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Dominant Gain-of-Function Mutations in Hsp104p Reveal Crucial Roles for the Middle Region

Eric C. Schirmer,*† Oliver R. Homann,‡ Anthony S. Kowal,§ and Susan Lindquist*‡§‖¶

*Department of Molecular Genetics and Cell Biology, ‡Committee on Genetics, and §Howard Hughes Medical Institute, The University of Chicago, Chicago, Illinois 60637

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Heat-shock protein 104 (Hsp104p) is a protein-remodeling factor that promotes survival after extreme stress by disassembling aggregated proteins and can either promote or prevent the propagation of prions (protein-based genetic elements). Hsp104p can be greatly overexpressed without slowing growth, suggesting tight control of its powerful protein-remodeling activities. We isolated point mutations in Hsp104p that interfere with this control and block cell growth. Each mutant contained alterations in the middle region (MR). Each of the three MR point mutations analyzed in detail had distinct phenotypes. In combination with nucleotide binding site mutations, Hsp104p^{T499I} altered bud morphology and caused septin mislocalization, colocalizing with the misplaced septins. Point mutations in the septin Cdc12p suppressed this phenotype, suggesting that it is due to direct Hsp104p–septin interactions. Hsp104p^{A503V} did not perturb morphology but stopped cell growth. Remarkably, when expressed transiently, the mutant protein promoted survival after extreme stress as effectively as did wild-type Hsp104p. Hsp104p^{A509D} had no deleterious effects on growth or morphology but had a greatly reduced ability to promote thermotolerance. That mutations in an 11-amino acid stretch of the MR have such profound and diverse effects suggests the MR plays a central role in regulating Hsp104p function.

INTRODUCTION

The AAA$^+$ proteins are *A*TPases associated with various cellular activities. They are important proteins with a great diversity of functions, including protein folding, membrane trafficking, organelle biogenesis, proteolysis, intracellular motility, and DNA replication (Neuwald *et al*., 1999; Vale, 2000; Ogura and Wilkinson, 2001). Little is known about how most AAA$^+$ proteins recognize substrates and use ATP binding and hydrolysis to remodel them. The intrinsic complexity and multiplicity of conformational states of the AAA$^+$ proteins make them difficult to study. The Clp/HSP100 proteins are members of the AAA$^+$ superfamily and have been the subject of intense biochemical analysis in vitro and genetic analysis in vivo (Wickner *et al*., 1999; Glover and Tkach, 2001). The functional unit of the yeast HSP100 heat-shock protein 104 (Hsp104p) is composed of six monomers, each with two ATP binding sites (nucleotide binding domains one and two; NBD1 and NBD2) flanked by amino-terminal, middle, and carboxy-terminal regions (Figure 1).

Hsp104p has remarkable functions, one of which is to allow survival after extreme stress. For example, yeast cells expressing Hsp104p are 1000 times more viable after exposure to temperatures ≥50°C or to an ethanol concentration of 20% than cells carrying deletions of *HSP104* (Sanchez and Lindquist, 1990; Sanchez *et al*., 1992). This survival capacity is directly attributable to Hsp104p's ability to resolubilize protein aggregates and, together with Hsp70p and Hsp40p, return them to their folded and active states (Parsell *et al*., 1994; Glover and Lindquist, 1998; Goloubinoff *et al*., 1999). This is in contrast to other heat shock proteins, which generally act by preventing aggregate accumulation or by promoting the degradation of misfolded proteins (Schirmer *et al*., 1996; Zolkiewski, 1999). During times of severe stress, the rate of protein aggregation exceeds the capacity of other heat shock proteins to prevent aggregate accumulation, and Hsp104p becomes critical to survival. This explains the observation that Hsp104p is not required for normal growth, or even growth at high temperatures, but is vital for surviving extreme conditions. The relationship between Hsp104p and thermotolerance is simple and direct: the more Hsp104p present, the higher the level of thermotolerance.

