The C-terminal Extension of *Saccharomyces cerevisiae* Hsp104 Plays a Role in Oligomer Assembly†

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ABSTRACT: The *Saccharomyces cerevisiae* protein Hsp104, a member of the Hsp100/Clp AAA+ family of ATPases, and its orthologues in plants (Hsp101) and bacteria (ClpB) function to disaggregate and refold thermally denatured proteins following heat shock and play important roles in thermotolerance. The primary sequences of fungal Hsp104's contain a largely acidic C-terminal extension not present in bacterial ClpB's. In this work, deletion mutants were used to determine the role this extension plays in Hsp104 structure and function. Elimination of the C-terminal tetrapeptide DDLD diminishes binding of the tetratricopeptide repeat domain cochaperone Cpr7 but is dispensable for Hsp104-mediated thermotolerance. The acidic region of the extension is also dispensable for thermotolerance and for the stimulation of Hsp104 ATPase activity by poly-L-lysine, but its truncation results in an oligomerization defect and reduced ATPase activity in vitro. Finally, sequence alignments reveal that the C-terminal extension contains a sequence (VLPNH) that is conserved in fungal Hsp104's but not in other orthologues. Hsp104 lacking the entire C-terminal extension including the VLPNH region does not assemble and has very low ATPase activity. In the presence of a molecular crowding agent the ATPase activities of mutants with longer truncations are partially restored possibly through enhanced oligomer formation. However, elimination of the whole C-terminal extension results in an Hsp104 molecule which is unable to assemble and becomes aggregation prone at high temperature, highlighting a novel structural role for this region.

The *Saccharomyces cerevisiae* heat shock protein Hsp104 (*1*), ClpB in bacteria (*2*), and Hsp101 in plants (*3*) are crucial determinants of induced thermotolerance in their respective organisms. Hsp104 belongs to the Hsp100/Clp subfamily of AAA+ proteins whose members form ring-shaped hexamers and are involved in ATP-dependent conformational remodeling and degradation of protein substrates (for review, see ref *4*). Instead of preventing protein aggregation as do other molecular chaperones, Hsp104 is remarkable in that it mediates the remodeling of protein aggregates (*5*). Incapable of refolding proteins alone, Hsp104 cooperates with the conventional Hsp70/40 system, which partially disassembles aggregates prior to the action of Hsp104 (*6*, *7*). Subsequently, ATP hydrolysis by Hsp104 is used to drive translocation of single polypeptide chains through its axial pore (*8*) where the Hsp70/40 system may act again to refold emerging chains into functional proteins. The synergistic action of this bichaperone network allows cells recovering from severe heat stress to resolubilize aggregates and therefore rescue essential protein activities (*5*, *7*, *9*).

Not only does Hsp104 function in resistance to extreme heat stress, at normal temperatures it is also involved in the propagation of yeast prions, self-replicating ordered aggregates of otherwise soluble proteins (reviewed in ref *10*). In *S. cerevisiae* three well-characterized prions, [*PSI*$^+$], [*URE3*], and [*PIN*$^+$], are lost when Hsp104 function is attenuated either by disruption of the *HSP104* gene (*11*−*13*) or when the ATPase activity of the protein is inhibited by guanidinium (*13*−*16*). In [*PSI*$^+$] strains, most of the translation termination factor Sup35 is sequestered in self-replicating aggregates, resulting in a loss of translation termination fidelity and thereby enhanced suppression of nonsense codons (*17*−*19*). Expression of Hsp104 at a level higher than is normally present in unstressed cells increases the solubility of Sup35 in [*PSI*$^+$] cells, restoring efficient termination (*11*). In fact, when Hsp104 is overexpressed to even higher levels, the rate of spontaneous loss of the [*PSI*$^+$] prion or "curing" is greatly enhanced.

Hsp104's roles in both thermotolerance and prion maintenance spurred further studies focusing on the protein's structure and function. The Hsp104 monomer can be subdivided into five domains. The bulk of the protein consists of two essential AAA+ modules that bind and hydrolyze ATP (*20*). Although hydrolysis at both sites is required to confer thermotolerance in yeast, the first AAA+ module has a much greater turnover number than the second (*21*). While the portion of the axial channel formed by the second AAA+ module is critical for protein disaggregation (*8*), mutations that affect nucleotide binding (*22*) or its folding stability (*23*) demonstrate that, relative to the first AAA+ module, the second AAA+ module plays a greater role in oligomer assembly. The first AAA+ module is interrupted by a coiled-coil domain that is required for the function of both Hsp104 (*24*) and ClpB (*25*). These proteins also contain a conserved

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N-terminal domain that is dispensable for the function of ClpB (*25*) and for thermotolerance in Hsp104 (*26*). Finally, at the C-terminal end of the protein there is an acidic extension found in both fungal Hsp104's and plant Hsp101's but not in bacterial ClpB's (Supporting Information Figure 1). Although the functional significance of the other domains of Hsp104 has been previously explored, the role of the C-terminal extension in Hsp104 structure and function is unknown.

In *S. cerevisiae* Hsp104, this extension consists of residues 870−908; for the purposes of this study, this 38-amino acid segment can be divided into three regions of interest. First, the extreme C-terminal tetrapeptide DDLD (residues 905−908) resembles the EEVD motif found in cytosolic members of the Hsp70 and Hsp90 families, which serves as a docking site for tetratricopeptide repeat (TPR)[1]-containing adaptor proteins (cochaperones) and functions in the assembly of multichaperone complexes (*27*). Indeed, Hsp104 physically interacts with the Hsp90 cochaperones Sti1, Cpr7, and Cns1 in vitro and in vivo in respiring cells (*28*). Furthermore, deletion of the last eight C-terminal residues of Hsp104 (including the DDLD tetrapeptide) abolishes the interaction with Sti1 in vitro. Second, the C-terminal extension of Hsp104 contains an abundance of negatively charged residues (Asp and Glu) from residue 886 to residue 908. It has been proposed that this region interacts electrostatically with the positively charged polypeptide poly-L-lysine (pLK), which binds to Hsp104 and stimulates its ATPase activity, a notion supported by the observation that the minimal pLK-binding fragment of Hsp104 consists of residues 773−908 (*29*). Finally, alignment of available fungal Hsp104 sequences (Supporting Information Figure 1) reveals a conserved VLPNH sequence (residues 878−883) proximal to the second AAA+ domain to which no function has been hitherto ascribed.

We used deletion analysis to determine the structural and functional significance of the C-terminal extension of Hsp104 both in vivo and in vitro. Removal of the extreme C-terminal DDLD sequence alone (Hsp104Δ4) greatly diminished the interaction of Hsp104 with the TPR-domain-containing cochaperone Cpr7 but had no significant effect on thermotolerance. Removal of the acidic portion of the C-terminal extension (Hsp104Δ22) did not abolish poly-L-lysine stimulation of Hsp104's ATPase activity; instead, size exclusion chromatography provided evidence that this segment plays a role in promoting oligomer assembly. Finally, a truncated Hsp104 lacking the entire extension including the conserved VLPNH sequence (Hsp104Δ38) failed to oligomerize in vitro, revealing that critical determinants for stable assembly of Hsp104 exist beyond the second AAA+ module.

