Amino Acid Substitutions in the C-terminal AAA⁺ Module of Hsp104 Prevent Substrate Recognition by Disrupting Oligomerization and Cause High Temperature Inactivation*

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Hsp104 is an important determinant of thermotolerance in yeast and is an unusual molecular chaperone that specializes in the remodeling of aggregated proteins. The structural requirements for Hsp104-substrate interactions remain unclear. Upon mild heat shock, Hsp104 forms cytosolic foci in live cells that indicated co-localization of the chaperone with aggregates of thermally denatured proteins. We generated random amino acid substitutions in the C-terminal 199 amino acid residues of a GFP-Hsp104 fusion protein, and we used a visual screen to identify mutants that remained diffusely distributed immediately after heat shock. Multiple amino acid substitutions were required for loss of heat-inducible redistribution, and this correlated with complete loss of nucleotide-dependent oligomerization. Based on the multiply substituted proteins, several single amino acid substitutions were generated by site-directed mutagenesis. The singly substituted proteins retained the ability to oligomerize and detect substrates. Intriguingly, some derivatives of Hsp104 functioned well in prion propagation and multiple stress tolerance but failed to protect yeast from extreme thermal stress. We demonstrate that these proteins co-aggregate in the presence of other thermolabile proteins during heat treatment both in vitro and in vivo suggesting a novel mechanism for uncoupling the function of Hsp104 in acute severe heat shock from its functions at moderate temperatures.

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1 The abbreviations used are: NBD, nucleotide-binding domain; GFP, green fluorescent protein; FFL, firefly luciferase; SD, small domain; PVDF, polyvinylidene difluoride; WT, wild type.
Hsp104 Substrate Recognition

53693

Stitutions into the second AAA+ module of Hsp104. Hsp104 variants that failed to redistribute in living cells following heat shock also failed to oligomerize and either contained multiple amino acid substitutions, including the introduction of polar residues in conserved hydrophobic positions, or were truncated by nonsense codons that eliminated the SD. The presence of multiple substitutions was required for these characteristics because previously engineered reduced proteins only showed recordations oligomerized and refolded proteins in vivo and in vitro. Three derivatives of Hsp104 that functioned well in prion propagation and low temperature stress tolerance failed to confer high temperature survival. We found that these had a conditional defect that was temperature-dependent and resulted in a decreased solubility of these proteins in vivo at high temperature. Rather than interacting directly with substrates, as has been suggested (24, 25), our analysis indicates that the second AAA+ module of Hsp104 contributes to substrate recognition by stabilizing the oligomeric structure of Hsp104.

MATERIALS AND METHODS

Yeast Strains—W303A104 (MATa, leu2-3, 112, trp1-1, ura3-1, his3-11, his3-15, ade2-1, can1-100, hsp104::HIS3) was created by replacing the HSP104 open reading frame with a PCR-amplified cassette containing the Schizosaccharomyces pombe HIS3 gene (26) and was used for immunofluorescence studies. YPH499A104 (MATa ura3-52, lys2-801, ade2-101, trp1-Δ63, his3-Δ200, leu2-3Δ, hsp104::LEU2) (27) was used for direct fluorescence microscopy, stress tolerance determinations, and in vivo luciferase refolding assays. For analysis of the interaction between HSP104 alleles and the yeast prion [PSI+], GT81-1C (28) [PSI+] MATa ade1-14, his3-Δ200, leu2-3, lys2, trp1-Δ, ura3-52) and GT234 (29) (psi+) MATa ade1-14, his3-Δ200, leu2-3, lys2, trp1-Δ, ura3-52) were used.

DNA Methods—A DNA segment encoding amino acids 710–908 of Hsp104 and including 116 bp of the 3′-untranslated region was inserted into pBlueScript II SK+ as an SpeI/SacI fragment and used as a template for error-prone PCR (30) using the T3 and T7 promoter primers. The PCR product was digested with SpeI/SacI and used to replace the corresponding fragment of a fusion between GFP and a modified Hsp104 open reading frame (Hsp104::GFP) (31) under the control of the Gal1-10 promoter in a pRS315 backbone (27). The ligations products were transformed into Escherichia coli DH5α, and library DNA was prepared from ~3000 colonies. To remove the GFP coding region, plasmids were digested with BamHI and closed using T4 DNA ligase (New England Biolabs). Rescued DNA was sequenced with a primer (5′-CGATGAAGTAGAAAAGGCACATC-3′) by dye termination at The Centre for Applied Genomics DNA Sequencing Facility (Hospital for Sick Children). Site-directed mutagenesis was performed using the QuickChange method according to the manufacturer’s instructions (Stratagene). Point mutations were inserted into the genome of GT234 by a pop-in/pop-out strategy (32). Integration of mutant sequences was confirmed by colony PCR.