Another remarkable activity of Hsp104p is prion maintenance (Chernoff *et al*., 1995; Patino *et al*., 1996; Paushkin *et al*., 1996; DebBurman *et al*., 1997; Moriyama *et al*., 2000). Prions are proteinaceous genetic elements that have the ability to undergo heritable, self-perpetuating changes in conformation. As with stress survival, prion maintenance is dependent on Hsp104p's control of protein aggregation, but in this case the relationship is more complex. For maintenance of the [*PSI*$^+$] prion, intermediate levels of Hsp104p are necessary: either deletion or overexpression of *HSP104* eliminates [*PSI*$^+$].

The importance of the ATP hydrolysis sites in the Hsp104p NBD regions is apparent from the debilitating effects of mutations in either NBD1 or NBD2 on thermotolerance and prion maintenance (Parsell *et al*., 1991; Chernoff *et al*., 1995; Patino *et al*., 1996; Schirmer *et al*., 2001) and from the well documented importance of these domains in other AAA$^+$ proteins (Ogura and Wilkinson, 2001; Lupas and

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Present addresses: †Department of Cell Biology, The Scripps Research Institute, La Jolla, CA 92037; ‖Whitehead Institute for Biomedical Research, Nine Cambridge Center, Cambridge, MA 02142.
¶ Corresponding author. E-mail address: lindquist_admin@wi.mit.edu.

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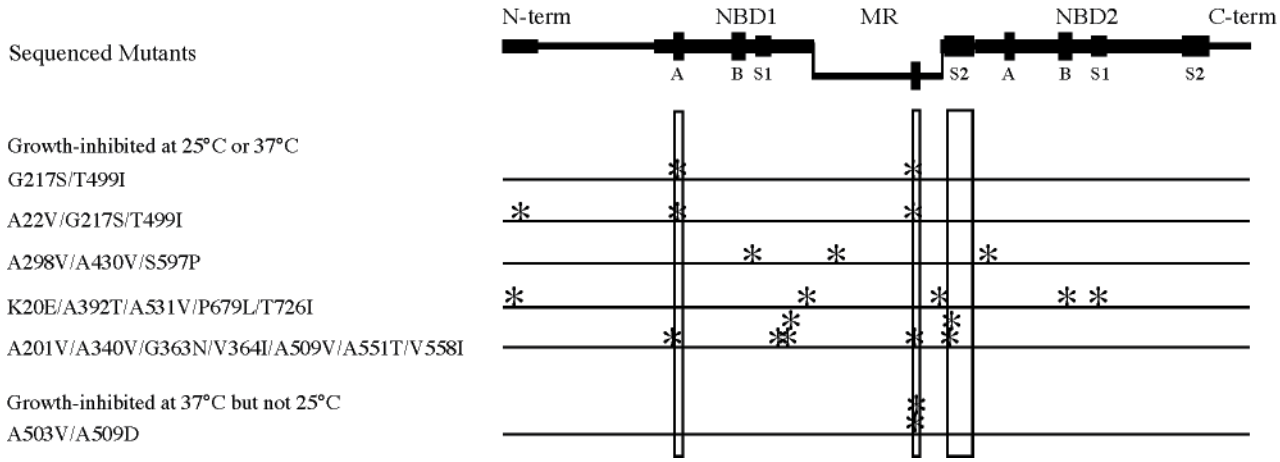

Figure 1. Mutations recovered in the screen for dominant lethal *HSP104* mutants. Hsp104p (908 amino acids) has two highly conserved but distinct NBDs (NBD1 and NBD2) flanked by less conserved N-terminal and C-terminal regions. The MR is an insertion within NBD1. The boundaries of NBD1 (according to an alignment of AAA$^+$ protein sequences; Neuwald *et al.*, 1999) are from residue 180 to residue 400 and again from residue 549 to residue 586 for the sensor 2 domain. The MR is the area bounded by these regions (residue 401 to residue 548). Areas of greater conservation are denoted by a thicker line (Schirmer *et al.*, 1996), and signature sequences of the NBDs are denoted: A and B, the Walker-type P-loop motifs; S1 and S2, the two sensor regions. Asterisks indicate the positions of the mutations in sequenced mutants, and the amino acid substitutions are specified on the left. Conserved sequence elements where mutations tended to cluster are further marked by open boxes. The mutants are grouped by phenotype.

