Chaperoning the Chaperone: A Role for the Co-chaperone Cpr7 in Modulating Hsp90 Function in *Saccharomyces cerevisiae*

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ABSTRACT Heat-shock protein 90 (Hsp90) of *Saccharomyces cerevisiae* is an abundant essential eukaryotic molecular chaperone involved in the activation and stabilization of client proteins, including several transcription factors and oncogenic kinases. Hsp90 undergoes a complex series of conformational changes and interacts with partner co-chaperones such as Sba1, Cpr6, Cpr7, and Cns1 as it binds and hydrolyzes ATP. In the absence of nucleotide, Hsp90 is dimerized only at the carboxy-terminus. In the presence of ATP, Hsp90 also dimerizes at the amino-terminus, creating a binding site for Sba1. Truncation of a charged linker region of yeast Hsp90 (Hsp82Δlinker) was known to disrupt the ability of Hsp82 to undergo amino-terminal dimerization and bind Sba1. We found that yeast expressing Hsp82Δlinker constructs exhibited a specific synthetic lethal phenotype in cells lacking CPR7. The isolated tetratricopeptide repeat domain of Cpr7 was both necessary and sufficient for growth in those strains. Cpr6 and Cpr7 stably bound the carboxy-terminus of wild-type Hsp82 only in the presence of nonhydrolyzable ATP and formed an Hsp82–Cpr6–Cpr7 ternary complex. However, in cells expressing Hsp82Δlinker or lacking CPR7, Cpr6 was able to bind Hsp82 in the presence or absence of nucleotide. Overexpression of CNS1, but not of other co-chaperones, in cpr7 cells restored nucleotide-dependent Hsp82–Cpr6 interaction. Together, our results suggest that the *in vivo* functions of Cpr7 include modulating Hsp90 conformational changes, mediating proper signaling of the nucleotide-bound state to the carboxy-terminus of Hsp82, or regulating Hsp82–Cpr6 interaction.

Hsp90 is an essential, abundant molecular chaperone critical for the folding and regulation of an estimated 5–10% of all cellular proteins, termed “client proteins” (Zhao *et al.* 2005; McClellan *et al.* 2007). The ability of Hsp90 to bind and hydrolyze ATP is essential for function, and many clients become destabilized or inactive in the presence of 17-AAG, a competitive inhibitor of ATP binding. Because a number of oncogenic signaling proteins, including Akt, Raf-1, Bcr-Abl, mutant p53, and HER-2/ErbB2, require Hsp90 for function, Hsp90 is a promising anti-cancer target, and Hsp90 inhibitors are currently in clinical trials (Pearl *et al.* 2008; Trepel *et al.* 2010).

Hsp90 contains three conserved domains: an amino-terminal ATP-binding domain, a middle domain, and a carboxy-terminal dimerization domain. Hsp90 binds clients

that possess some secondary structure, and all three domains have been implicated in client binding (Jakob *et al.* 1995; Vaughan *et al.* 2006; Richter and Buchner 2011; Street *et al.* 2011). Hsp90 undergoes significant conformational changes upon nucleotide binding and hydrolysis (Ali *et al.* 2006; Shiau *et al.* 2006). Nucleotide-free Hsp90 dimerized at the carboxy-terminus is in an open conformation. ATP binding induces dimerization of amino-terminal domains. ATP hydrolysis requires contact between the amino-terminal domains and a flexible loop in the middle domains. Mutations in Hsp90 that disrupt the ability of Hsp90 to undergo conformational changes cause defects in ATPase and client activity. One such mutation, Hsp82Δlinker, is the deletion of a charged linker region (CLR) between the amino-terminal and middle domains. Increasingly larger deletions of the linker cause growth defects, ranging up to inviability, as well as defects in the ability of Hsp90 to undergo ATP-induced amino-terminal dimerization (Louvion *et al.* 1996; Hainzl *et al.* 2009).

Hsp90 functions with a set of co-chaperone proteins that modulate the ATPase activity of Hsp90 and/or mediate

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interactions with client proteins (Pearl *et al.* 2008). Co-chaperones bind different conformations of Hsp90 and/or compete for the same binding site (Siligardi *et al.* 2004; Harst *et al.* 2005). Many co-chaperones contain tetratricopeptide repeat (TPR) domains and compete for binding to the conserved MEEVD sequence found at the carboxy-terminus of Hsp90 (Owens-Grillo *et al.* 1995; D'Andrea and Regan 2003). In *Saccharomyces cerevisiae*, there are at least six co-chaperones that bind Hsp90 through their TPR domains: Sti1, Cpr6, Cpr7, Ppt1, Cns1, and Tah1 (Wandinger *et al.* 2008). Despite competing for the same binding site, TPR-containing co-chaperones have differing effects on Hsp90 activity. Sti1 is a potent inhibitor of Hsp90's ATPase activity, while Cpr6 and Tah1 weakly stimulate its ATPase activity (Prodromou *et al.* 1999; Panaretou *et al.* 2002; Millson *et al.* 2008).

Differential binding of co-chaperones is linked to conformational changes in Hsp90 as it binds and hydrolyzes ATP (Pearl *et al.* 2008). Hop/Sti1 preferentially binds Hsp90 in the absence of nucleotide (Prodromou *et al.* 1999; Richter *et al.* 2003), while p23/Sba1 binds the ATP-bound, closed conformation (Ali *et al.* 2006). ATP binding also results in interaction with FKBP51/FKBP52 and Cyp40/Cpr6, which displaces Hop/Sti1 (Johnson and Toft 1994; Prodromou *et al.* 1999; Johnson *et al.* 2007). A ternary complex consisting of an Hsp90 dimer bound to both Sti1 and Cpr6 or FKBP51 (occupying different TPR-binding sites) may be a key intermediate in the Hsp90-folding cycle (Li *et al.* 2011). The structural basis for the ability of TPR-containing co-chaperones such as FKBP51/52 and Cyp40/Cpr6 to specifically recognize the ATP-bound form of Hsp90 is unknown, as the carboxy-terminal domain does not appear to undergo large conformation changes upon ATP binding (Ali *et al.* 2006).