Fluorescence Microscopy Techniques and Visual Screen—Library DNA was transformed into yeast strain YPH499A104. Individual colonies were grown to saturation in 2% glucose minimal synthetic media (SD) lacking histidine and diluted in media supplemented with 2% galactose and 0.1% glucose (SGD) for growth to mid-log phase (~2 × 10⁶ cells/ml) at 30 °C. Live cells from each strain were examined using a polyclonal goat anti-Hsp104 antibody (1:5000) after a heat shock of 37 °C for 1 h, 1 ml of the culture was transferred to sterile glass tubes warmed to 50 °C in a water bath. For multistress tolerance, induced cultures were pretreated at 37 °C for 30 min and then exposed to a temperature of 40 °C and 6% (v/v) ethanol (3). In both cases, aliquots of cells were removed at various time points and serially diluted in 5-fold steps in YPD to a 98% soluble state. A pin replicator was used to transfer cells to YPD agar. Cell survival was assessed after incubation at 30 °C for 2–3 days.

Polycanal Anti-Hsp104 Antibody—To minimize variability in detection of Hsp104 molecules as a result of altering or deleting epitopes, New Zealand White rabbits were immunized subcutaneously with full-length recombinant Hsp104. The specificity of the immune serum was assessed by Western blotting yeast lysates prepared from wild type and hsp104 mutant yeast and by immunofluorescence microscopy of the same strains. For Western blot analysis, 10 μg of protein from glass bead lysates was separated by SDS-PAGE and transferred to PVDF. Hsp104 antiserum was used at a 1:15,000 dilution. Immunocomplexes were detected with a horseradish peroxidase-conjugated secondary antibody (AP Biotech) and visualized using enhanced chemiluminescence (AP Biotech).

Protein Purification—DNAs encoding full-length Hsp104 and the mutantized variants were subcloned into a modified pPROEX-HT-b (Invitrogen) vector, as BamHI/SacI fragments, and transformed into BL21 codon plus (Stratagene). Polyhistidine-tagged Hsp104 was isolated by chromatography on Ni²⁺-nitrilotriacetic acid (Qiagen). Following tobacco etch virus protease (Invitrogen) cleavage of the tag, the protein was re-applied to Ni²⁺-nitrilotriacetic acid. The unbound (cleaved) fraction was further purified by anion exchange chromatography. Peak fractions were analyzed by SDS-PAGE, pooled, and dialyzed into an ATP-regenerating system consisting of 20 mM phosphocreatine, and 0.02 units of phosphocreatine kinase, 2.5 μM each of Hsp104, recombinant human Hsp70, and recombinant yeast Ydj1. After 90 min of incubation at 30 °C for 2 min, aliquots of the 100,000 g supernatant were used in FFL refolding experiments, control (nonheat-shocked) and heat-shocked cells (37 °C) were disrupted by glass beads in refolding buffer containing protease inhibitors. Unbroken cells and debris were removed by centrifugation at 5,000 × g for 10 min. The extract was further centrifuged at 100,000 × g for 1 h. 100 μg of the 100,000 × g supernatant was used in FFL refolding assays. For the heat-treated lysates, separate refolding reactions were supplemented with 5 μg of purified Hsp104. For in vivo FFL experiments, yeast were transformed with pJH907 (E. coli BamHI/HindIII) with a derivatives peroxidase (37) was inserted into p426GPD (38) for expression in yeast. Cells were grown to saturation in SD lacking histidine and uracil and diluted for re-growth to midlog phase in SGD lacking histidine and uracil. Cycloheximide was added to a final concentration of 10 μg/ml immediately before FFL inactivation at 44 °C for 20 min or 50 °C for 5 min. Cultures were recovered to 37 °C. FFL activity was determined as described (37) by the addition of 0.5 mM 3-b-glucuronide (Sigma) to equal volumes of intact cells immediately before heat shock, immediately after heat shock, and at indicated intervals during recovery.

ATPase Assays—2.5 μg of protein was added to ice-cold XLB (20 mM HEFES-KOH, pH 7.6, 200 mM KCl, 10 mM MgCl₂, 2 mM EDTA, 2 mM DTT) containing 100 μM Mg⁰⁰°C. The unfolded protein was rapidly dispersed 1:100 into ice-cold refolding buffer, incubated on ice for 30 min, and aliquoted into pre-cooled tubes, flash-frozen in liquid N₂, and stored at 80 °C. Frozen, aggregated FFL was thawed on ice immediately before use. Refolding reactions contained refolding buffer, 5 μM ATP, an ATP-regenerating system consisting of 20 μM phosphocreatine, and 0.02 units of phosphocreatine kinase, 2.5 μM each of Hsp104, recombinant human Hsp70, and recombinant yeast Ydj1. After 90 min of incubation at 25 °C, duplicate 1-μl aliquots were dispersed into luciferase assay reagent (Promega), and light emission was recorded in a lumino-

2 Mosser, D. D., Ho, S., and Glover, J. R. (2004) Biochemistry 43, 8107–8115.
reaction volumes were 25 μl. After 3–10 min, the reactions were stopped by the addition of Malachite Green. All activities were determined through the linear range of ATP hydrolysis and background subtracted for phosphate present in reactions containing ATP alone. For kinetic analysis 25 μl of ATP was added to 2 μg of protein in 25 μl of XLB20 (XLB with 20 mM KCl instead of 200 mM) to initiate the reaction. All solutions were warmed to 25 °C.