MATERIALS AND METHODS

Plasmid Construction. PCR products were generated from pBluescript Cas5 (*30*) using the T7 promoter primer and one of three unique primers (Hsp104Δ4, 5′-AGCTCTCGAGTTAATCAATTTCCATACTGTCCTC-3′; Hsp104Δ22, 5′-ACTGCTCGAGTTAAGTAGCTTCGTGATTTGGTAG-3′; Hsp104Δ38, 5′-AGCTCTCGAGTTAAGGAACATTTTTCATCACGAG-3′) and were inserted into the p413GAL1 vector (*31*). Coding sequences for each protein were also subcloned into the pPROEX-HT-b vector (Invitrogen) as *Bam*HI/*Xho*I fragments for bacterial expression (see below). C-terminal truncation mutants containing the Y662W probe were created by replacing the *Nde*I/*Spe*I fragments of pPROEX-HT-b Hsp104Δ22 and Hsp104Δ38 with that of the Hsp104Y662W coding sequence (*8*). The Y819W probe was incorporated into the Hsp104Δ22 and Hsp104Δ38 sequences by PCR, amplifying the Hsp104Y819W (*32*) sequence using the forward primer 5′-ATAATCGTAGCTTGTTTAATGATGAGGACATG-3′ and the appropriate C-terminal truncation primer described above. PCR products were digested with *Spe*I/*Xho*I and ligated into pPROEX-HT-b plasmids containing Hsp104 coding sequences cut with the same enzymes. All plasmids were sequenced to verify the fidelity of the PCR amplification step.

Strain Construction. The kanamycin resistance gene in BY4741 *cpr7*::KAN^R was replaced with the nourseothricin resistance gene using the switcher plasmid p4339 (*33*). Genomic DNA from the resulting strain, BY4741 *cpr7*::NAT^R, was used as the template for PCR using primers that anneal approximately 350 bp upstream and downstream of the *CPR7* locus. The resulting PCR product was used to transform BY4741 *hsp104*::KAN^R from the yeast deletion array set, giving rise to BY4741 *hsp104*Δ*cpr7*Δ. The expected genotype of the strain was verified by antibiotic resistance and by PCR analysis of genomic DNA.

Thermotolerance Assay. YPH499Δ104 cells (*5*, *34*) were transformed with plasmids encoding the full-length protein and each truncation mutant. Cells were grown to saturation in CSM lacking histidine supplemented with 2% galactose and 0.1% glucose, diluted into 50 mL of the same medium, and grown to a density of approximately 2.0×10^6 cells/mL. Cultures were preconditioned at 37 °C for 1 h, after which 1 mL aliquots were transferred to preheated, sterile glass tubes and incubated at 50 °C. Samples were withdrawn at various time points and grown on YPD agar for 2 d at 30 °C, and colonies were counted and compared to numbers of colonies formed by cultures sampled prior to the 50 °C heat treatment.

Western Blot. Cells were grown as above to a similar density. After glass bead lysis, 25 μg of protein was separated by SDS−PAGE, transferred to PVDF, and probed with a 1:100 000 dilution of rabbit polyclonal anti-Hsp104 (*23*). Immunocomplexes were detected using HRP-conjugated goat anti-rabbit IgG (BioShop) and visualized with enhanced chemiluminescence.

Protein Purification. BL21 gold codon plus cells (Stratagene) were transformed with pPROEX Hsp104, Hsp104Y662W, Hsp104Y819W, Hsp104Δ4, Hsp104Δ22, Hsp104Δ22Y662W, Hsp104Δ22Y819W, Hsp104Δ38, Hsp104Δ38Y662W, or Hsp104Δ38Y819W. Fresh transformants were used to inoculate 1 L of Circlegrow (MP Biomedicals) containing 100 μg/μL ampicillin and 34 μg/μL chloramphenicol. Cultures were grown to an OD_{600} of ∼0.7. Expression was induced by adding IPTG to 1 mM, and cultures were grown overnight at 18 °C. Cells were harvested and lysed by a French press, and His_6-tagged Hsp104 was purified by Ni^{2+}−NTA (Qiagen)

[1] Abbreviations: CSM, complete synthetic medium; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GSH, glutathione; HRP, horseradish peroxidase; IPTG, isopropyl β-D-1-thiogalactopyranoside; NTA, nitrilotriacetic acid; pLK, poly-L-lysine; PVDF, poly(vinylidene difluoride); TPR, tetratricopeptide repeat; YPD, yeast extract peptone dextrose.

chromatography. The polyhistidine tag was removed using tobacco etch virus protease (Invitrogen); cleaved tags and uncleaved Hsp104 were removed by binding to Ni^{2+}−NTA agarose. The proteins Hsp104, Hsp104Y662W, Hsp104Y819W, and Hsp104Δ4 underwent an additional anion exchange chromatography step. Purity was assessed by SDS−PAGE, and peak fractions were pooled, dialyzed in 50 mM Tris−HCl, pH 8.0, 500 mM NaCl, 10% (v/v) glycerol, and 1.4 mM β-mercaptoethanol, frozen, and stored at −80 °C. All proteins exhibited similar purity.

BL21 gold codon plus cells were transformed with pPROEX Ssa1 or pPROEX Ssa1Δ4. Fresh transformants were used to inoculate 2 L Circlegrow (MP Biomedicals) containing 100 μg/mL ampicillin and 34 μg/mL chloramphenicol. Cultures were grown at 37 °C to an OD_{600} of ∼0.6. IPTG (100 μM) was added, and cultures were grown overnight at 18 °C. Cells were harvested, washed once with cold water, and snap frozen in liquid nitrogen. Cell pellets were thawed and resuspended to 40 mL in nickel start buffer (NSB; 20 mM Tris−HCl, pH 8.0, 500 mM NaCl, 10 mM imidazole, and 1.4 mM β-mercaptoethanol) containing 0.5 mM PMSF and a protease inhibitor cocktail (0.1 μg/mL each aprotinin, pepstatin A, and leupeptin). Cells were disrupted by sonication, and the soluble fraction was collected after centrifugation at 30000*g*. The cell lysate was applied to Ni^{2+}−NTA agarose, washed extensively with NSB, and eluted with NSB containing 200 mM imidazole. Purified protein was exhaustively dialyzed versus 10 mM HEPES−KOH, pH 7.6, 150 mM KCl, 10 mM $MgCl_2$, 1.4 mM β-mercaptoethanol, and 10% (v/v) glycerol.

BL21 gold cells were transformed with pGEX Cpr7 (gift of D. Picard). A fresh transformant was used to inoculate 20 mL of LB containing 100 μg/mL ampicillin. After growth at 30 °C overnight, the culture was diluted into 1 L Circlegrow containing 100 μg/mL ampicillin and grown to an OD_{600} of ∼0.8 at 30 °C. IPTG (2 mM) was added and the culture grown at 30 °C for an additional 3 h. Cells were harvested, washed once with cold water, and snap frozen. The thawed cell pellet was resuspended to 20 mL in PBS containing 10% (v/v) glycerol, 5 mM DTT, 0.5 mM PMSF, and protease inhibitor cocktail and disrupted by sonication. After sonication, Triton X-100 was added to 1% (v/v). The soluble fraction was collected after centrifugation at 30 000*g* and incubated with 3 mL of equilibrated GSH−agarose. The resin was washed extensively with PBS containing 200 mM NaCl, 10% (v/v) glycerol, 1 mM DTT, and 0.5 mM PMSF and bound proteins eluted with 50 mM Tris−HCl, pH 8.0, 10 mM GSH, and 10% (v/v) glycerol. Peak fractions were pooled and dialyzed extensively versus 10 mM HEPES−KOH, 100 mM KOAc, 1 mM EDTA, and 10% (v/v) glycerol.