Recent studies suggest that the co-chaperone Aha1 plays an active role in mediating conformational changes in Hsp90 that are required for ATP hydrolysis (Hessling *et al.* 2009). To gain insights into other factors governing conformational changes in Hsp90, we determined whether cells expressing Hsp82Δlinker constructs that reduce conformational flexibility but do not result in inviability exhibit synthetic growth defects when combined with deletion of genes encoding co-chaperones. We observed severe growth defects only upon combination of Hsp82Δlinker and deletion of CPR7. Deletion of CPR7 or truncation of the CLR also resulted in loss of nucleotide-specific interaction of Cpr6 with Hsp90. Together, these results suggest that growth defects caused by the loss of CPR7 are linked to a role in mediating conformational or signaling changes in Hsp90.

Materials and Methods

Media, chemicals, antibodies, and plasmids

Standard yeast genetic methods were employed (Burke *et al.* 2000). Yeast cells were grown in either YPD (1% Bacto yeast extract, 2% peptone, and 2% dextrose) or defined synthetic

complete media. Growth was examined by spotting 10-fold serial dilutions of yeast cultures on appropriate media, followed by incubation for 2 days at 25°, 30°, or 37°. 5-FOA was obtained from Toronto Research Chemicals. α-Flag monoclonal antibody, G418, and 5'-adenylylimidodiphosphate (AMP-PNP) were obtained from Sigma. Polyclonal antibodies against Hsc82/Hsp82 have been described (Flom *et al.* 2006). Polyclonal antisera was raised against keyhole limpet hemocyanin-conjugated peptides corresponding to amino acids 81–96 of Cpr6, 211–225 of Cpr7, 118–131 of Sba1, and 492–509 of Ppt1. Antibodies were validated by establishing that they recognize a protein of the correct size in yeast lysates and fail to recognize a protein of that size in strains lacking that co-chaperone (not shown).

Yeast strains

All strains are isogenic to W303. Strains JJ816 (*hsc82::LEU2 hsp82::LEU2/YEp24-HSP82*) and JJ832 (*sti1::MET2 hsc82::LEU2 hsp82::LEU2/YEp24-HSP82*) have been described (Flom *et al.* 2006). Additional yeast strains were constructed by transforming the appropriate gene disruption cassette from the yeast genome collection (Open Biosystems) into a *hsc82::LEU2 hsp82::LEU2/YEp24-HSP82* diploid (JJ928). Colonies that grew in the presence of G418 were sporulated to obtain haploid versions of the desired strain. We constructed the following strains: *hsc82::LEU2 hsp82::LEU2/YEp24-HSP82* strains lacking *PPT1* (JJ146, *MATa*), *CPR6* (JJ110, *MATa*), *CPR7* (JJ149, *MATa*), *SBA1* (JJ94, *MATa*), *AHA1* (JJ95, *MATa*), *HCH1* (JJ111, *MATa*), or *TAH1* (JJ464, *MATa*). We also constructed the *CPR7* disruption strain JJ567 (*MATa trp1-1 ura3-1 leu2-3,112 his3-11,15 ade2-1 met2-Δ1 lys2-Δ2 cpr7::kanr*).

HSP82 plasmids and constructs

In *S. cerevisiae*, Hsp90 is encoded by two genes, *HSC82* and *HSP82*, one of which must be present for viability (Borkovich *et al.* 1989). Plasmids expressing wild-type (WT) *HSP82* under the endogenous promoter (*YEp24-HSP82*) or *GPD* promoter (*pTGPDHSP82*) were gifts from Susan Lindquist (Whitehead Institute). Amino acid mutations were constructed using site-directed mutagenesis (QuikChange, Stratagene, La Jolla, Ca). Sequences of mutagenic oligonucleotides are available on request. A plasmid expressing Hsp82 with an amino-terminal 6× His tag and Xpress epitope tag under the constitutive *GPD* promoter was constructed as described for Hsc82 (Johnson *et al.* 2007). The following amino acid alterations were constructed within pRS314GPDHISHSP82 and/or pTGPDHSP82: Δ211–259, Δ211–264, and ΔMEEVD, which deletes the last five amino acids of Hsp82 and Δ211–264ΔMEEVD. All mutant constructs were sequenced completely using automated DNA sequencing.