**RESULTS**

Subcellular Redistribution of Hsp104 in Response to Heat Shock—The accumulation of Hsp104 around the periphery of heat-induced aggregates has been observed previously by using immunoelectron microscopy (22, 23). By using indirect immunofluorescence, we first tested whether a change in Hsp104 localization could be observed at the level of light microscopy. Because Hsp104 expression is barely detectable in unstressed cells, wild type Hsp104 and two Hsp104 mutants with amino acid substitutions in each of the two NBDs for Hsp104, Hsp104K218T and Hsp104K620T, were expressed in an hsp104 deletion strain under the control of a copper-inducible promoter (33). In all nonstressed cells Hsp104 was diffusely distributed (Fig. 1A). After heat shock, Hsp104WT formed 1–3 foci in most cells. In contrast, Hsp104K218T and Hsp104K620T formed numerous bright foci. To facilitate a visual screening procedure, we examined the subcellular distribution of Hsp104 with GFP fused to its N terminus. GFP-tagged Hsp104 complemented the survival defect of an hsp104 yeast strain exposed to combined heat shock and ethanol stresses (40 °C and 6% ethanol) but not a high temperature (50 °C) treatment (data not shown). GFP itself remained diffuse after a mild heat shock at 37 °C, whereas the GFP-Hsp104 fusion protein formed a few concentrated foci comparable with those observed for the unmodified wild type Hsp104 by immunofluorescence produced under the same conditions (Fig. 1B).

A library of plasmids encoding GFP-Hsp104 fusion proteins with random mutations in the C-terminal region of Hsp104 was generated by error-prone PCR and transformed into a yeast strain deleted for HSP104. In a screen of 400 individual transformants, we identified 20 clones that remained diffuse after heat shock (Fig. 1C). Sequencing the mutagenized segment of HSP104 DNA revealed the existence of multiple base substitutions predicted to result in 2–7 amino acid substitutions per clone, and in some cases nonsense codons were detected (Table I). Of the initial 20 clones we focused our attention on hsp104-27, hsp104-101, and hsp104-138 (Table I).

To gain insight into the contributions of individual amino acid substitutions to the targeting defect of the multiply substituted Hsp104 mutants, we studied five singly substituted proteins in detail: Hsp104T228T, Hsp104F772S, and Hsp104K774E were derived from hsp104-138 and Hsp104L814S and Hsp104L840Q were derived from hsp104-101. First we examined the subcellular distribution of the singly substituted proteins as GFP fusions before and after heat shock (Fig. 1C). In contrast to the multiply substituted parental clones, these proteins formed foci that resembled those formed by GFP-Hsp104WT (Fig. 1C). Thus the defect in heat-induced redistribution requires more than one substitution and could not be recapitulated by any single amino acid substitution we introduced into the wild type protein.

Oligomerization of Hsp104 Mutants—We reasoned that amino acid substitutions could interfere with substrate binding directly, by perturbing a substrate-binding site, or indirectly,
by preventing the proper folding and assembly of Hsp104. To differentiate between these possibilities, we undertook an analysis of Hsp104 assembly by gel filtration using purified recombinant proteins expressed without the GFP fusion domain used in the initial screening procedure. Previously, Hsp104 oligomerization was analyzed by using stringent, high ionic strength buffers (140–200 mM KCl) (18, 19) that enabled the detection of gross oligomerization defects. To increase our ability to detect subtle oligomerization differences between Hsp104 molecules, we used a low ionic strength buffer (50 mM KCl). Under these conditions, Hsp104WT was partially assembled in the absence of nucleotide and completely assembled into hexamers in the presence of nucleotide (Fig. 2). The multiply substituted proteins, Hsp104–101 and Hsp104–138 and the truncated protein Hsp104–27 were unassembled in the absence of nucleotide and did not respond to the presence of nucleotide in the elution buffer.

For comparison with other singly substituted proteins, we incorporated into our analysis an Hsp104 derivative with a Walker A lysine substitution in NBD2, Hsp104K620T, that is defective in nucleotide-dependent assembly (19). Under our conditions, Hsp104K620T assembled into hexamers with a shoulder of lower molecular weight species in the presence of ATP. The elution profiles obtained for Hsp104F772S and Hsp104K774E most closely resembled this pattern. Hsp104I722T was indistinguishable from the wild type protein (data not shown). Hsp104K620T and Hsp104K774E were almost completely assembled with a smaller shoulder of incompletely assembled species. Thus, although the point mutants have assembly defects, they still retain some ability to assemble.