Pull-Down Assays. (1) In Vitro. A 50 pmol portion of His_6-tagged Ssa1 or Ssa1Δ4 was incubated with 500 pmol of GST-Cpr7 or 1 nmol of GST in RFBE50X (20 mM HEPES−KOH, pH 7.6, 50 mM KOAc, 10 mM $Mg(OAc)_2$, 0.5 mM EDTA, 2 mM DTT, 0.05% (v/v) Triton X-100) in a total volume of 250 μL for 2 h on ice. An 80 μL portion of a 50% GSH−agarose (Sigma) slurry equilibrated in RFBE50X was added, and the reactions were rotated at 4 °C for 1 h. GSH−agarose was collected by centrifugation (500*g*). The supernatants containing unbound proteins were removed, and the GSH−agarose was washed with 1 mL of RFBE50X three times. Bound proteins were solubilized by the addition of 60 μL of 1.5× SDS−PAGE sample buffer and incubation at 95 °C for 5 min. His_6-tagged Ssa1 proteins were detected using the HisProbe-HRP detection kit (Pierce) and ECL plus (GE Healthcare) according to the manufacturers' instructions.

(2) In Vivo. BY4741*hsp104*Δ*cpr7*Δ was transformed with p2UGST Cpr7 (gift of D. Picard) and p413 Hsp104 or p413 Hsp104Δ4. Transformants were grown in 5 mL of CSM lacking uracil and histidine supplemented with 2% galactose and 0.1% glucose for 2 d at 30 °C. Cells were harvested, washed once with cold H_2O, and resuspended in 300 μL of RFBE100X (RFBE50X containing 100 mM KOAc) containing 0.5 mM PMSF and protease inhibitor cocktail. Cells were disrupted by glass bead lysis, and the supernatant was collected after centrifugation at 16 000*g* for 10 min. A 300 μg portion of protein from the supernatant was diluted to 250 μL with RFBE100X, and an aliquot was withdrawn for analysis of the input. A 50 μL portion of an equilibrated 50% GSH−agarose slurry was added, and the reactions were rotated at 4 °C for 2 h. GSH−agarose was collected and washed with 1 mL of RFBE100X three times. Bound proteins were solubilized by the addition of 60 μL of 1.5× SDS−PAGE sample buffer and incubation at 95 °C for 5 min.

ATPase Assays. Proteins were dialyzed overnight into 10 mM Tris−HCl, pH 7.5, 5 mM $MgCl_2$, 150 mM NaCl, and 0.02% (v/v) TX-100, and the concentration was determined using a dye-binding assay (BioRad). ATP (0−1 mM for the wild-type and Hsp104Δ4, 0−3 mM for Hsp104Δ22 and Hsp104Δ38) was added to 2 μg of protein in a total volume of 25 μL, yielding a final salt concentration of 36 mM. Reactions were incubated at room temperature for 1 min (Hsp104Wt, Hsp104Δ4) or 15 min (Hsp104Δ22, Hsp104Δ38). Reactions were quenched by the addition of malachite green, and color development was stopped 1 min later by the addition of 34% (w/v) citric acid (*30*). Absorbance at 645 nm was measured, and the inorganic phosphate concentration was calculated by comparison to a standard curve. The initial reaction rates were calculated and fit to the Michaelis−Menten equation

$$V_{initial} = V_{max}[S]/(K_m + [S])$$

where $V_{max} = k_{cat}E_{total}$, to determine the kinetic parameters K_m and k_{cat} for each mutant. Enzyme activities were reported as k_{cat}/K_m ratios.

Poly-L-*lysine Stimulation*. ATPase assays were performed and analyzed as described above, but at 150 mM NaCl instead of 36 mM to lower the basal hydrolysis activity. Assays were carried out in the presence and absence of 4 μM 15 kDa pLK (Sigma). In the absence of pLK, the wild-type and Hsp104Δ4 were incubated with 0−1 mM ATP for 5 min, while Hsp104Δ22 and Hsp104Δ38 were incubated with 0−3 mM ATP for 15 min at room temperature. In the presence of pLK, all proteins were incubated with 0−3 mM ATP for 1 min.

Molecular Crowding. ATPase assays were performed as described above, at 150 mM NaCl and 25% (w/v) 66.9 kDa dextran (Sigma-Aldrich (item D-1537), produced by *Leuconostoc mesenteroides*). Hsp104WT, Hsp104Δ22, and Hsp104Δ38 proteins were all incubated with 0−1 mM ATP for 5 min at room temperature prior to addition of malachite green.

Size Exclusion Chromatography. Proteins were dialyzed overnight in ATPase buffer containing 2 mM DTT and diluted to 0.6 μg/μL. Samples were incubated at room temperature for 10 min in the presence or absence of 2 mM ADP, and 100 μL of were chromatographed on a Superose 6 column (Pharmacia) at a flow rate of 0.2 mL/min. Fractions of 400 μL were collected between 7 and 20 mL elution volume. In the case of samples containing ADP, 2 mM ADP was also added to the ATPase buffer used for elution. The protein content of each fraction was measured using a dye-binding assay (BioRad).

High-Temperature Solubility Assay. Proteins were dialyzed overnight in ATPase buffer and diluted to 0.6 μg/μL. ADP was added to 2 mM, and proteins were incubated at room temperature for 10 min prior to further incubation at 50 °C for 15 min. Solutions were subjected to differential ultracentrifugation at 100 000*g* and 25 °C for 30 min. Supernatants were collected, and pellets were washed twice with ATPase buffer. Finally, pellets were resuspended, and samples were analyzed by SDS−PAGE and stained with Coomassie blue. Loading volumes were adjusted for direct comparison of the protein content of each fraction.

Influence of Hsp104 on [PSI+]. The yeast strain GT81-1C (*MAT*a *ade1−14 (UGA) his3-Δ200 leu2−3,112 lys2−801* 14 *trp1−289 ura3−52[PSI$^+$] [PIN$^+$]*) (*35*) was transformed with an empty vector or plasmids encoding wild-type Hsp104, Hsp104Δ22, and Hsp104Δ38 under control of a galactose-inducible promoter. Cells were spotted on CSM lacking histidine with 2% glucose and CSM lacking histidine with 0.1% glucose and 2% galactose plates and incubated for 3 d at 30 °C.

Multiple-Sequence Alignment. Sequences were retrieved from the RefSeq database using the BLAST algorithm. For bacteria, *Escherichia coli* ClpB was used as the search query, for plants, *Arabidopsis thaliana* Hsp101 was used, and for fungi, *S. cerevisiae* Hsp104 was used. For bacterial proteins, only the top 30 hits were used in the alignment. Alignment was performed using the Blosum62 matrix in the program BioEdit (*36*).