Co-chaperone plasmids

CPR7 was cloned into pRS313 (Sikorski and Hieter 1989) and pRS316ADE3 (Flom *et al.* 2005) using engineered *SacI*

Figure 1 Hsp82Δlinker constructs exhibit specific synthetic growth defects upon loss of *CPR7*. (A) *hsc82hsp82/YEp24-HSP82* strains containing deletions in the indicated co-chaperones were transformed with plasmids expressing His-tagged versions of Hsp82 WT, Hsp82Δ211-259, or Hsp82Δ211-264. Resultant colonies were grown in the presence of 5-FOA for 2 days at 30°. (B) Colonies that appeared in JJ816 (*hsc82hsp82*) and JJ149 (*cpr7hsc82hsp82*) strains plated on 5-FOA were grown overnight, serially diluted 10-fold, dropped onto selective media, and grown for 2 days at the indicated temperatures.

and *XbaI* sites at the 5' and 3' ends, respectively. *CPR6* was cloned into pRS316ADE3 using engineered *SacI* and *BamHI* sites at the 5' and 3' ends, respectively. *Cpr6* was also cloned into pRS416GPD to introduce an amino-terminal 6× His and Xpress epitope tag. Flag-*Cpr6* and Flag-*Cpr7* were cloned into the *XmaI* and *XhoI* sites of pRS413GPD (Mumberg *et al.* 1995). To create 413GPDFlag-6PPI/7TPR and 413GPDFlag-7PPI/6TPR, *ClaI* sites were introduced near the end of the peptidyl-prolyl isomerase (PPIase) domains of *Cpr6* and *Cpr7*, resulting in the insertion of isoleucine and aspartic acid after amino acid 197 of *Cpr7* and 176 of *Cpr6*. 413GPDFlag-6TPR expresses amino acids 171–371 of *Cpr6* and 413GPDFlag-7TPR expresses amino acids 193–393 of *Cpr7*. *CNS1* was cloned into YEp24 as a 2.0-kb *EcoRI* fragment. pRS316ADE3*STI1* has been previously described (Flom *et al.* 2005). *PPT1* and *TAH1* were amplified from WT yeast and cloned into pRS316ADE3 using engineered *SacI/XbaI* and *SacI/BamHI* sites at the 5' and 3' ends, respectively.

Isolation of His-Hsp82 and His-Cpr6 complexes

His-Hsp82 complexes were isolated as described for His-Hsc82 complexes (Johnson *et al.* 2007). Briefly, a plasmid expressing His-tagged WT or mutant Hsp82 was transformed into strain JJ816 (or other strain as indicated), and resultant colonies were grown in the presence of 5-FOA to counterselect for the plasmid expressing WT untagged Hsp82 (YEp24-HSP82). Cells were harvested at an OD₆₀₀ of 1.2–2.0 and lysed in 20 mM Tris (pH 7.5), 100 mM KCl, and 5 mM MgCl₂ plus protease inhibitors. As indicated, lysate was adjusted to contain 5 mM AMP-PNP and incubated 5 min at 30°. Hsp82 complexes were isolated by incubation with nickel resin (1 hr with rocking at 4°) followed by washes with lysis buffer plus 0.1% Tween-20 and 35 mM imidazole. Nickel resin was boiled in SDS-PAGE sample buffer, and protein complexes were separated by gel electrophoresis (10% acrylamide unless noted otherwise) followed by Coomassie Blue staining or immunoblot analysis. Chemiluminescence immunoblots were performed according to the manufacturer's suggestions (Pierce,

Rockford, IL). His-Cpr6 complexes were isolated in a similar manner.

Results

Hsp90 contains two features that are not present in HtpG, the Hsp90 of *Escherichia coli*: (1) the extended CLR between the amino-terminal and middle domains and (2) the carboxy-terminal pentapeptide MEEVD (Louvion *et al.* 1996). The MEEVD sequence is the only established binding site for TPR-containing co-chaperones such as Hop/Sti1, Ppt1, Cpr7, and Cpr6. Two recent studies highlight the importance of the CLR in mediating nucleotide-induced changes in Hsp90 structure and co-chaperone interactions. Although partial deletion of the CLR of Hsp82 (Δ211–259) did not significantly affect Hsp90 function, Hsp82 containing a longer deletion (Δ211–266) was unable to support viability. Purified Hsp82 containing deletions in the CLR was able to bind ATP but was unable to undergo amino-terminal dimerization and bind p23/Sba1 in the presence of ATP, indicating that it was conformationally restricted (Hainzl *et al.* 2009; Tsutsumi *et al.* 2009).

We took a genetic approach to determine whether any co-chaperones become essential when Hsp82 is conformationally restricted due to deletion of the CLR. Starting with strain JJ816 (*hsc82hsp82/YEp24-HSP82*), we introduced disruptions in genes encoding nonessential co-chaperones. We transformed in a plasmid expressing WT or mutant HSP82 and grew the cells in the presence of 5-FOA so that the introduced form of HSP82 was the only Hsp90 present in the cell. In the starting strain (JJ816), *hsp82Δ211-259* exhibited WT growth and *hsp82Δ211-264* displayed a growth defect at 37°. Both Hsp82 mutants were expressed at WT levels (Supporting Information, Figure S1). As shown in Figure 1A, there was a strong specific synthetic growth defect upon combination of deletion of *CPR7* with *hsp82Δ211-259*. The combination of *hsp82Δ211-264* and *cpr7* was inviable. To better illustrate the enhanced growth defect, cells that survived on 5-FOA were tested for growth. As shown in Figure 1B, loss of *CPR7* results in decreased

Figure 2 No synthetic growth defects of Hsp82Δlinker constructs were observed upon loss of other co-chaperones. The indicated *hsc82hsp82* co-chaperone deletion strains were transformed with plasmids expressing His-tagged versions of Hsp82Δ211-264 or Hsp82Δ211-259. Growth of colonies that grew in the presence of 5-FOA was assayed as described for Figure 1B.

growth at 25° and slow growth at 30° and 37°, which is evident by the formation of smaller colonies at those temperatures (Duina *et al.* 1996). It is unclear why the growth defect of *cpr7* cells is more pronounced at lower temperatures. Cells expressing *hsp82Δ211-259* grew like cells expressing WT *HSP82* (Louvion *et al.* 1996). Upon combination of *hsp82Δ211-259* and *cpr7*, cells exhibited enhanced growth defects at all three temperatures. We conducted similar tests to examine the growth of *hsp82Δ211-259* or *hsp82Δ211-264* in the absence of other co-chaperones (Figure 2). No synthetic growth defects were observed upon combination of either *hsp82Δ211-259* or *hsp82Δ211-264* with deletion of other co-chaperones. This result indicates a specific requirement for *CPR7* in cells expressing Hsp82Δlinker constructs.