**ATPase and in Vitro Protein Refolding Activities of Hsp104 Mutants**—We next examined the kinetics of ATP hydrolysis by Hsp104WT and the singly substituted derivatives (Table II). The ATPase activities of the multiply substituted and truncated Hsp104 derivatives were too low to obtain reliable kinetic parameters. For the wild type protein we obtained a K_m of 120 μM. Although there are slight differences in buffer composition and assay temperature, our data agree with the published kinetic constants for NBD1 of Hsp104 (39). Most interesting, Hsp104I722T displayed a 3-fold increase in V_max, whereas the other mutations had moderate effects on this parameter. The most profound effect of the other single amino acid substitutions was to increase the apparent K_m for ATP hydrolysis by 3–35-fold. The elevated K_m values are consistent with the mild oligomerization defects observed with these proteins.

We also examined the effect of temperature on the ATPase activity of Hsp104. As observed previously (18), Hsp104WT ATPase activity increased with increasing temperature (Fig. 3). We found that, with the exception of Hsp104I722T that had elevated ATPase activity over all temperatures assayed, the ATPase activities of the singly substituted proteins increased only marginally, or not at all, when assayed at 30 °C and were nearly undetectable at 37 °C.

**TABLE I**

| Clone     | Amino acid substitutions |
|-----------|--------------------------|
| Hsp104–27 | S745P, F772S, E774Term   |
| Hsp104–101| K747R, L814S, L840Q, E878G|
| Hsp104–138| I722T, F772S, K774E, L775F, L845I |

The elution profiles were obtained in the absence (- - -) and presence (——) of 5 mM ATP. The elution profile of Hsp104I722T (not illustrated) was indistinguishable from the wild type (wt) protein. The elution volume of Hsp104WT in the presence of ATP is indicated with a [caret] on all axes.

**ATPase and in Vitro Protein Refolding Activities of Hsp104 Mutants**—We next examined the kinetics of ATP hydrolysis by Hsp104WT and the singly substituted derivatives (Table II).

**TABLE II**

| Protein | K_m [μM] | V_max [μmol min⁻¹ μg⁻¹] |
|---------|----------|-------------------------|
| WT      | 0.12 ± 0.01 | 216 ± 7    |
| K620T   | 0.21 ± 0.05 | 32 ± 2    |
| F772S   | 0.31 ± 0.00 | 684 ± 19  |
| K774E   | 4.4 ± 0.06 | 330 ± 27  |
| L814S   | 3.8 ± 1.4  | 121 ± 25  |
| L840Q   | 0.89 ± 0.14 | 172 ± 14  |
| 138     | 0.56 ± 0.14 | 188 ± 13  |

a Values are given as m M ± S.D. (n = 3).

b Values are given as nmol min⁻¹ μg⁻¹ ± S.D. (n = 3).

NA indicates not applicable; best fit was obtained using non-cooperative kinetics.

![Analytical gel filtration analysis of Hsp104 assembly.](image)

**FIG. 2. Analytical gel filtration analysis of Hsp104 assembly.** The elution profiles were obtained in the absence (—) and presence (——) of 5 mM ATP. The elution profile of Hsp104I722T (not illustrated) was indistinguishable from the wild type (wt) protein. The elution volume of Hsp104WT in the presence of ATP is indicated with a [caret] on all axes.

The point mutants displayed robust FFL refolding activities (Fig. 4A). Only the NBD2 Walker A mutant Hsp104K620T, as observed previously (35), produced significantly less refolding activity than Hsp104WT.

To determine whether oligomerization defects influenced the refolding capabilities of Hsp104 point mutants, we conducted FFL refolding assays with various concentrations of Hsp104 (Fig. 4B). The maximal amount of refolding for each protein was reached at ~0.4 μM. Although the concentration required for half-maximal refolding by Hsp104WT and most of the point mutants was ~0.09 μM, the concentration of Hsp104K620T and Hsp104F772S was ~0.3 μM. The concentration of Hsp104K774E required for half-maximal refolding was slightly increased compared with the wild type protein. These results are consistent with the idea that these molecules have reduced abilities to assemble but that their assembly is not completely blocked.

Given the substantial differences in ATPase activities between the mutant proteins and the wild type protein at 37 °C, we predicted that we would observe a pronounced effect on the refolding activity of Hsp104 derivatives at that temperature. Because FFL is poorly reactivated at 37 °C, we used E. coli β-galactosidase as a model refolding substrate. As observed previously (35), spontaneous refolding of β-galactosidase was significant in the absence of chaperones and in the presence of Hsp70 and Ydj1 only (Fig. 4C). Surprisingly, despite their low ATPase activity at 37 °C, Hsp104L814S and Hsp104L840Q medi-
ated wild type levels of protein refolding, whereas Hsp104I722T refolding was significantly impaired. Hsp104K620T, which was a poor chaperone for FFL refolding at 25 °C, exhibited relatively robust refolding of β-galactosidase at 37 °C. To examine the possibility that the presence of aggregated β-galactosidase might restore ATPase activity of Hsp104 at 37 °C, we carried out ATPase assays at various temperatures in the presence of a molar excess of refolding substrate. No restoration of ATPase activity was detected (data not shown).