Fluorescence Spectroscopy. All proteins containing tryptophan probe mutations were dialyzed overnight in buffer containing 25 mM HEPES−NaOH, pH 7.5, and 200 mM NaCl. Proteins were diluted to 1 μM into reactions containing increasing concentrations of guanidine hydrochloride (BioShop) which were incubated overnight at room temperature to establish equilibrium. Fluorescence measurements were made using the SPEX FluoroLog-3 (FL3-22, Jobin Yvon Inc.). For the proteins containing the Y662W mutation, emission was measured at 360 nm and averaged over 1 min with an excitation wavelength of 295 nm and a 5 nm band-pass. All values were buffer-subtracted. For the Y819W mutations, two emission spectra were collected from 330 to 390 nm and averaged. Peaks of buffer-subtracted spectra were found by determining the highest fluorescence value and values that were within 1% of the peak value. The respective wavelengths were averaged to determine the peak wavelength.

Denaturation Curve Analysis. Data obtained via fluorescence spectroscopy were converted into fraction of unfolded protein using the equation

$$f_U = (y_F - y)(y_F - y_U)$$

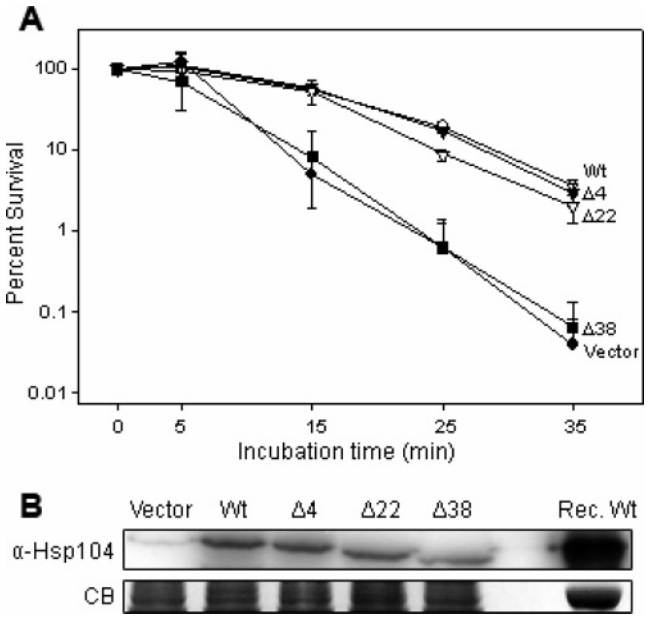

FIGURE 1: Effect of C-terminal truncations on Hsp104-mediated thermotolerance. (A) Preconditioned cells expressing full-length and truncated Hsp104 from a galactose-inducible promoter were incubated at 50 °C for up to 35 min. Aliquots of cells were plated and grown on YPD, and the percent survival was measured over time. Short designations for wild-type Hsp104, Hsp104Δ4, Hsp104Δ22, and Hsp104Δ38 are Wt, Δ4, Δ22, and Δ38, respectively. "Vector" indicates the control strain transformed with plasmid alone and therefore lacking Hsp104. Data presented are mean values of two independent experiments; error bars represent the standard deviation. (B) Cell lysates of strains expressing Hsp104 and mutants were analyzed by Western blot with polyclonal α-Hsp104 antibody (top). Lysate lanes each contain 25 μg of protein; the "Rec. Wt" lane contains 5 μg of recombinant wild-type Hsp104. A duplicate SDS−PAGE gel stained with Coomassie blue verifies equal loading (bottom). Data presented are representative of two independent experiments.

where f_U represents the fraction of unfolded protein, y_F the value that most closely represents the folded state, y_U the value for the unfolded state, and y the signal obtained at a particular denaturant concentration (*37*). The denaturation curves obtained for the mutants containing the domain-specific probe Y662W were fitted to a two-state unfolding model:

$$F360 = (y_n + y_u)(K)/(1 + K)$$

where K represents $\Delta G_{D-N}^{H_2O} + m$[GuHCl]/$RT$ and y_n and y_u represent linear dependencies of the folded and unfolded states, respectively (*32*).

RESULTS

Truncation of the Entire C-Terminal Extension Abolishes Thermotolerance. We first assessed the ability of each C-terminal truncation mutant to confer thermotolerance to an *hsp104* deletion strain. Preconditioned cells were exposed to a lethal heat shock (50 °C), and cell survival was normalized to the cell density of the culture prior to heat shock and plotted over time (Figure 1A). The smallest truncation mutant, Hsp104Δ4, did not significantly differ from the wild-type protein in terms of in vivo function. Cells expressing Hsp104Δ22 exhibited only a slight decrease in survival relative to wild-type cells, but survived 35 min of heat stress 100-fold better than cells lacking Hsp104. Thus,

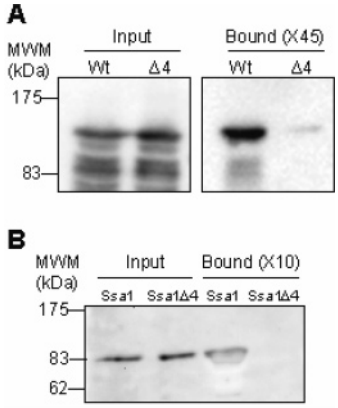

FIGURE 2: Role of C-terminal tetrapeptides in the interaction between the TPR domain cochaperone Cpr7 and Hsp104 and Ssa1. (A) In vivo pull-down of GST−Cpr7 coexpressed in yeast with either wild-type Hsp104 (Wt) or Hsp104Δ4 (Δ4). Hsp104 was detected by Western blot. The amount of the bound fraction is equivalent to 45× the input. (B) In vitro pull-down of GST−Cpr7 with His-tagged Ssa1 and Ssa1Δ4 lacking the C-terminal EEVD motif. The amount of the bound fraction loaded is equivalent to 10× the input fraction. His-tagged Ssa1 was detected with a Ni^{2+}-activated HRP conjugate.

neither the DDLD sequence nor the acidic region of the C-terminal extension are essential for the function of Hsp104 in thermotolerance. The largest truncation mutant, Hsp104Δ38, however, did not confer thermotolerance; instead, cells expressing this protein were just as susceptible to lethal heat stress as cells lacking Hsp104.

To ensure that any differences in thermotolerance were occurring at the level of protein activity and not expression, Western blots were performed to measure the expression levels of each protein (Figure 1B). All proteins were expressed at approximately equal levels, except for Hsp104Δ38, which was only slightly diminished. However, even a 50% reduction in expression under these conditions is not sufficient to reduce Hsp104-dependent refolding in vivo (J. M. Tkach and J. R. Glover, in press).