TPR domain of *Cpr7* specifies its *in vivo* functions

Cpr6 and *Cpr7* share 38% amino acid sequence identity, but overexpression of *CPR6* does not rescue growth phenotypes or defects in Hsp90 client activity in cells lacking *CPR7* (Dolinski *et al.* 1998; Marsh *et al.* 1998), indicating that they have different functions *in vivo*. *Cpr6* and *Cpr7* contain two main domains: an amino-terminal PPIase domain and a carboxy-terminal TPR domain. The PPIase domain of *Cpr7* is dispensable for growth (Duina *et al.* 1998b). We made chimeras in which the PPIase domains were swapped, creating

Cpr6 PPIase domain/*Cpr7* TPR domain (6PPI/7TPR) and *Cpr7* PPIase domain/*Cpr6* TPR domain (7PPI/6TPR). Since *Cpr7* is expressed at roughly fivefold lower levels than *Cpr6* (Ghaemmaghami *et al.* 2003), Flag-tagged constructs were expressed under the constitutive *GPD* promoter to ensure similar levels of expression (Figure 3A and Figure S2). The PPIase domain of *Cpr7* is ~20 amino acids larger than that of *Cpr6*, accounting for the reduced mobility of those constructs in SDS-PAGE. The growth of cells expressing Flag-*Cpr7* expressed under the *GPD* promoter was indistinguishable from cells expressing *CPR7* under its own promoter (not shown). Consistent with prior studies (Tescic *et al.* 2003), only Flag-*Cpr7* or Flag-6PPI/7TPR was able to fully rescue the growth defect of a *cpr7* strain (Figure 3B). Similarly, only Flag-*Cpr7* or Flag-6PPI/7TPR was able to support viability in the *cpr7hsc82hsp82* strain expressing *hsp82Δ211-264* (Figure 3C).

The TPR domains of *Cpr6* and *Cpr7* share ~33% amino acid sequence identity. To determine whether the TPR domain of *Cpr7* was sufficient for viability in the *cpr7hsc82hsp82* strain expressing *hsp82Δ211-264*, we constructed plasmids expressing the isolated Flag-tagged TPR domain of *Cpr7* (amino acids 193–393) or *Cpr6* (amino acids 171–371) under the *GPD* promoter. These constructs were expressed at levels similar to full-length proteins (Figure S3). Flag-7TPR, but not Flag-6TPR, was able to rescue growth (Figure 3D), indicating that the TPR domain of *Cpr7* is necessary and sufficient for viability in the strain expressing *hsp82Δ211-264*.

Hsp82Δ211-264 exhibits altered interaction with *Cpr6* and *Cpr7*

Cpr6 bound His-Hsc82 in cell lysates only in the presence of nonhydrolyzable ATP, AMP-PNP (Johnson *et al.* 2007). To examine *Cpr7*–Hsp82 interaction, cells expressing His-Hsp82 were transformed with the plasmid expressing Flag-*Cpr7*. His-Hsp82 complexes were isolated by incubating cell extracts with nickel resin, followed by SDS-PAGE and immunoblot analysis using peptide-specific antibodies that we raised against *Sba1*, *Cpr6*, *Cpr7*, or *Ppt1*. As expected, binding of *Cpr6* and *Sba1* to His-Hsp82 was observed only in the presence of AMP-PNP (Figure 4A). In contrast, *Ppt1* bound Hsp82 in the presence or absence of AMP-PNP. Flag-*Cpr7* bound His-Hsp82 only in the presence of AMP-PNP, and the presence of overexpressed Flag-*Cpr7* did not affect *Ppt1*, *Cpr6*, or *Sba1* interaction.

Using the same assay, we examined the ability of His-Hsp82Δ211-264 to interact with co-chaperones (Figure 4B). As previously observed, *Sba1* exhibited reduced interaction with His-Hsp82Δ211-264 due to the reduced ability of this mutant form to adopt the closed conformation (Ali *et al.* 2006; Hainzl *et al.* 2009). In contrast, *Cpr6* bound His-Hsp82Δ211-264 in the presence or absence of AMP-PNP. This suggests that deletion of the CLR also disrupts proper signaling of the nucleotide-bound state to the carboxy-terminus, resulting in loss of nucleotide-specific Hsp82–*Cpr6*

Figure 3 Expression and function of Cpr6/Cpr7 chimeras. (A) Chimeric versions of Cpr6 and Cpr7 expressed under the GPD promoter and containing an amino-terminal Flag tag were transformed into a WT (JJ762) strain. Cell lysates were separated by SDS-PAGE (7.5% acrylamide) and probed with the α -Flag antibody. The asterisk (*) represents a band that cross-reacts with the Flag antibody and thus serves as an internal loading control. (B) Indicated plasmids were transformed into the *cpr7* deletion strain (JJ567) and growth was assayed as described. (C) Indicated plasmids were transformed into the *cpr7hsc82hsp82* strain (JJ149) along with a plasmid expressing His-Hsp82 Δ 211-264. Resultant colonies were grown in the presence of 5-FOA for 2 days at 30°. (D) Plasmids expressing Flag-Cpr6, Flag-Cpr7, or isolated TPR domains of Cpr6 and Cpr7 were assayed for growth as described above.

interaction. The shorter CLR truncation, Hsp82 Δ 211-259, which did not cause notable growth defects, did not exhibit altered Cpr6 interaction (not shown).