Martin, 2002). The function of Hsp104p's other domains, which are much more variable between different members of the AAA$^+$ superfamily, and how they cooperate with the NBDs to accomplish varied protein-remodeling actions, remain mysterious.

As an alternative to using site-directed mutagenesis to investigate the functions of domains of predefined importance, here we undertook the first genetic screen to identify other critical residues. The mutations recovered, and their analysis, establish that the middle region (MR) has crucial roles in Hsp104p function.

MATERIALS AND METHODS

Yeast Strains and Media

Plasmids (see below) were introduced into *Saccharomyces cerevisiae* strain LP112, W303a (an isogenic haploid), or SL304A (a W303a derivative with *HSP104* codons 1–321 replaced by *LEU2*) (Table 1). To allow use of *LEU2* as an auxotrophic marker in suppressor screens, a new Δ*hsp104* strain, A3224, was created in which *HSP104* codons 18–892 in W303a were replaced by *kan*^{r} by using pFA6a-kanMX4 (Wach *et al.*, 1994) as template to generate the desired polymerase chain reaction (PCR) product; the screen itself was conducted in a modified variant, A3330, with a galactose-inducible *LacZ* reporter integrated at the *HIS3* locus. The pRS303-LacZ reporter was created for this study by transferring *LacZ* (*Bam*HI/*Xba*I) from pCM171 (Gari *et al.*, 1997) to pRS303:GAL1–10. Strains A3685 and A3686 were derived from the originally isolated suppressor strains by mating each to W303α, sporulating, and selecting strains that maintained the suppressor phenotype but had lost the transposon insertion and *LacZ* reporter.

Transformants were maintained on synthetic dextrose medium (SD) (Schirmer *et al.*, 1994). Raffinose (SR) or galactose (SG) replaced dextrose in this medium to preadapt and induce *GAL1*-regulated plasmids, respectively. (Raffinose relieves glucose repression, allowing more rapid induction by galactose.) In strains that also carried plasmid GAL4.ER.VP16 (encoding a chimeric transcriptional activator), galactose-regulated 104b-U1 plasmids were induced in SD by 10 nM β-estradiol. *MET14*-regulated genes were induced by washing and incubation in SD lacking methionine.

Plasmids

A *URA3*-selected plasmid carrying *GAL1*-regulated *HSP104* was used for the mutagenesis screen (Table 2, pYS-GAL104). Subsequent experiments used a

Table 1. Strains used in this study

Strain	Genotype	Source
E. coli		
KC8	*r-m+ leu B600 trpC9830 ΔLacX74 strA galUK pyrF::Tn5 his B463*	M. Casadaban
DH10B	*F-mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80dlacZΔM15 ΔlacX74 deoR recA1 endA1 araΔ139 D(ara, leu)7697 galU galK λ-rpsL nupG*	Invitrogen
S. cerevisiae		
LP112	*MAT a/α leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3-1/ura3-1 ade2-1/ade2-1 his3-11,15/his3-11,15 lys2Δ/lys2Δ can1-100/can1-100*	R. Rothstein
W303a	*MAT a leu2-3,112 trp1-1 ura3-1 ade2-1 his3-11,15 lys2Δ can1-100*	R. Rothstein
W303α	*MAT α leu2-3,112 trp1-1 ura3-1 ade2-1 his3-11,15 lys2Δ can1-100*	R. Rothstein
SL304A	*MAT a leu2-3,112 trp1-1 ura3-1 ade2-1 his3-11,15 lys2Δ can1-100 hsp104::LEU2*	Sanchez and Lindquist, 1990
A3224	*MAT a leu2-3,112 trp1-1 ura3-1 ade2-1 his3-11,15 lys2Δ can1-100 hsp104::KanMX4*	See text
A3330	*MAT a leu2-3,112 trp1-1 ura3-1 ade2-1 his3-11,15::HIS3 (GAL1-LACZ) lys2Δ can1-100 hsp104::KanMX4*	See text
A3685	*MAT a leu2-3,112 trp1-1 ura3-1 ade2-1 his3-11,15 lys2Δ can1-100 hsp104::KanMX4 cdc12^{K351N}*	See text
A3686	*MAT a leu2-3,112 trp1-1 ura3-1 ade2-1 his3-11,15 lys2Δ can1-100 hsp104::KanMX4 cdc12^{E368Q}*	See text

