon loss of the resident URA3-marked CNS1 plasmid. b, inability of Cpr7K67P,N68G to suppress the lethality of cpr7Δ cns1-1. cpr7Δ cns1-1 cells harbored a URA3-marked plasmid expressing Cns1- and LEU2-marked plasmids expressing wild-type Cpr7, Cpr7K67P,N68G, or vector alone. These cells were replica-plated to YNB as control and to 5-FOA to test the ability to grow in the absence of the URA3-marked plasmid.
interaction between Cpr7 and Cns1 (data not shown). Consistent with our results shown above, these experiments reveal that the PPIase domain is required for viability in cells with compromised Cns1 function, but in a manner independent of the catalytic activity of the domain.

Cns1-Cpr7 Complexes—Cns1 and Cpr7 can be found in the same complexes (20, 21), although it is not known whether the interaction between them is direct or indirect. Using recombinant GST-Cpr7 and His6-Cns1 fusion proteins expressed in E. coli, we performed GST pull-down experiments but were unable to detect direct binding between the two co-chaperones (data not shown). Because Cns1 and Cpr7 bind directly to Hsp90, we tested the possibility that Hsp90 mediates the interaction between the two co-chaperones. Hsp82ΔEEVD was used in this approach, because the interaction between Cns1 or Cpr7 and Hsp90 is primarily mediated by the carboxyl-terminal EEVD of Hsp90 (50). In this experiment, we examined the ability of FLAG-Cpr7 from wild-type or hsp82ΔEEVD lysates to bind immobilized GST-Cns1. As shown in Fig. 7a, the interaction between Hsp90 and GST-Cns1 was significantly reduced in the absence of the EEVD terminus. However, the ability of GST-Cns1 to precipitate Cpr7 from these cells was unaffected. Therefore, the formation of Cns1-Cpr7 complexes is independent of the ability of either co-chaperone to interact with the EEVD region of Hsp90.

As an alternative way to examine the effect of deleting the Hsp90 EEVD terminus on the ability of Cns1 and Cpr7 to associate, size exclusion chromatography was performed. Extracts from wild-type or hsp82ΔEEVD cells were prepared and resolved on a gel filtration column. Collected fractions were analyzed by SDS-PAGE, and Western blotting was performed to detect the presence of the Hsp90 complex components, including Hsp90, Cns1, Cpr7, Cpr6, and Sti1 (Fig. 7b). The elution profile of Hsp90 did not differ between extracts from wild-type and hsp82ΔEEVD cells, with peaks between fractions 22 and 24 in both cases. In wild-type cells, the peaks of Cpr6 and Sti1 elution profiles coincided with the peak of Hsp90. However, in extracts from hsp82ΔEEVD cells, Cpr6 eluted primarily in the fraction 31, which corresponds to the molecular mass of ~42 kDa, the predicted size of the Cpr6 monomer. The peak of the Sti1 elution was shifted down to fraction 27, which corresponds to about 160 kDa.

In contrast to Sti1 and Cpr6, Cns1 and Cpr7 did not co-elute with the major peak of Hsp90 in extracts from either wild-type or hsp82ΔEEVD cells. Rather, the peak of Cns1 elution was between fractions 28 and 30, and Cpr7 eluted between fractions 29 and 31. These results indicate that, unlike Sti1 and Cpr6, Cns1 and Cpr7 are not primarily associated with Hsp90. However, we cannot rule out the possibility that their interaction with Hsp90 fails to withstand these particular lysis and dilution conditions. The migration of Cpr7 is consistent with a monomeric protein. Although the molecular masses of fractions in which Cns1 migrates (~85–110 kDa) could suggest that Cns1 homodimerizes, we believe that this is unlikely. Using sedimentation equilibrium analysis of purified Cns1, we determined a molecular mass of 42.500 ± 1.300 kDa, corresponding to the monomer. Whether the interaction between Cns1 and Cpr7 is direct or mediated by an unknown partner, it is clearly not affected by the deletion of EEVD.

Genetic Interaction between cns1-1 and hsp82ΔEEVD—Since Hsp82 EEVD is apparently not needed for the formation of Cns1-Cpr7 complexes, we investigated the in vivo significance of this region in cells with compromised Cns1 function. cns1-1 cells were crossed with hsp82ΔEEVD cells to generate double mutant recombinants. In contrast with either single mutant, cns1-1 hsp82ΔEEVD progeny exhibited extreme slow growth at 23 °C and lethality at or above 30 °C (Fig. 8a). This phenotype was suppressed by the introduction of plasmids carrying wild-type alleles of CNS1 or HSP82. The genetic interaction between CNS1 and HSP82 further supports the notion that Cns1 plays a vital role in the proper functioning of Hsp90. It demonstrates for the first time the in vivo requirement for the EEVD region, revealing that EEVD is critical when the function of Cns1 is compromised.