To confirm that Cpr6 binding to His-Hsp82 was dependent on the MEEVD sequence, we compared the interaction of Cpr6 with WT His-Hsp82 and His-Hsp82 Δ MEEVD (Figure 4B). As expected, disruption of the TPR acceptor site disrupted binding of Cpr6 and Ppt1 to Hsp90 without altering Sba1 interaction. Hsp82 Δ MEEVD also failed to interact with Flag-Cpr7 (data not shown). We also constructed His-Hsp82 Δ 211-264 Δ MEEVD ($\Delta\Delta$). The growth of this mutant was similar to growth of Hsp82 Δ 211-264 (Figure S1). Cpr6 did not interact with Hsp82 Δ 211-264 Δ MEEVD, indicating that altered interaction of Cpr6 with Hsp82 Δ 211-264 was not due to formation of a second binding site that obviates the requirement for the MEEVD binding site.

We next determined whether Cpr6 and Cpr7 exhibit similar binding to His-Hsp82 Δ 211-264. Flag-Cpr7 or Flag-Cpr6 were expressed in cells expressing His-tagged Hsp82 or His-Hsp82 Δ 211-264. Endogenous Cpr6 bound WT Hsp82 only in the presence of AMP-PNP, and both Flag-Cpr6 and Flag-Cpr7 bound Hsp82 Δ 211-264 in the presence or absence of AMP-PNP (Figure 4C). This is consistent with a prior report that Cpr6 and Cpr7 have similar interactions with purified Hsp82 (Mayr *et al.* 2000).

Cpr6 and Cpr7 form a ternary complex with Hsp90

Li *et al.* (2011) recently demonstrated that the two TPR acceptor sites within an Hsp90 dimer may be occupied by Sti1 and Cpr6 either *in vivo* or *in vitro*. We determined whether a ternary complex forms between Cpr6, Cpr7, and Hsp82 *in vivo*. Strain JJ110 (*cpr6hsc82hsp82*/YEp24-HSP82) expressing untagged Hsp82, Hsp82 Δ MEEVD, or Hsp82 Δ 211-264 was transformed with a plasmid expressing WT or His-tagged Cpr6 in addition to a plasmid expressing Flag-Cpr7. His-Cpr6 complexes were isolated in the presence of AMP-PNP to determine whether Flag-Cpr7 and

His-Cpr6 bound to the same dimer of Hsp82 (Figure 5). Flag-Cpr7 was isolated in His-Cpr6 complexes when cells expressed WT Hsp82, but not Hsp82 Δ MEEVD. The amount of Flag-Cpr7 present in complex with Cpr6 was not significantly affected by the Hsp82 Δ 211-264 mutation. This indicates the presence of an Hsp82-Cpr6-Cpr7 ternary complex and provides evidence that Cpr6 and Cpr7 do not stably interact in the absence of Hsp82. Under these conditions, very little Sti1 was present in the His-Cpr6 complexes (not shown).

Loss of CPR7 results in altered Hsp82-Cpr6 interaction

The synthetic growth phenotype observed upon combination of *hsp82* Δ 211-264 and deletion of *CPR7* suggests that the two mutations cause similar defects or affect the same functions of Hsp82. Since Hsp82 Δ 211-264 exhibited altered Cpr6 interaction, we examined the effect of *CPR7* deletion on Hsp82 interaction with other co-chaperones (Figure 6). His-Hsp82 complexes were isolated from strain JJ816 (*hsc82hsp82*) or JJ149 (*cpr7hsc82hsp82*) as described. Similar to the results observed with Hsp82 Δ 211-264, Cpr6 bound to Hsp82 in the presence or absence of AMP-PNP. Unlike Hsp82 Δ 211-264, no reduction in Sba1 binding to Hsp82 in the presence of AMP-PNP was observed. Thus, Cpr7 may be involved in mediating conformational or signaling changes that result in nucleotide-specific Cpr6 interaction with the carboxy-terminus of Hsp82, but it does not appear to play a role in amino-terminal dimerization and/or Sba1 interaction.

Overexpression of CNS1 restores nucleotide-specific Hsp82-Cpr6 interaction in *cpr7* cells

Loss of *CPR7* resulted in slow growth and defects in activity of clients such as the glucocorticoid receptor and the heat-shock transcription factor Hsf1 (Duina *et al.* 1996, 1998a). These defects were rescued by the overexpression of *CNS1*, which encodes an essential TPR-containing co-chaperone

Figure 4 Hsp82 Δ 211-264 exhibits altered interaction with Cpr6 and Cpr7. (A) *hsc82hsp82* cells expressing untagged or His-tagged wild-type Hsp82 were transformed with pRS413GPDFlag-Cpr7 (+) or pRS413GPD (-). His-Hsp82 complexes isolated in the presence or the absence of AMP-PNP were subjected to SDS-PAGE and/or immunoblot analysis. (Top) Coomassie Blue-stained gel. (Middle panels) Immunoblot analysis of copurifying proteins. (Bottom) Expression of Flag-Cpr7 in whole-cell lysate. (B) WT and mutant His-Hsp82 complexes were isolated in the presence or absence of AMP-PNP as described. (C)

hsc82hsp82 cells expressing His-Hsp82 WT or His-Hsp82 Δ 211-264 were transformed with pRS413GPD (-), pRS413GPDFlag-Cpr7, or pRS413GPDFlag-Cpr6. (Top) Coomassie Blue-stained gel. (Middle panels) Immunoblot analyses of Cpr6 and Cpr7. The asterisk denotes the migration of Flag-Cpr6 relative to endogenous Cpr6. (Bottom) Expression of Flag-Cpr7 or Flag-Cpr6 in whole-cell extract. All gels were 10% acrylamide except the bottom panel in C comparing the migration of Flag-Cpr6 and Flag-Cpr7 in whole-cell lysates, which was done using a 7.5% acrylamide gel.