Table 2. Plasmids used

Name	Gene[a]	Promoter	Features[b]	Use	Source
pYS-GAL104	*HSP104*	*GAL1*	CEN, *URA3*	Original screen	Sanchez and Lindquist 1990
pLA1	*none*	*GAL1*	CEN, *HIS3*	Vector control for pGALSc104	L. Arwood and S. Lindquist
pGALSc104	*HSP104/mutants*	*GAL1*	CEN, *HIS3*	Directed mutants	See text
pRS316:GAL1-10	*none*	*GAL1*	CEN, *URA3*	Vector control for 104b-U1	J. Taulien and S. Lindquist
I04b-U1	*HSP104/mutants*	*GAL1*	CEN, *URA3*	Directed mutants	See text
pRS315:GAL1-10	*none*	*GAL1*	CEN, *LEU2*	Vector control for HSP104-b/Leu	J. Taulien and S. Lindquist
HSP104-b/Leu	*HSP104/mutants*	*GAL1*	CEN, *LEU2*	Suppressor screen	Ter-Avanesyan *et al.*, 1993
TM104b	*HSP104/mutants*	*MET14*	CEN, *TRP1*	Alternative induction system	E. Schirmer and S. Lindquist
GAL4.ER.VP16	*GAL4/ER/VP16*	*ADH*	CEN, *HIS3*	Estradiol induction of *GAL1* plasmids	Louvion *et al.*, 1993
pEMBL-Δ3AUG	*SUP35 C-terminus*	*SUP35*	2μm, *URA3*	Test suppression	Ter-Avanesyan *et al.*, 1993
pRS303-LacZ	*LacZ*	*GAL1*	none, *HIS3*	Integration into suppressor strain	See text
CDC12-WT	*CDC12/mutants*	*CDC12*	CEN, *URA3*	Suppressor screen	See text
GAL-CDC12	*CDC12/mutants*	*GAL1*	CEN, *URA3*	Suppressor screen	See text
GFP-CDC3	*GFP-CDC3*	*CDC3*	CEN, *URA3*	Septin visualization	M. Longtine
GFP-CDC12	*GFP-CDC12*	*CDC12*	CEN, *URA3*	Septin visualization	See text

[a] Plasmids containing directed mutants are indicated in the text by the plasmid name with the mutation in parentheses, e.g., pGALSc104 carrying a mutant G217S/T499I allele is designated pGALSc104(G217S/T449I).

[b] Features listed are yeast replication (CEN is low copy number and 2μm is high copy number) and auxotrophic selection.

modified *HSP104* plasmid that had three guanosine nucleotides inserted before the ATG to lower basal expression in both yeast and *Escherichia coli*. This plasmid also had additional unique restriction endonuclease sites every ~500 base pairs that facilitated insertion of directed mutations but did not change the protein sequence ($HSP104_R$; Schirmer and Lindquist, 1998). The *GAL1:10* promoter was inserted between the *Bam*HI and *Eco*RI sites of the polylinker of the pRS300 series vectors (Sikorski and Hieter, 1989), converting pRS303 into pRS303:GAL1–10, pRS313 into pLA1, pRS315 into pRS315:GAL1-10, and pRS316 into pRS316:GAL1-10. $HSP104_R$ (*Bam*HI to the *Sac*I site after the poly A) was then inserted behind the *GAL1* promoter in the modified vectors, converting pLA1 into pGALSc104, pRS315:GAL1-10 into HSP104-b/Leu, and pRS316:GAL1-10 into 104b-U1.

Individual substitutions were introduced into each ~500-base pair segment from $HSP104_R$ in pBluescript KS+ by using the Muta-Gene M13 in vitro mutagenesis kit (Bio-Rad, Hercules, CA). The segments were completely sequenced and then replaced into the corresponding region of pGALSc104, HSP104-b/Leu, or 104b-U1.