Deletion of the C-Terminal Tetrapeptide DDLD Greatly Reduces Physical Interaction with Cpr7. Removal of the C-terminal DDLD tetrapeptide from Hsp104 had no significant impact on the ability of Hsp104 to confer thermotolerance, suggesting that the interaction between Hsp104 and Hsp90 cochaperones is dispensable for this process. Others have previously demonstrated that the interaction between Hsp104 and the TPR-domain-containing cochaperone Sti1 is dependent on the C-terminal eight amino acid residues of Hsp104 (*28*). To demonstrate that DDLD alone was important for Hsp104's interaction with Hsp90 cochaperones, we coexpressed either full-length Hsp104 or Hsp104Δ4 along with a GST−Cpr7 fusion protein in yeast lacking both endogenous Hsp104 and Cpr7. GST−Cpr7 was able to complement the slow-growth phenotype of a *cpr7*Δ strain (*38*), demonstrating that the fusion protein is biologically active (data not shown). When GST−Cpr7 was pulled down on glutathione beads, full-length Hsp104 was coprecipitated whereas Hsp104Δ4 was barely detectable (Figure 2A). To further demonstrate the specificity of the pull-down, recombinant GST−Cpr7 was pulled down from a reaction containing His-tagged full-length Ssa1 or His-tagged Ssa1Δ4, which lacks the C-terminal EEVD motif common to both Hsp70 and Hsp90. In these reactions only full-length His-tagged Ssa1 could be detected in the bound fraction (Figure 2B). While this illustrates the role of the Hsp104 DDLD sequence for its interaction with Cpr7, further in vitro studies were carried out on recombinant Hsp104's to better understand the structural and functional consequences of truncating or eliminating the C-terminal extension.

Truncation of the C-Terminal Extension Does Not Significantly Affect the Protein Fold or Stability. A standard purification protocol was used for the wild-type and Hsp104Δ4, but was altered for Hsp104Δ22 and Hsp104Δ38, which were aggregation prone in low salt buffers, with Hsp104Δ38 being significantly more likely to precipitate (data not shown). As measured by circular dichroism spectroscopy (Supporting Information Figure 2), the wild-type protein, Hsp104Δ4, and Hsp104Δ22 appeared to possess the same global fold. Hsp104Δ38's spectrum shared the same overall shape with the aforementioned proteins' spectra, yet was slightly less intense. This reduced intensity may result from a lower protein concentration caused by spontaneous aggregation or could indicate that truncation of the entire C-terminal extension perturbs protein folding.

To investigate the possibility that this observation was caused by a folding defect, domain-specific tryptophan probes were introduced into the second AAA+ module and C-terminal small domain to establish the effect of C-terminal truncations on the stability of these domains (Supporting Information Figure 3). The unfolding of the second AAA+ module of full-length Hsp104, Hsp104Δ22, and Hsp104Δ38 was monitored using a tryptophan probe at residue 662 (*8*). Cooperative unfolding was observed, and the denaturant concentration at which half-maximal unfolding occurred was similar for all three proteins, ranging between 2.0 and 2.25 M guanidine hydrochloride. This indicates that the folding stability of the second AAA+ module is not significantly affected by C-terminal truncations (Supporting Information Figure 3a). Similarly, a probe specific for the C-terminal small domain (Y819W) (*32*) showed little difference in full-length Hsp104, Hsp104Δ22, and Hsp104Δ38 stability (Supporting Information Figure 3b). While cooperative unfolding was not observed, half-maximal unfolding for each protein occurred between 1.5 and 1.8 M guanidine hydrochloride. Together these results indicate that truncation of the entire C-terminal extension has little effect on the folding of either the second AAA+ module or the C-terminal small domain. These data suggest that the slight difference in CD spectra between Hsp104Δ38 and the other proteins is more likely to be caused by aggregation of a small population of molecules than protein misfolding.

The C-Terminal Extension Is Required for Normal in Vitro ATPase Activity. Since the C-terminal truncations did not appear to affect the folding of recombinant Hsp104's, several in vitro functional assays were performed to further characterize these mutants. The kinetics of ATP hydrolysis for each protein was measured using an in vitro assay (Figure 3 and Table 1). Since ATP hydrolysis is required for refolding activity in vitro and in vivo (*21*), Hsp104Δ38 was expected to have a low ATPase activity, while Hsp104Δ4 and Hsp104Δ22 were expected to have approximately wild-type levels of activity. Unexpectedly, both Hsp104Δ22 and Hsp104Δ38 had drastic ATPase defects; they were only 1.6% and 0.3%, respectively, as active as the wild-type protein, as determined by k_{cat}/K_m ratios. The mutant lacking the

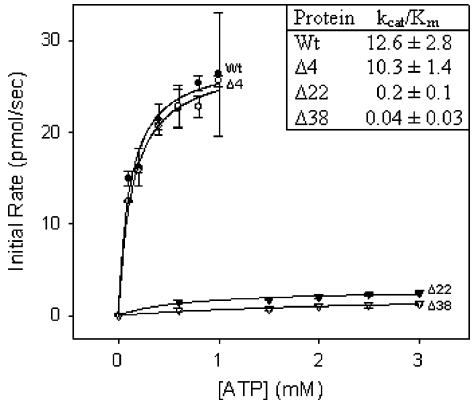

FIGURE 3: Effect of C-terminal truncations on Hsp104 in vitro ATPase activity. Initial rates of ATP hydrolysis were measured and fit to the Michaelis−Menten equation to determine kinetic parameters for each mutant. Data points are means of duplicate determinations, and error bars represent the standard deviation. Inset: Catalytic efficiency of ATP hydrolysis by Hsp104 and truncation mutants represented by k_{cat}/K_m.

Table 1: Effects of Poly-L-lysine and Dextran on the Catalytic Efficiency of ATP Hydrolysis by Hsp104 and Truncation Mutants[a]

	unstimulated	+ 4 μM poly-L-lysine		+ 25% dextran	
protein	k_{cat}/K_m	k_{cat}/K_m	fold stim	k_{cat}/K_m	fold stim
Wt	0.7 ± 0.2	2.3 ± 0.4	3.2 ± 0.9	12 ± 2.08	17 ± 4.8
Δ4	0.6 ± 0.1	2.1 ± 0.5	3.8 ± 1.0	not determined	
Δ22	0.2 ± 0.1[b]	2.2 ± 0.8	14 ± 7.6	2.7 ± 0.5	17 ± 7.4
Δ38	0.02 ± 0.01[b]	2.4 ± 2.3[b]	150 ± 190	1.6 ± 0.4	100 ± 86

[a] Catalytic efficiency of ATP hydrolysis by Hsp104 and truncation mutants represented by K_{cat}/K_m. Fold stimulation represents the catalytic efficiency of Hsp104 in the presence of poly-L-lysine or dextran relative to that in standard buffer containing 150 mM NaCl. [b] Due to very low ATPase activities kinetic parameters could not be determined with high precision and are listed to demonstrate the proteins' inefficiency in ATP hydrolysis.

DDLD tetrapeptide (Hsp104Δ4) was 80% as active as the full-length protein. While these observations began to explain why Hsp104Δ38 did not provide thermotolerance, it was unclear as to why Hsp104Δ22 was able to confer thermotolerance despite having such a low ATPase activity.