**Biological Functions of Hsp104 Mutants**—To evaluate the biological properties of Hsp104 mutants, we first conducted thermotolerance tests. In an hsp104 deletion strain, the expression of Hsp104 restores enhanced survival after exposure to an extreme temperature (50 °C) (Fig. 5A). As expected, the multiply substituted clones Hsp104–138 and Hsp104–101 did not restore thermotolerance. Surprisingly, given the high protein refolding activities of the singly substituted derivatives of Hsp104, only the expression of Hsp104I722T fully restored thermotolerance to the hsp104 deletion strain.

Because the mutants exhibited temperature-sensitive ATPase activities, we considered the possibility that they may not function, or may lose function, at 50 °C. We therefore tested the ability of the mutants to complement survival of the hsp104 deletion strain under the dual stress of 6% ethanol and 40 °C (3). Although viability is lost over a longer incubation period than in severe heat treatment, survival is still largely dependent on Hsp104 (Fig. 5B). We found the results corresponded more closely to the chaperone activities measured in *in vitro* refolding assays. Only Hsp104I722T was defective in multistress tolerance and was comparable with cells expressing Hsp104K620T.

In addition to its role in stress protection, Hsp104 is required for the stable maintenance of yeast prions at normal temperatures (4). In *psi* cells, a red pigment accumulates as the result of faithful translation termination at a nonsense codon located in the *ade1-14* mRNA. In *PSI* strains, where a sufficient quantity of the termination factor Sup35 (eRF3) is sequestered in self-seeding aggregates to permit translational read-through of nonsense codons, the colonies are unpigmented (40). As *PSI* is inherited as a cytoplasmic, non-Mendelian element, co-segregation of a *psi* phenotype with a mutant *hsp104* allele can be indicative of the ability of the protein to support the maintenance of the *PSI* prion.

As a test of Hsp104 function at ambient temperatures, we replaced the wild type HSP104 allele in the haploid genome of a *psi* yeast strain with point-mutated alleles (no replacement with *hsp104I722T* was obtained for undetermined reasons), and these strains were mated to an isogenic *PSI* strain. The resulting diploids were sporulated and the tetrads analyzed. When the *HSP104/hsp104I722T* diploid was sporulated, two unpigmented and two red colonies were formed (Fig. 6A). PCR analysis indicated that the *psi* phenotype co-segregated with *hsp104I722T* allele. When pigmented *hsp104I722T*...
proteins to various temperatures for 10 min, the proteins were stable of purified Hsp104s activities of these molecules. First we tested the functional imparted by the apparent temperature sensitivity of the ATPase distinct temperature-sensitive property. This idea was sup-

indicated dilutions. Plating of cells prior to the 50 °C treatment indicated that each culture had equal cell densities (data not shown). The 20-min time point is shown. B, multistress tolerance. Equal numbers of cells were pretreated at 37 °C for 30 min then exposed to 6% (v/v) ethanol and 40 °C for 40 min. Survival at the 90-min time point is shown.

haplotypes were backcrossed to the [psi−] strain, 4 of 10 tetrad showed a 2:2 segregation indicating that some hsp104K774E hsp104 haplotypes retained the prion whose unpigmented phenotype re-emerged (Fig. 6B). Haploid strains derived from the remaining 6 tetrad were all pigmented indicating that the prion was lost. Diploids of HSP104/hsp104K774E and hsp104K774E hsp104L814S spores were slightly pink indicating that the mutant Hsp104 caused mild antisuppression. HSP104/hsp104L814S and HSP104/hsp104L840Q diploids and germinated spores were indistinguishable from the wild type controls (Fig. 6A).

High Temperature Stability of Hsp104 Mutants in Vitro and in Vivo—In tests of biological functions of Hsp104, we observed that Hsp104K774E, Hsp104L814S, and Hsp104L840Q were functional in prion propagation and multistress tolerance but not in thermostability indicating that these variants might have a distinct temperature-sensitive property. This idea was supported by the apparent temperature sensitivity of the ATPase activities of these molecules. First we tested the functional stability of purified Hsp104s in vitro. After heating the Hsp104 proteins to various temperatures for 10 min, the proteins were cooled and used in FFL refolding assays at 25 °C. All proteins tested in this manner were stable at 50 °C but were all irreversibly inactivated at 55 °C to slightly different extents (Fig. 7A).