(Marsh *et al.* 1998). We determined whether overexpression of *CNS1* or other TPR-containing co-chaperones restored nucleotide-dependent interaction of Cpr6 and Hsp82 in cells lacking *CPR7* (Figure 7A). *cpr7hsc82hsp82* cells expressing untagged Hsp82 or His-Hsp82 were transformed with a plasmid expressing *CPR7*, *CNS1*, *PPT1*, *STI1*, or *TAH1*. His-Hsp82 complexes were isolated from cells in the absence of exogenous nucleotide. In the absence of *CPR7*, significant amounts of Cpr6 bound Hsp82, but the presence of *CPR7* resulted in loss of Cpr6 binding. In the presence of *CNS1*, the level of Cpr6 binding to Hsp82 was similarly decreased. In contrast, overexpression of *STI1*, *PPT1*, or *TAH1* had no effect on Hsp82-Cpr6 interaction (Figure 7A). *CNS1* overexpression also rescued the growth defect of *cpr7hsc82hsp82* cells expressing *hsp82* Δ 211-259 (Figure 7B). Overexpression of *STI1*, *PPT1*, or *TAH1* had no effect on the slow-growth defect of *cpr7* cells or *cpr7hsc82hsp82* cells expressing *hsp82* Δ 211-259 (not shown). Thus, the specific ability of *CNS1* to restore nucleotide-dependent Hsp82-Cpr6 interaction suggests that this effect is not due to displacement of Cpr6 by binding of another overexpressed TPR-containing protein. This provides additional evidence that the *in vivo* functions of Cpr7 and Cns1 are related to changes in Hsp90 that regulate Cpr6-Hsp82 interaction.

Discussion

Ever since the first Hsp90 co-chaperones were identified, a main goal has been to identify their specific roles in the function of the Hsp90 molecular chaperone machine. The Hsp90-binding sites for most co-chaperones have been identified, and most co-chaperones have been analyzed to determine whether they affect the ATPase activity of Hsp90 (Pearl *et al.* 2008; Wandinger *et al.* 2008). Despite these advances, there are still many questions about the diverse roles of the 12 known co-chaperones of *S. cerevisiae*, including questions about whether co-chaperones have overlapping or unique functions. Recent studies suggest that co-chaperones

may modulate conformational changes in Hsp90. Aha1 was shown to interact with both the middle and the amino-terminal domains of Hsp90, promoting conformational changes required for ATP hydrolysis. In this way, Aha1 was proposed to play a key role in regulating the timing of the ATP-dependent Hsp90-client interactions (Koulov *et al.* 2010; Retzlaff *et al.* 2010).

We took a genetic approach to determining whether any co-chaperones play a role in mediating conformational changes in Hsp90. We started with Hsp82 Δ linker mutants that are capable of ATP binding but defective in ATP-dependent dimerization of the amino-termini, resulting in reduced p23/Sba1 binding (Hainzl *et al.* 2009; Tsutsumi *et al.* 2009). We found that these “conformationally restricted” forms of Hsp82 exhibited specific growth defects when combined with loss of the gene encoding Cpr7. Deletion of *CPR7* or the CLR resulted in loss of ATP-dependent Hsp82-Cpr6 interaction, suggesting that both of these mutations may affect the proper transmission of information about the state of bound nucleotide to the carboxy-terminus. Overexpression of *CNS1*, which was previously shown to rescue defects of *cpr7* cells (Dolinski *et al.* 1998; Marsh *et al.* 1998), restored nucleotide-specific interactions between Hsp82 and Cpr6, providing further evidence that this is an important *in vivo* function of Cpr7. These results do not appear to be a consequence of direct competition between Cpr6 and other co-chaperones for binding to Hsp82, since overexpression of *CPR7* did not alter the interaction of Cpr6 with WT or mutant Hsp82. Additionally, overexpression of *STI1*, *TAH1*, or *PPT1* was unable to restore nucleotide-dependent Hsp82-Cpr6 interactions in cells lacking *CPR7*. Although the functions of Cpr7 and Cns1 are apparently related to the function of the CLR, overexpression of neither *CPR7* nor *CNS1* was able to rescue the growth defects of cells expressing *hsp82* Δ 211-264 (not shown).

Hsp90 and Hsp70 contain carboxy-terminal EEVD residues that bind TPR domains in what is described as a “carboxylate clamp” in which the aspartic acid residues bind

Figure 5 Ternary complex between Hsp90, Cpr6, and Cpr7. *cpr6hsc82hsp82* cells expressing untagged WT or mutant Hsp82 were transformed with either untagged or His-tagged Cpr6 and pRS413GPD or 413GPDFlag-Cpr7 as indicated. His-Cpr6 was isolated from cells incubated in the presence of AMP-PNP. (Top) Coomassie Blue-stained gel showing isolation of His-Cpr6 and copurifying Hsp82. (Bottom) The asterisk in the lysate samples represents an unknown protein that cross-reacts with the α -Cpr7 antisera, serving as an internal loading control.

basic residues within a groove formed by the helical structure of the TPR domains (Scheufler *et al.* 2000; Pratt and Toft 2003). From structural and mutational evidence, it is clear that differing TPR co-chaperones such as Hop, Cyp40, and FKBP51/52 have similar interactions with the terminal MEEVD residues of Hsp90 and that basic residues within the TPR domains that are required for efficient binding to the MEEVD sequence are highly conserved (Ward *et al.* 2002; Wu *et al.* 2003; Carrigan *et al.* 2004). In yeast lysates as well as in rabbit reticulocyte lysates, Cpr6, Cpr7, and Cyp40 bind Hsp90 preferentially in the presence of AMP-PNP or ATP (Johnson and Toft 1994; Johnson *et al.* 2007). The structural basis for nucleotide-specific binding of some, but not all, TPR-containing co-chaperones to Hsp90 is unknown.