The C-Terminal Extension Is Not Required for ATPase Stimulation by Poly-L-lysine. A previous study had suggested that the positively charged polypeptide pLK resembled the surface of aggregated proteins and was a potential substrate mimic or recognition factor for Hsp104 (*29*). The authors had shown that the ATPase activity of Hsp104 could be specifically stimulated by the addition of micromolar concentrations of pLK and concluded that the effect was mediated through an interaction with the C-terminal domain (residues 773−908). To test the function of the acidic C-terminal tail, all proteins were subjected to ATPase assays in the presence and absence of 4 μM 15 kDa poly-L-lysine at 150 mM NaCl (Figure 4). The initial hydrolysis rates were measured, and kinetic parameters were calculated (Table 1). Fold stimulation was calculated as the ratio of the k_{cat}/K_m value in the presence of pLK to the same value in the absence of pLK. While the addition of 15 kDa pLK had a stimulatory effect on each protein's ATPase activity, Hsp104Δ22 and Hsp104Δ38 were more strongly affected (14- and 150-fold stimulation, respectively) than the wild-type or Hsp104Δ4 (approximately 3- and 4-fold stimulation each). This shows that, under these conditions, the acidic residues in the

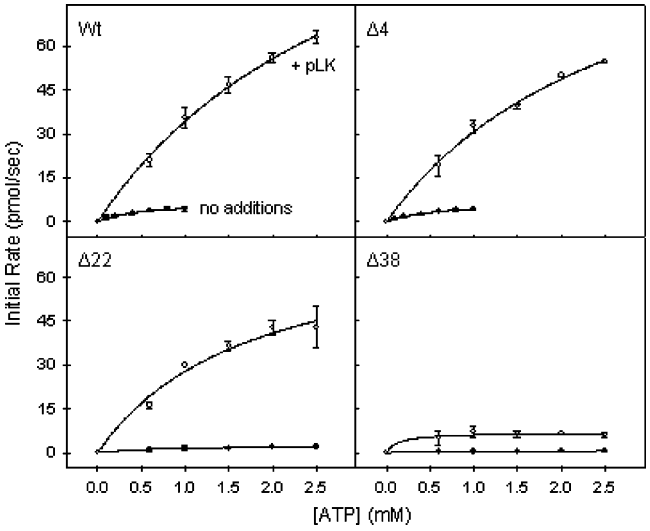

FIGURE 4: Effect of C-terminal truncations on poly-L-lysine stimulation of Hsp104 in vitro ATPase activity. ATPase assays were performed in the presence (empty circles) and absence (filled circles) of 4 μM 15 kDa poly-L-lysine. Assays were performed in 150 mM NaCl to lower the basal activity to observe a greater stimulatory effect. Data points are means of duplicate determinations, and error bars represent the standard deviation.

C-terminal extension are not required for stimulation by pLK and that stimulation is likely mediated by another part of Hsp104.

The VLPNH Sequence Is Essential for Oligomer Assembly. While the in vitro assembly of Hsp104 hexamers is spontaneous in buffers of low ionic strength (*21*), in buffers of higher ionic strength full assembly requires the addition of adenine nucleotides (*22*). Each mutant was subjected to size exclusion chromatography in the presence and absence of ADP to probe its ability to oligomerize. In buffer containing 150 mM NaCl, the wild-type protein eluted from the column at approximately 14.2 mL in the absence of ADP (Figure 5), the same volume as that of the 440 kDa globular standard, suggesting that, at this salt concentration, the protein is not fully assembled. Addition of 2 mM ADP shifted the wild-type elution peak to 13 mL, close to that of the 669 kDa standard, corresponding to the fully assembled hexamer (*22*). Both Hsp104Δ4 and Hsp104Δ22 exhibited a similar peak shift upon addition of ADP, indicating that both proteins are capable of forming hexamers. In contrast to those of wild-type Hsp104 and Hsp104Δ4, the Hsp104Δ22 elution profile contained an additional shoulder reminiscent of the peak observed in the absence of nucleotide. It seems likely that this shoulder represents a population of incompletely assembled molecules. Unlike those of the other mutants, the elution profile of Hsp104Δ38 did not undergo the characteristic peak shift upon addition of ADP, indicating that this protein was unable to fully assemble in the presence of nucleotide. Instead, the shape of the profile remained unchanged but decreased in intensity slightly. These observations indicate that the C-terminal extension of Hsp104 plays a role in hexamer assembly that could account for the reduced ATPase activities of Hsp104Δ22 and Hsp104Δ38, even though the assembly defect of Hsp104Δ22 is less pronounced than that of Hsp104Δ38.

Molecular Crowding Enhances in Vitro ATPase Activity. While Hsp104Δ22 was able to provide robust thermotolerance, Hsp104Δ38 did not, even though both proteins had

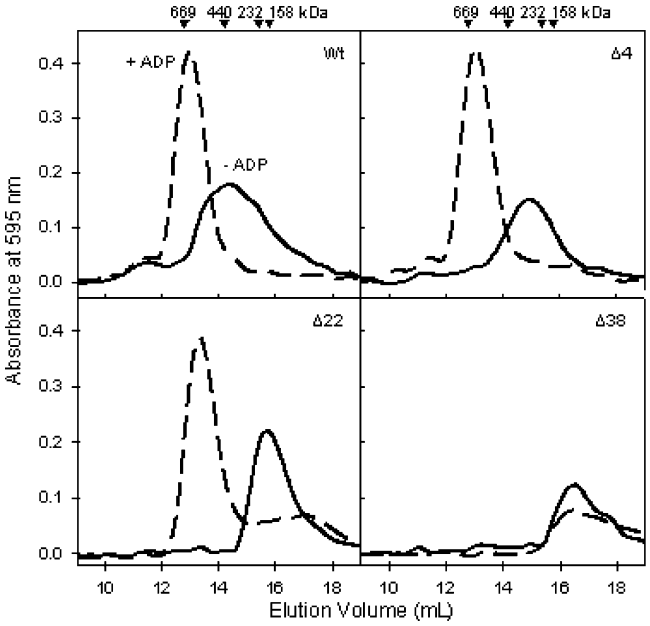

FIGURE 5: Effect of C-terminal truncations on Hsp104 in vitro oligomerization. Different assembly states of Hsp104 and truncation mutants in the presence (dotted line) and absence (solid line) of 2 mM ADP were analyzed using size exclusion chromatography. Arrows indicate the elution volume of globular molecular mass standards. Data presented are the mean values of two independent experiments for each condition.