Because the chaperone activities of these proteins were sta-

ble at 50 °C in vitro, we considered it possible that they have impaired function at high temperatures but function normally during recovery. However, it is also possible that the stability to high temperatures observed in vitro might be different from exposure to high temperature in vivo. To differentiate these possibilities, we monitored protein refolding in vivo using FFL. To inactivate FFL, cells were heated at high temperatures, and the restoration of FFL activity was monitored in intact cells during recovery at 25 °C in the presence of cycloheximide to inhibit the accumulation of newly synthesized active FFL. The ability of Hsp104 to reactivate heat-inactivated FFL in vivo was assessed under two conditions, after inactivating treatments of 44 °C for 20 min and after 50 °C for 5 min. After the 44 °C/20-min treatment, −7% of the FFL activity present prior to heat shock was restored in the presence of Hsp104WT (Fig. 7B). As observed previously using bacterial luciferase as an in vivo reporter (2), recovery of FFL activity was poor in cells lacking Hsp104 (vector only) and in cells expressing Hsp104K620T. The time course of reactivation and the final yield of active FFL in cells expressing Hsp104K774E, Hsp104L814S, and Hsp104L840Q were comparable with cells expressing Hsp104WT. Hsp104K774E supported an intermediate level of FFL reactivation.

After the 50 °C/5-min treatment FFL reactivation was much slower with a lag time of 1 h before significant refolding was observed (Fig. 7C). Approximately 35% of the original FFL activity was reactivated in Hsp104WT and Hsp104K774E cells after 5 h of recovery. Under these conditions, Hsp104K774E was completely inactivated, whereas Hsp104K774E, Hsp104L814S, and Hsp104L840Q retained some activity, but significant refolding was delayed.

Because the singly substituted proteins that do not provide thermostability failed to mediate protein refolding after exposure to 50 °C in vivo, we could not rule out the possibility that nonthermotolerant cells were incapable of supporting protein refolding even if the Hsp104 molecule remained active. Nonthermotolerant cells may accumulate overwhelming amounts of unfolded proteins or lose the activity of other chaperones required for Hsp104-mediated refolding. We therefore prepared protein extracts from untreated (control) and heat-shocked (37 °C pretreatment followed by 50 °C for 15 min) and

![Figure 5. Hsp104-mediated stress tolerance.](image)

![Figure 6. The effect of mutations in HSP104 on [psi+].](image)
insoluble components were removed first by a low speed centrifugation (5000 × g, 5 S) and then by high speed centrifugation (100,000 × g, 100 S). These supernatants were then used to refold FFL in vitro (Fig. 8A). The relative refolding activity detected using control lysates closely matched the activities detected using purified components (Fig. 8A versus Fig. 4A). However, refolding by 100 S fractions from severely heat-shocked cells correlated with the ability of each protein to confer thermotolerance (Fig. 8A versus Fig. 5A). Addition of purified recombinant Hsp104 to the inactive 100 S fractions restored refolding activity indicating that the function of Hsp104 is the only defect in the refolding capacity of extracts produced from nonthermotolerant cells. Western blot analysis of low speed (5 S) and high speed supernatants from control cells indicated that all proteins were expressed at levels comparable with that of Hsp104WT (Fig. 8B). In contrast, with the exception of Hsp104WT and, to a lesser extent, Hsp104K620T, all other Hsp104 variants that do not function in thermotolerance were largely depleted from both the low speed and high speed supernatants in heat-shocked cells (Fig. 8B).

Paradoxically, in vitro stability assays suggested that the singly substituted mutants were stable for brief periods at 50 °C, yet those same proteins were depleted from the soluble fraction of yeast heated at the same temperature. We hypothesized that the singly substituted proteins partially unfolded at high temperature but did not aggregate in vitro in the absence of other aggregation prone proteins. To test this hypothesis, we performed a co-aggregation assay. Wild type Hsp104 and the singly substituted proteins were heated at 50 °C for 10 min alone or in the presence of a soluble yeast lysate. Consistent with our functional assessment of Hsp104 stability, all Hsp104 variants remained in the soluble fraction when heated at 50 °C in the absence of yeast lysate (Fig. 9C). In contrast, in the presence of yeast lysate, all the singly substituted mutants preferentially accumulated in the pellet fraction of samples heated at 50 °C, whereas significantly more wild type protein remained soluble.