Sti1/Hop binds Hsp90 in the nucleotide-free form and prevents amino-terminal dimerization. In addition to the MEEVD sequence, Sti1 also contacts the middle domain (Southworth and Agard 2011; Schmid *et al.* 2012). Displacement of Hsp90–Sti1 complexes by Cpr6, including formation of an Hsp90–Sti1–Cpr6 ternary complex has been proposed to be a key component of the Hsp90-folding cycle (Prodromou *et al.* 1999; Li *et al.* 2011). Our results indicate that an Hsp90–Cpr6–Cpr7 ternary complex also exists. In one model (Figure 8A), formation of an Hsp90–Sti1–Cpr6 complex is followed by formation of an Hsp90–Cpr6–Cpr7 complex that is required for Cpr6 and Cpr7 dissociation. A requirement for Cpr7 in dissociating Hsp82–Cpr6 complexes could explain the persistence of Hsp82–Cpr6 complexes in the absence of nucleotide (Figure 8B).

The above model does not address the important role of ATP in regulating Hsp90 interaction with Cpr6 and Cpr7. It is possible that binding of ATP to the amino-terminus of Hsp90 results in a conformational change or other structural

Figure 6 Deletion of *CPR7* results in altered interaction of Cpr6 with Hsp82. *hsc82hsp82* or *cpr7hsc82hsp82* cells transformed with plasmids expressing untagged or His-tagged Hsp82 WT were grown on 5-FOA to lose the YEp24-HSP82 plasmid. (Top) Coomassie Blue-stained gel. (Middle panels) Immunoblot analysis of proteins that copurify with His-Hsp82 in the presence or absence of AMP-PNP. (Bottom panels) Immunoblot of whole-cell extract incubated with the α -Cpr7 antisera. The asterisk represents an unknown protein that cross-reacts with the α -Cpr7 antisera, serving as an internal loading control.

modification near the carboxy-terminus of Hsp90 that results in formation or unmasking of the Cpr6/7 binding site. In cells expressing Hsp82 Δ 211-264 or lacking *CPR7*, regulation of this signal is lost, resulting in Hsp82–Cpr6 interaction in the presence or absence of nucleotide. Alternatively, Cpr7 or the CLR of Hsp90 may be required to promote a conformational change in Cpr6 that allows it to distinguish the ATP-bound conformation. It is possible that Hsp90 interaction with another protein or co-chaperone normally prevents Cpr6 interaction in the absence of nucleotide and that other protein is unable to bind Hsp82 Δ 211-264. However, it is unclear why loss of Cpr7 would cause the same effect since Cpr7 was able to bind Hsp82 Δ 211-264.

Although both Sba1 and Cpr6 preferentially bind Hsp90 in the presence of AMP-PNP, the two co-chaperones have distinct requirements for Hsp90 binding since we identified mutations that selectively altered either Sba1 or Cpr6 interaction (Johnson *et al.* 2007). The current study supports our prior results that Cpr6 and Sba1 bind Hsp90 independently. However, the specific requirement for Cpr6 binding to Hsp90 remains unclear. Although Cpr6 stably bound WT Hsp82 only in the presence of AMP-PNP, Hsp82 Δ 211-264 was able to bind Cpr6 in the absence of AMP-PNP. Our studies are unable to distinguish whether this reflects binding of Cpr6 to the open nucleotide-free form of Hsp82 Δ 211-264 or an inability of Cpr6 to dissociate from Hsp82 Δ 211-264 after ATP hydrolysis. Additional studies are required to determine how nucleotide-induced conformational changes in Hsp90 affect the extreme carboxy-terminus. The carboxy-terminal domain of Hsp90 does not appear to undergo large

Figure 7 Overexpression of *CNS1* restores nucleotide-specific interaction of Hsp82 with Cpr6. (A) *cpr7hsc82hsp82* cells expressing His-tagged Hsp82-WT were transformed with pRS316, pRS316ADE3CPR7, YEp24-CNS1, pRS316ADE3STI1, pRS316ADE3PPT1, or pRS316ADE3TAH1. His-Hsp82 was isolated from cells lacking exogenous nucleotide, and complexes were analyzed as described. (Bottom) An immunoblot of whole-cell extract incubated with the α -Cpr7 antisera. The asterisk represents an unknown protein that cross-reacts with the α -Cpr7 antisera, serving as an internal loading control. (B) *cpr7hsc82hsp82* cells expressing His-tagged Hsp82 Δ 211-259 were transformed with pRS316, pRS316ADE3CPR7, or YEp24-CNS1. Growth in selective media was monitored after 2 days on rich media at the indicated temperatures.

conformational changes upon nucleotide binding. However, the most complete structure of yeast Hsp82 available lacks both part of the charged linker region and the last 32 amino acids, including the MEEVD residues (Ali *et al.* 2006). Our results suggest that signaling of nucleotide-induced changes in the amino-terminus of Hsp90 that results in stable Cpr6 binding to the carboxy-terminus is disrupted by either deletion of the CLR or loss of Cpr7. Further studies will be necessary to determine the extent of these changes as well

as the mechanism of Cpr7 function. Additional studies will be needed to establish the function of the CLR, although a recent report indicates that the specific sequence, rather than the size, of the linker is critical for function (Tsutsumi *et al.* 2012).