It remains unclear why there is a requirement for EEVD in cns1 mutant cells. If the interaction between TPR proteins and Hsp90 can be fully explained by a model describing contacts between amino acids in the groove of the TPR domain and EEVD, an Hsp90 mutant lacking the EEVD motif and a mutation in the Cns1 TPR domain should not exhibit a synthetic interaction. To explore the possibility that Cns1 might interact
functions of Hsp90, regulation of the pheromone response and negative regulation of HSF, were largely unaffected by cns1
mutations suggests involvement of Cns1 in only a subset of Hsp90 functions.

We demonstrated that the TPR domain of Cns1 binds Hsp90 directly and that some mutations in CNS1 that confer temperature sensitivity alter sites within the TPR domain and disrupt this interaction. When the TPR-containing amino terminus of Cns1 is overexpressed, it is sufficient for cell viability at normal temperatures. Combined, these findings support the notion that the interaction between these two proteins is essential. Interestingly, the extent of physical interaction between Cns1 and Hsp90 corresponds with the viability of cells containing temperature-sensitive alleles of CNS1, suggesting that the inviability of cells at elevated temperatures can be attributed to the loss of this interaction. A genetic interaction between Cns1G9OD and the form of Hsp90 lacking the EEVD terminus further supports the idea that a Cns1-Hsp90 interaction is essential.

The temperature-sensitive phenotype was also conferred by a mutant harboring substitutions in the carboxyl terminus of Cns1 (cns1-3 allele). The steady-state level of Cns13C was significantly reduced compared with wild-type. Therefore, the phenotypes associated with the cns1-3 mutations may partly be attributed to the decrease in Cns1 protein levels. However, this mutant reveals more than simply the requirement for normal protein levels. We have found that cns1-3 cells exhibit both a temperature-sensitive phenotype and a decrease in Hsp90 function while maintaining a significant degree of Cns1-Hsp90 interaction. Furthermore, although overexpression of the amino terminus can suppress the lethality of a cns1Δ null allele, it is not sufficient for normal growth at elevated temperatures. These results underscore a requirement for the carboxyl terminus of Cns1 in the overall function of the protein, a requirement particularly pronounced at elevated temperatures.

Our genetic investigation of the function of Cns1 has revealed a previously unknown involvement of the Hsp90 co-chaperone, Cpr7. Overexpression of Cpr7 can suppress the temperature sensitivity of cns1Δ mutants, and deletion of CPR7 exhibits synthetic lethality in cns1Δ cells. These results strongly support the notion that Cns1 and Cpr7 share an essential function in vivo. It is also apparent that their cellular functions are not simply redundant and only partially overlapped. Loss of CNS1 causes lethality, whereas loss of CPR7 leads only to a moderate slow growth phenotype. Deletion of CPR7 leads to the loss of negative regulation of HSF (35), whereas mutations in CNS1 appear to have no effect on HSF-dependent signaling (this work). Although normal growth and full Hsp90 activity require Cpr7, they do not require the Cpr7 isomerase domain. In contrast, an essential Cns1-Cpr7 function requires the presence of this domain. Furthermore, although cns1 mutations lead to synthetic inviability of cells in which the Hsp90 EEVD sequence is deleted, a similar effect is not observed in analogous cpr7Δ hsp82ΔEEVD cells. The cross-suppression of mutant cns1 and cpr7 by overexpression of CPR7 and CNS1, respectively, and the synthetic lethality between cns1 and cpr7 mutations demonstrate a considerable functional overlap between the two genes and indicate that they are involved in a common and essential cellular role.

We have discovered that the PPIase domain of Cpr7 is required for viability in cells in which Cns1 function is compromised. This was unexpected, given that the PPIase domain of Cpr7 is not required for normal growth or full Hsp90-related
activity (29). Equally surprising is the evidence suggesting that, although the PPIase domain is necessary for viability in cns1 cells, the catalytic activity of this domain is not. First, the catalytically more active PPIase domain of Cpr6 (28) is unable to restore viability to cpr7Δ cns1-1 cells, even when fused to the TPR domain of Cpr7. Second, cns1-1 cells are not hypersensitive to the potent cyclophilin inhibitor CsA, and CsA does not prevent suppression of Cns1<sup>G90D</sup> temperature sensitivity by Cpr7<sup>overexpression</sup>. Finally, a mutation in the isomerase domain of Cpr7 predicted to drastically reduce PPIase catalytic activity does not result in a synthetic phenotype with cns1 mutant cells. In contrast, substitutions of two hydrophilic residues on the predicted surface of the Cpr7<sup>PPIase</sup> domain exhibit strong genetic interaction with cns1<sup>-1</sup>. It is possible therefore that the PPIase domain is involved in mediating protein-protein interactions and that these interactions are essential for viability in cns1 mutant cells. Such a role for an isomerase domain is not unprecedented; the isomerase domain of another immunophilin co-chaperone of Hsp90, FKBP52, interacts with dynem in an FK506-independent manner (51). Our data suggest that the isomerase domains of cyclophilins may perform similar functions. Because mutations in the PPIase domain of Cpr7 do not affect the interaction with Cns1 (data not shown), they may disrupt interaction with a novel binding partner.