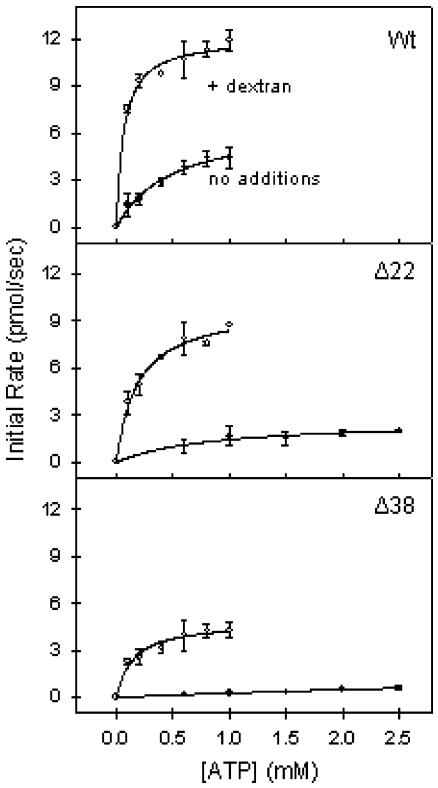

FIGURE 6: Effect of molecular crowding agents on Hsp104 truncation mutants' in vitro ATPase activity. ATPase assays were performed in the presence (empty circles) and absence (filled circles) of 25% dextran at 150 mM NaCl. Data points are means of duplicate determinations, and error bars represent the standard deviation.

only a fraction of the ATPase activity of the full-length protein. Since the oligomerization defect associated with Hsp104Δ22 was far less profound than that of Hsp104Δ38, we speculated that Hsp104Δ22 might be induced to fully assemble in vivo where molecular crowding is a significant factor in enhancing protein−protein interactions (*39*). Because the ATPase activity of Hsp104 is sensitive to the oligomeric state of the protein (*40*), we determined the effect of molecular crowding on the ATPase activity of Hsp104 by simulating the macromolecular milieu of the cell with an artificial crowding agent (70 kDa dextran). The ATPase activities of wild-type Hsp104, Hsp104Δ22, and Hsp104Δ38 were each enhanced in the presence of crowding agent (Figure 6 and Table 1). Most importantly the activities of both Hsp104Δ22 and Hsp104Δ38 were comparable and exceeded the activity of wild-type Hsp104 measured in the absence of dextran. Thus, on the basis of these results, we exclude the possibility that molecular crowding selectively enhances the function of Hsp104Δ22 that has a partial assembly defect over that of Hsp104Δ38 with a more pronounced assembly defect.

Hsp104 Lacking the VLPNH Sequence Provides Limited in Vivo Activity at 30 °C but Is Aggregation Prone at Elevated Temperatures. Since the ATPase defects of both Hsp104Δ22 and Hsp104Δ38 were partially recovered by the addition of dextran in vitro, we examined the in vivo function of the truncation mutants relative to the wild-type under nonstress conditions. Wild-type Hsp104, Hsp104Δ22, and Hsp104Δ38 were expressed in the [*PSI*$^+$] strain GT81-1C using the same plasmids and conditions used for the thermotolerance assay. In this strain the *ade1−14* allele containing a UGA nonsense codon provides a convenient readout of suppression based on colony pigmentation. [*PSI*$^+$] cells, in which the translation termination factor Sup35 is largely insoluble (*18*), are white, indicating efficient readthrough. Pigment accumulation therefore signals an increase in translation fidelity, correlating with increased solubility of Sup35 (*11*) as a consequence of an overall increase in functional Hsp104 hexamers either by the co-oligomerzation of plasmid-borne Hsp104's with the endogenous protein or by formation of hexamers comprised entirely of galactose-induced Hsp104's. Since Hsp104Δ22 had a weaker oligomerization defect than Hsp104Δ38 and was able to confer thermotolerance at a level close to that of wild-type Hsp104, it was predicted that overexpression of Hsp104Δ22 would exhibit an antisuppression phenotype similar to that of the wild-type protein. Overexpression of Hsp104Δ38, incapable of nucleotide-dependent oligomerization in vitro, was therefore expected to exhibit a weaker phenotype than either the wild-type or Hsp104Δ22.

When [*PSI*$^+$] cells were cultured on glucose medium to prevent the expression of plasmid-borne Hsp104, the cells remained white. As expected, galactose-induced expression of wild-type Hsp104 caused an increase in red pigmentation, indicative of antisuppression (Figure 7A). Cells expressing Hsp104Δ22 were indistinguishable from those expressing the wild-type protein, supporting the idea that the minor oligomerization defect of Hsp104Δ22 is ameliorated in the crowded cellular environment. However, cells expressing Hsp104Δ38 displayed weak antisuppression compared to cells expressing either wild-type Hsp104 or Hsp104Δ22. This supports the idea that, in vivo, the activity of Hsp104Δ38 can be recovered, albeit to a limited extent, as suggested by the ability of molecular crowding agents to stimulate its ATPase activity.

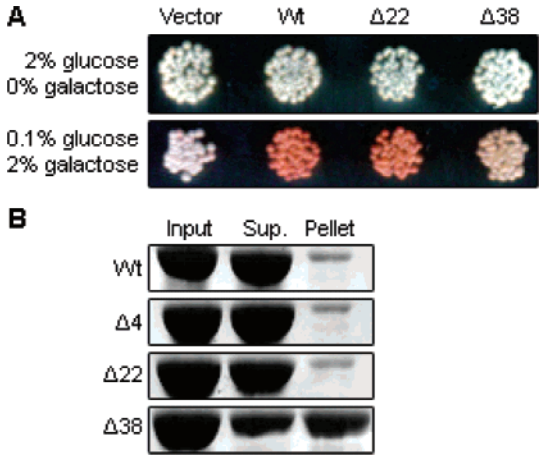

FIGURE 7: Effect of C-terminal truncations on Hsp104 high-temperature stability and in vivo activity. (A) Yeast strain GT81-1C was transformed with galactose-inducible vector, Hsp104, Hsp104Δ22, and Hsp104Δ38. Cells were grown on selective media containing either 2% glucose or 2% galactose and 0.1% glucose to induce protein expression. The extent of antisuppression was determined by red pigmentation of the resulting colonies. Images presented are representative of four independent clones. (B) Proteins were incubated with 2 mM ADP at room temperature before further incubation at 45 °C for 15 min. Samples were subjected to differential ultracentrifugation, yielding supernatant and pellet fractions which were analyzed by SDS−PAGE and stained with Coomassie blue. The "input" lane represents an equal volume of samples prior to ultracentrifugation. Data presented are representative of two independent experiments.

We have previously shown that some Hsp104 mutants that function well in prion propagation but which fail to provide thermotolerance are inactivated and become aggregation prone in vivo at high temperatures (*23*). To address whether high-temperature inactivation might account for the failure of Hsp104Δ38 to provide any detectable thermotolerance, we examined the aggregation propensity of each truncation mutant relative to the wild-type under the same conditions as the size exclusion runs in the presence of ADP. After incubation at 50 °C, the wild-type protein, Hsp104Δ4, and Hsp104Δ22 all remained largely soluble with limited aggregation (Figure 7B). In the case of Hsp104Δ38, however, a significant amount of protein was rendered completely insoluble under these conditions, suggesting that indeed this mutant's failure to provide thermotolerance in vivo is caused by its aggregation at high temperatures.

DISCUSSION

The only detailed structural data for Hsp104/ClpB at the atomic level can be derived from the model of *Thermus thermophilus* ClpB (*41*) that, like other bacterial ClpB's, extends only a handful of amino acid residues C-terminal to the final β-strand. Thus, the currently available data do not provide clues as to the structural role that the C-terminal extension might play in Hsp104. As for its functional role, the C-terminal extension of Hsp104 has been previously implicated in cochaperone binding and ATPase stimulation by pLK, but the biological significance of these findings has not been investigated further. In this work we examined the functional and structural consequences of removing progressively longer segments of the C-terminal extension and find that the role of this domain is primarily involved in oligomer assembly.