CPR7 deletion leads to growth defects and reduced activity of Hsp90 clients. However, no growth defects or client activity defects are known to occur in cells lacking CPR6 (Duina *et al.* 1996, 1998a; Lee *et al.* 2004). Purified Cpr6 and Cpr7 exhibited differences in PPIase activity and molecular chaperone activity yet bound WT Hsp82 with the same affinity (Mayr *et al.* 2000). We established that sequences outside the PPIase domain are responsible for Cpr7 function. Cpr7-R64A, which alters a residue predicted to be required for catalytic activity (Duina *et al.* 1998b), was able to support viability in *cpr7hsc82hsp82* cells expressing *hsp82* Δ 211-264 (not shown). The chimeric constructs as well as the isolated TPR domain (amino acids 193–393) that supported Cpr7 functions included the TPR domain as well as a linker region postulated to be important for chaperone activity (Mok *et al.* 2006). Although the importance of Cpr7's chaperone activity with respect to Hsp90 functions is unclear, it is of interest that Cpr7 was found to have elevated chaperone activity relative to Cpr6 (Mayr *et al.* 2000). FKBP51 and FKBP52, which share ~70% sequence similarity, have opposing effects on the activity of steroid hormone receptors and differing patterns of associations with different steroid hormone receptors (Riggs *et al.* 2004). Prior studies by the Smith lab suggested that the terminal 20–30 amino acids are critical for differences in binding of FKBP51 and FKBP52 to Hsp90 (Cheung-Flynn *et al.* 2003). Further studies are required to establish the critical residues of Cpr7 and Cns1 that regulate Hsp90–Cpr6 interaction and viability in cells expressing Hsp82 Δ linker constructs.

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Figure 8 Model of Cpr6 and Cpr7 interaction with Hsp90. (A) In the absence of nucleotide, Sti1 binds the open conformation of Hsp90. Upon ATP binding by Hsp90, Cpr6 binds the carboxy-terminus of Hsp90, resulting in formation of an Hsp90–Sti1–Cpr6 complex. Cpr6 binding displaces Sti1, resulting in the closed conformation characterized by Sba1 interaction. Formation of an Hsp90–Cpr6–Cpr7 ternary complex promotes dissociation of Cpr6, allowing for another cycle of Sti1 binding. (B) In the absence of Cpr7, Cpr6 fails to dissociate from Hsp90 after ATP hydrolysis, resulting in the persistence of Hsp82–Cpr6 complexes. N, amino-terminal domain; M, middle domain; C, carboxy-terminal domain.

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GENETICS

Supporting Information

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Chaperoning the Chaperone: A Role for the Co-chaperone Cpr7 in Modulating Hsp90 Function in *Saccharomyces cerevisiae*

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Figure S1 Growth of Hsp82Δlinker constructs. (A) JJ816 (*hsc82hsp82*/YEp24Hsp82) was transformed with plasmids expressing His-tagged versions of Hsp82 WT, Δ211-259, Δ211-264, ΔMEEVD or Δ211-264ΔMEEVD. Colonies were grown on 5-FOA to lose the YEp24-*HSP82* plasmid. Resultant colonies were grown overnight, serially diluted 10-fold and grown on selective media plates at the indicated temperatures for two days. (B) Extracts from *hsc82hsp82* cells expressing the indicated mutant were separated using SDS-PAGE (10% acrylamide) and probed using antibodies specific for Hsp90 or Ppt1 (as a loading control).

Figure S2 Expression of Cpr6 and Cpr7 chimeras. Left panel. Cpr6 and Cpr7 coding sequences containing an amino-terminal Flag tag were cloned into the same restriction sites of pRS413GPD. A *ClaI* site was introduced after the PPIase domain to facilitate domain swapping. The location of Cpr6 and Cpr7 peptides used to generate specific antibodies is shown. Right panel. Chimeric versions of Cpr6 and Cpr7 were transformed into a wild-type strain (JJ762). Cell extracts were separated using SDS-PAGE (7.5% acrylamide) and probed using antibodies specific for Hsp90 (as a loading control), Flag, Cpr6, or Cpr7. An asterisk (*) denotes the presence of endogenous Cpr6. Flag-Cpr7 is overexpressed relative to native Cpr7, which is not visible in this exposure. As expected, the Cpr6 antisera recognizes native Cpr6, Flag-Cpr6 and Flag-6PPI/7TPR, and the Cpr7 antisera recognizes Flag-Cpr7 and Flag-6PPI/7TPR.

Figure S3 Expression and growth of isolated Cpr6/Cpr7 TPR domains. **A.** Plasmids expressing Flag-tagged TPR domains of Cpr6 (amino acids 171-371) or Cpr7 (amino acids 193-393) under the GPD promoter were transformed into strain JJ816 expressing His-Hsp82WT. Cell lysates were separated using SDS-PAGE (15% acrylamide) and probed with the α -Flag antibody as well as the α -Hsp90 antibody which served as a loading control. **B.** Indicated plasmids were transformed into the *cpr7* deletion strain (JJ567). Resultant colonies were grown overnight, serially diluted 10-fold, dropped onto selective media plates and grown at the indicated temperatures for two days.