The number of co-chaperones that Hsp90 can accommodate simultaneously has been difficult to establish. Competition studies suggested that only one TPR protein can bind to a Hsp90 dimer (52, 53). However, purified Sti1 exists in solution as a dimer and binds Hsp90 in a 1:1 molar ratio (5). Cpr6, which is monomeric, binds Hsp90 with the same molar ratio (5), suggesting that a dimer of Hsp90 could bind to two different TPR domain proteins simultaneously. Given the overall relatedness of Cpr6 and Cpr7, it is likely that Cpr7<sup>-1</sup> is also a monomer in solution. In this work, we established that purified Cns1 is monomeric. Therefore, we tested the possibility that the existence of Cns1-[Hsp90]<sup>2</sup>Cpr7<sup>complexes</sup> might explain the physical association between Cns1 and Cpr7. Although a weak interaction between Cns1 and Hsp90 remains in the absence of the Hsp90 EEVD carboxyl terminus, this region of Hsp90 provides the major contribution to the interactions between TPR domains and Hsp90. Because deletion of EEVD had no detectable effect on Cns1-Cpr7<sup>association</sup>, the interaction between the two co-chaperones can occur in the absence of the EEVD region and possibly even without Hsp90 itself.

Protein complexes containing Hsp90 and its co-chaperones were investigated by gel filtration chromatography using wild-type and hsp82ΔEEVD cells. Although Cns1 and Cpr7 partially co-eluted in the same fractions, they did not co-elute with the major Hsp90 peak. This finding is consistent with the observation that deletion of the EEVD sequence does not affect the formation of Cns1-Cpr7<sup>complexes</sup>. In contrast, Sti1 and Cpr6 co-eluted with the Hsp90 peak and shifted to lower molecular weight fractions in hsp82ΔEEVD cells. Thus, at least under these conditions, binding of Cpr6 and Sti1 to Hsp90 <em>in vitro</em> appears to depend entirely on the EEVD sequence. Although all four TPR-containing co-chaperones investigated here (Sti1, Cns1, Cpr6, and Cpr7<sup>-1</sup>) presumably interact with Hsp90 primarily through highly conserved sites in the TPR domains, there is a striking difference in the extent of their interaction with Hsp90 <em>in vivo</em>. Our findings illustrate the difference between co-chaperones that exhibit strong interactions with Hsp90, such as Cpr6 and Sti1, and those that associate weakly, such as Cns1 and Cpr7. The detection of Cns1-Cpr7<sup>complexes</sup> in common fractions does not exclude their independent binding to Hsp90 but suggests that this interaction is transient.

The synthetic slow growth of cns1-1 hsp82ΔEEVD cells is the first demonstration of a functional requirement for the Hsp90 EEVD sequence in <em>vivo</em> and reveals that interactions with co-chaperones are important for proper function of Hsp90. An important question remains: why is EEVD essential only when Cns1 function is impaired? Although several explanations are possible, the detection of a weak association between Cns1 and Hsp90<sup>Cpr6</sup>,<sup>Cpr7</sup> suggests the existence of binding determinants in the Hsp90-Cns1 interaction beyond the EEVD sequence. Multiple sites of interaction between Hsp90 and Hop have been suggested by a study in which a substitution of EEVD for AAVA did not disrupt the interaction with Hop (54). Recent identification of another TPR protein that can bind to a form of Hsp90 in which the carboxyl terminus has been truncated also supports the idea that there are additional binding determinants for TPR proteins in the Hsp90 molecule. Although binding is significantly reduced in the ΔEEVD mutant, the level of interaction that remains is apparently sufficient for viability. This argument is bolstered by our finding that Cns1-Hsp90 interaction is transient and that Cns1 mostly exists in complexes distinct from those containing Hsp90. Overall, these results may help explain the lack of an obvious phenotype of Hsp90<sup>ΔEEVD</sup> cells (26).

Our findings underscore the complexity of interactions between Hsp90 co-chaperones and demonstrate that regulation of Hsp90 can occur on multiple levels, with different co-chaperones acting both independently and in concert to ensure proper signaling through the complex.

Acknowledgments—We thank Sricharan Bandhakavi, Amy DeHart, Herman Dierick, Sally McFall, and Joshua Schneck for the critical reading of the manuscript. We thank S. Lindquist, L. Pearl, D. Winge, J. Heitman, D. Picard, L. Minvielle-Sebastia, A. Caplan, D. Toft, L. Hicke, and P. Hieter for reagents.

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