**Discussion**

To date, it has been difficult to detect stable interactions between Hsp104 and aggregates of misfolded protein in vitro. A prolonged interaction between the bacterial Hsp104 ortholog ClpB and one of its substrates, TrfA, was only recently demonstrated in vitro using a ClpB “trap” molecule that contained amino acid substitutions in the Walker B motif of both NBDs (41). To further our understanding of substrate interaction by Hsp104, we developed a novel visual screen that exploited the co-localization of Hsp104 with one of its natural substrates, thermally inactivated proteins, in living cells. By using this screen, we obtained mutant Hsp104 molecules that failed to interact with substrates. All mutant proteins obtained from this screen were either full-length proteins with multiple amino acid substitutions or truncated proteins. In all cases, the mutant proteins that failed to interact with substrates also failed to oligomerize under any conditions we tested. In previous studies, Hsp104K620T was found to have an oligomerization defect (19) that could be overcome at high protein concentrations (20). In this study we observed that when gel filtration analysis is conducted under lower ionic strength conditions, Hsp104K620T is not as profoundly affected in its ability to assemble as the multiply substituted or truncated proteins isolated by our screening procedure. Thus the Hsp104 variants isolated in our screen represent the first report of Hsp104 molecules that are truly unassembled. Furthermore, when amino acid substitutions from the multiply substituted Hsp104 derivatives were introduced as individual mutations into full-length Hsp104, nucleotide-dependent oligomerization was reduced but not eliminated, and their ability to redistribute in response to mild heat stress was restored. Together, these results indicate that oligomerization of Hsp104 is a key determinant of substrate recognition. Notably, our screen did not identify any Hsp104 mutants that retained the ability to assemble (this work).3 This suggests that the second AAA+ module of Hsp104 contributes to substrate recognition by stabilizing the oligomerized molecule rather than directly recognizing substrates as has been suggested previously (24, 25).

An oligomerization constraint for substrate interaction by the Hsp100 molecules ClpA and ClpX (42, 43), and for protein refolding by ClpB (44, 45) has already been established. We speculate that Hsp104 oligomerization is required to mediate an interaction with Hsp70 or with aggregated proteins themselves. A number of AAA+ molecules use adaptor proteins to enhance or modify their substrate specificity (reviewed in Ref. 46). Although Hsp70 has not been generally regarded as an
adaptor, it may initially interact with aggregated proteins and direct Hsp104 to its substrates. Following the initial submission of this manuscript, it was demonstrated by others that Thermus thermophilus ClpB must be properly assembled to interact with DnaK (47); thus we cannot exclude the possibility that our oligomerization-deficient mutants fail to interact with Hsp70 in vivo resulting in the observed defect in relocalization.

However, there are indications that ClpB interacts directly with substrates. Using the E. coli ClpB trap mutant, it was shown that ClpB interacts directly with one of its physiological substrates, TrfA, in the absence of additional chaperones (41). Furthermore, the same ClpB trap mutant inhibits the DnaK-mediated refolding of non-native malate dehydrogenase captured by small Hsps suggesting that ClpB directly interacts with non-native substrates and competes with DnaK (41). Thus, we favor a model wherein Hsp104 contacts protein aggregates directly.

In this context, we suggest two reasons for the observed oligomerization constraint. First, Hsp104 might require the contribution of partial binding sites from two or more subunits. Most interesting, based on the recent crystal structure of ClpB, it was suggested that two coiled-coil “middle domains” (unique to Hsp104 and its orthologs) from adjacent subunits might contact aggregated substrates (13). Alternatively, each subunit might possess one or more substrate-binding sites with weak binding activity in the context of a monomeric subunit but that, when displayed together in the assembled hexamer, are capable of conferring a stable interaction.

The exact role of ATP hydrolysis in the Hsp104 disaggregation mechanism has not been defined. A number of mutation-based studies have demonstrated that Hsp104 requires two functional NBDs for biological function (18, 39, 48, 49), but it is unknown whether these substitutions affect the initial binding of Hsp104 to its substrates or impair subsequent events required for disaggregation and refolding. We found that Hsp104<sup>WT</sup> formed weak foci after a mild heat shock, whereas foci formation by the Walker A mutants Hsp104<sup>K218T</sup> and Hsp104<sup>K620T</sup> was robust (Fig. 1A). These results suggest that substrate recognition is not directly dependent on the function of the NBDs, providing the molecules retain some ability to oligomerize. Most likely, NBD function is required only for subsequent steps in disaggregation and protein refolding, and the inability of NBD mutants to process aggregated proteins forces these molecules to accumulate at sites of aggregation.

This conclusion is consistent with the recent characterization of the ClpB trap mutant in which mutations in the Walker B motif that abrogate ATP hydrolysis still permit, and in fact stabilize, the interaction of the protein with aggregated substrate (41). Additionally, our results suggest that a wild type level of ATPase activity is not stringent required for function. For example, we obtained Hsp104 molecules that had el-

![Fig. 8. Activity and solubility of Hsp104 mutants in vivo and in vitro.](image-url)
Hsp104 Substrate Recognition

The spatial relationship between Phe-772 and Lys-620 is illustrated by the corresponding residues in ClpB, Phe-754, and Lys-601. A portion of a ClpB monomer composed of residues 537–850 is shown. B, ClpB has been rotated and enlarged to accommodate a closer view of the residues. In each view, the indicated amino acids are labeled according to the corresponding Hsp104 residue and are shown in space fill mode. ADP is shown as a ball and stick model. The ClpB x-ray crystal structure (Protein Data Bank access code 1QVR) (13) was used. The illustrations are generated using Swiss-PDB Viewer (52) and POV-Ray (www.povray.org).