A previous study showed that Hsp104 interacts with the Hsp90 cochaperones Cns1, Cpr7, and Sti1 (*28*). The interaction between Hsp104 and Sti1 is dependent on the last eight C-terminal residues, including the DDLD sequence. Interestingly, this sequence resembles the conserved EEVD motif found in yeast and mammalian Hsp90's, which when removed abolishes the ability of Hsp90 to bind to the TPR domains of Sti1 (*42*). The same motif is also found at the C-termini of Hsp70's of the Ssa family of cytosolic chaperones in *S. cerevisiae* (*27*). Recruitment of disparate components of multichaperone complexes in this way (*27*) can facilitate the transfer of substrates between chaperones (*43*). We have speculated in the past that the interaction of TPR-domain-containing cochaperones with the C-terminus of Hsp104 could help to recruit Ssa−Hsp70's to the exit site of Hsp104's axial channel and thereby facilitate the transfer of polypeptides extracted from protein aggregates to the Hsp70/40 system to prevent reaggregation of the unfolded protein and promote refolding (*8*). Even though biochemical analysis of refolding reactions with ClpB and the DnaK system point to a crucial role of the Hsp70 before the action of the Hsp100 disaggregase (*7*), an additional role for Hsp70 after the Hsp100 cannot be excluded, and this additional interaction may be even more critical in vivo rather than in vitro. Given the potential importance of the DDLD tetrapeptide in *S. cerevisiae* Hsp104-mediated thermotolerance, we analyzed the consequences of truncating these last four residues.

Although we explicitly demonstrated that Hsp104Δ4 fails to interact with Cpr7, we would predict that the same deletion would abolish interactions with other Hsp90 cochaperones as well. The observation that Hsp104Δ4 has no defect in thermotolerance suggests that the Hsp104−Hsp90 cochaperone interactions are dispensable, at least for thermotolerance. Speculating that if the extreme C-terminal amino acids served as a crucial docking site for cochaperones, we predicted that we would be able to detect evidence of its conservation within the multiple-sequence alignment (Supporting Information Figure 1). In fact, this analysis shows that the extreme C-terminal residues of fungal Hsp104's are not identical to DDLD but generally resemble a sequence that begins with an acidic residue, followed by an aliphatic residue and a final acidic residue (which in some cases is preceded by a single aromatic residue). It is possible that C-terminal peptides with divergent sequences can still bind TPR domains (*27*). However, a survey of the available protein sequence data indicates that the EEVD motif of cytosolic Hsp70's and Hsp90's in the fungi is conserved among all of the fungal Ssa-type Hsp70's and Hsp90's (data not shown), supporting the idea that TPR−chaperone interaction motifs of these chaperones have not likely diverged in isolated lineages. Further analysis will be required to determine whether these interactions are physiologically significant for fungal Hsp104's or whether the interaction is simply incidental.

Both plant Hsp101's and fungal Hsp104's possess C-terminal extensions not found in bacteria. Although dissimilar in sequence among fungi, this region is conserved in terms of amino acid composition, containing an abundance of acidic residues. We find no evidence that this acidic region is required for pLK stimulation of Hsp104's ATPase activity. Experiments in which the ATPase activity of full-length

Hsp104 and Hsp104Δ38 was measured as a function of pLK concentration indicated that there was no significant difference in the amount of pLK required for half-maximal stimulation (data not shown), which further supports our conclusion that the C-terminal extension has only a minimal if any role at all in this phenomenon. This is consistent with the fact that bacterial ClpB, which has no C-terminal extension, is also stimulated by pLK (*44*). While our observation does not support a role for the C-terminal extension in the stimulation of Hsp104's ATPase activity, we cannot rule out the possibility that pLK−Hsp104 interaction mimics a physiologically relevant process.

Complete removal of the Hsp104 C-terminal extension also removes a region of high sequence identity among fungal Hsp104's. Under conditions used in our size exclusion experiments where Hsp104Δ22 assembles to a significant extent in the presence of nucleotide, Hsp104Δ38 remains unassembled. Previously both amino acid substitutions that influence nucleotide binding in the second AAA+ module of Hsp104 (*22*) and those that insert a polar or charged residue into the hydrophobic core of the C-terminal small domain (*23*) have shown that the integrity of the C-terminal AAA+ domain is required for normal oligomer assembly. In this work, we propose a role in assembly for residues beyond the second AAA+ domain.

It is possible that the short segment of the conserved sequence in fungi together with the acidic region contributes to oligomer assembly either independently or as an intrinsic structural part of the AAA+ small domain that is absent in prokaryotic ClpB's. Sequence alignments demonstrate that plants have an even longer conserved sequence proximal to the AAA+ small domain. Little is known about the structure−function relationship of Hsp101's except that, like Hsp104, nucleotide binding in the second but not the first AAA+ domain is required for assembly (*45*). It will be interesting to know whether the C-terminal extension of Hsp101's also plays a role in oligomer assembly.

While Hsp104Δ22 displayed only a marginal defect in thermotolerance, the in vitro ATPase activity of Hsp104Δ22 was very low and closer to that of Hsp104Δ38 than to the full-length protein. The partial restoration of ATPase activity of assembly-deficient mutants by molecular crowding supports the idea that reduced assembly of purified proteins in dilute solution may not directly correlate with their in vivo functions unless the effects of excluded volume on the apparent dissociation constants of interacting macromolecules can be taken into account (for a review, see ref *46*). Interestingly, the ATPase activity of even wild-type Hsp104 is enhanced under conditions of molecular crowding. Further work will be required to understand the significance of these observations with respect to the function of Hsp104 but also other proteins that function in oligomeric complexes.

Enhanced assembly in the crowded cytosol offers a possible explanation as to why Hsp104Δ22 is nearly as functional as the full-length protein in thermotolerance and indistinguishable in antisuppression in [*PSI*$^+$] cells at 30 °C. It could also explain how Hsp104Δ38 is able to retain at least some in vivo function in antisuppression; however, it does not explain why this protein fails to provide thermotolerance. We have previously demonstrated that Hsp104 mutants with assembly defects and low in vitro ATPase activities function well in in vivo and in vitro refolding and the propagation of the [*PSI*$^+$] prion, but fail to provide thermotolerance and become aggregation prone at the extreme temperatures used to test thermotolerance in *S. cerevisiae* (*23*). This explanation can also provide a rationale for Hsp104Δ38's failure to function even partially in thermotolerance; only Hsp104Δ38 exhibited a pronounced propensity for aggregation on exposure to high temperatures. The observation that there were no dramatic differences in the denaturant concentration required for half-maximal unfolding for either the second AAA+ module or the C-terminal small domain of full-length Hsp104, Hsp104Δ22, or Hsp104Δ38 suggests that the C-terminal extension confers resistance to high-temperature-induced aggregation without dramatically altering the folding stability of Hsp104.

Presumably at the time of acquisition of the Hsp104 C-terminal extension by the primitive ancestor of modern fungi, the ancestral Hsp104 could function well without it. At least in *S. cerevisiae* Hsp104, this extension has become pivotal to the assembly of the Hsp104 hexamer and resistance to aggregation at high temperatures and thereby plays a critical role in Hsp104 function at both normal and high temperatures.

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SUPPORTING INFORMATION AVAILABLE

Three figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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