**FIG. 9. Model of Phe-772 (F772) and Lys-620 (K620) interaction.** The spatial relationship between Phe-772 and Lys-620 is illustrated by the corresponding residues in ClpB, Phe-754, and Lys-601. A portion of a ClpB monomer composed of residues 537–850 is shown. B, ClpB has been rotated and enlarged to accommodate a closer view of the residues. In each view, the indicated amino acids are labeled according to the corresponding Hsp104 residue and are shown in space fill mode. ADP is shown as a ball and stick model. The ClpB x-ray crystal structure (Protein Data Bank access code 1QVR) (13) was used. The illustrations are generated using Swiss-PDB Viewer (52) and POV-Ray (www.povray.org).

...Hsp104 mutant Hsp104K620T. It is possible that the Ser substitution for Phe-772 may incorrectly position Lys-620 within the NBD and mimics a substitution at Lys-620 itself and thereby impairs the binding stability and/or hydrolysis of ATP in NBD2. Alternatively, the conformational change induced by nucleotide binding in AAA⁺ NBDs could be propagated to other elements in the module (SD for example) by the interaction between the P-loop lysine and its hydrophobic partner residue.

Hsp104K774E, Hsp104L814S, and Hsp104L840Q derivatives were unable to complement the thermotolerance defect in yeast lacking hsp104 despite their ability to substitute for Hsp104WT in *in vitro* refolding of model proteins, in survival following combined ethanol and mild heat stress, and in [PSI⁺] propagation. Typically, high temperatures (50–52 °C) are used in thermotolerance assays to rapidly kill yeast. Because we observed temperature-dependent defects in ATPase activities of Hsp104K774E, Hsp104L814S, and Hsp104L840Q, we surmised that exposure to extreme temperature inactivated these particular Hsp104 derivatives. Most interesting, when these molecules were incubated alone at the same high temperature *in vitro*, they remained soluble and active for FFL refolding at lower temperatures. The key to this apparent paradox is that Hsp104, and especially the mutants generated in this study, can co-aggregate with other thermolabile proteins resulting in an irreversible loss of activity. Intriguingly, Hsp104WT and Hsp104K774T that function in thermotolerance and that were at least partially resistant to sedimentation in extracts derived from heat-shocked cells were also partially insolubilized by co-aggregation *in vitro*. Conceivably, other factors such as the accumulation of the compatible solute trehalose in thermotolerant yeast (37) contribute to Hsp104 stability and function at extreme temperatures. Nonetheless, temperature sensitivity, albeit at a temperature far above the range normally associated with functional impairment of temperature-sensitive proteins used in genetic analysis in yeast, represents a novel mechanism for the uncoupling of Hsp104 function in low temperature multistress survival and [PSI⁺] propagation from thermotolerance measured by survival following severe acute heat shock.

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**REFERENCES**

1. Ogura, T., and Wilkinson, A. J. (2001) *Genes Cells* 6, 575–597
2. Parsell, D. A., Kowal, A. S., Singer, M. A., and Lindquist, S. (1994) *Nature* 372, 475–478
3. Lindquist, S., Patino, M. M., Chernoff, Y. O., Kowal, A. S., Singer, M. A., Liebman, S. W., Lee, K. H., and Blake, T. (1995) *Cold Spring Harbor Symp. Quant. Biol.* 60, 451–460
4. Chernoff, Y. O., Lindquist, S. I., Ono, B., Inge-Vechtomov, S. G., and Liebman, S. W. (1995) *Science* 269, 881–884
5. Patino, M. M., Liu, J. J., Glover, J. R., and Lindquist, S. (1996) *Science* 273, 622–626
6. Moriyama, H., Edekes, H. K., and Wickner, R. B. (2000) *Mol. Cell. Biol.* 20, 8916–8922
7. Sondheimer, N., and Lindquist, S. (2000) *Mol. Cell* 5, 163–172
8. Bochtler, M., Hartmann, C., Song, H. K., Bourenkov, G. P., Bartunik, H. D., and Huber, R. (2000) *Nature* 403, 800–805
9. Souza, M. C., Trane, C. B., Tsuruta, H., Wilbanks, S. M., Reddy, V. S., and McKay, D. B. (2000) *Cell* 103, 633–643
10. Wang, J., Song, J. J., Franklin, M. C., Kantekar, S., Im, Y. J., Rho, S. H., Seong, I. S., Lee, C. S., Chung, C. H., and Eom, S. H. (2001) *Structure (Land.* 9, 177–184
11. Wang, J., Song, J. J., Seong, I. S., Franklin, M. C., Kantekar, S., Eom, S. H., and Chung, C. H. (2001) *Structure (Land.*) 9, 1107–1116
12. Gus, F., Maurizi, M. R., Esser, L., and Xia, D. (2002) *J. Biol. Chem.* 279, 46743–46752
13. Lee, S., Sowa, M. E., Watanabe, Y. H., Sigler, P. B., Chiu, W., Yoshida, M., and Tsai, F. T. (2003) *Cell* 115, 229–240