The wild-type *CDC12* and suppressor $cdc12^{K351N}$ and $cdc12^{E368Q}$ genes were amplified with and without 266 base pairs of upstream sequence (this 266 base pairs contains at least the partial endogenous promoter) from strains A3330, A3685, and A3686, respectively (Table 1). Using primers that added *Bam*HI and *Xba*I sites, the amplified genes were cloned into pRS316 (266 base pairs endogenous promoter; plasmid CDC12-WT) and pRS316:GAL1–10 (exogenous promoter; plasmid GAL-CDC12). GFP-CDC12 was constructed by introducing a silent mutation into codon 2 of *CDC12* (AGT → AGC) in plasmid CDC12-WT to create an *Eco*47 III site into which green fluorescent protein (GFP) (amplified from plasmid GFP-CDC3) was blunt-end cloned.

Mutagenesis Screen

pYS-GAL104 (10 μg of DNA/500-μl reaction) was mutagenized by incubation with 1 M hydroxylamine (pH 5.5–6.5) at 75°C for 45–90 min (Busby *et al.*, 1982). After phenol-chloroform purification, 1/100 of each preparation was transformed into KC8 bacteria (Table 1), and colonies were replica plated to +/− uracil plates to assess the mutagenesis frequency in the *URA3* marker. (*URA3* rescues the bacterial *pyrF* mutation, similar to *URA3* auxotrophic selection in yeast.) The remainders of two preparations, with only small (1–3%) losses of *URA3* function, were transformed into yeast strain LP112, and it was predicted from the *URA3* mutation rate that only single substitutions would be isolated in *HSP104* (Sikorski and Boeke, 1991). Transformants were selected on −Ura glucose plates and replica plated onto two −Ura glucose (for *HSP104* repression) and two −Ura galactose (for *HSP104* induction) plates. Cells were incubated at 25 or 37°C for 3–5 d, and strains exhibiting a significant reduction in growth on galactose compared with strains containing unmutagenized plasmid were retested quantitatively by growing in liquid SD to mid-log phase, equalizing cell densities, and spotting 5 μl of fivefold serial dilutions onto −Ura glucose and −Ura galactose plates.

To eliminate one class of false positives (growth inhibition on galactose due to general respiratory deficiency), colonies on glucose plates were overlaid with 20 ml of 0.75% Bacto agar in 0.067 M phosphate buffer, pH 7.0, containing 0.1% 2,3,5-triphenyltetrazolium chloride (Colson *et al.*, 1974). After 3 h, colonies were scored as respiration positive (red) or negative (white). A second class of false positives (carrying extraneous growth-inhibiting genomic mutations) was eliminated by testing to ensure that the growth-inhibition phenotype depended upon maintenance of the *HSP104* plasmid, using 5-fluoro-orotic acid to select for plasmid loss (Boeke *et al.*, 1984).

To determine the DNA sequences of the mutant plasmids, two overlapping fragments were amplified from yeast by using *HSP104* and vector primer combinations. PCR products were completely sequenced on both strands, and regions containing mutations were resequenced from independent amplifications.

Suppressor Screens

Two different suppressor screens were conducted in strain A3330 (Table 1) carrying plasmid HSP104(G217S/T499I)-b/Leu. For the first screen, $\sim 4 \times 10^9$ cells were transformed with 200 μg of a galactose-inducible genomic library (ATCC 87311; Ramer *et al.*, 1992), plated on −Leu glucose and then replica-plated to −Leu galactose. Screening of $\sim 10^6$ colonies yielded 534 suppressor candidates. None passed a secondary screen for restoration of the $Hsp104p^{G217S/T499I}$ phenotype upon loss of the library plasmid. For the second screen, $\sim 10^8$ cells were transformed with the mTN-3 × HA/*lacZ* library (provided by M. Snyder; Ross-Macdonald *et al.*, 1997), plated on −Ura −Leu glucose, and then replica plated to −Ura −Leu galactose. Screening $\sim 10^5$ transformants yielded 90 strong candidate suppressors. Only nine of these strains retained expression of the mutant Hsp104p, and seven could not suppress a freshly introduced HSP104(G217S/T499I)-b/Leu plasmid.

The two remaining bona fide suppressors were due to random genomic mutations and were not dependent on an integrated transposon. To isolate these, a library was constructed from suppressor strain A3685 to identify the dominant suppressor. Genomic DNA was partially digested with *Sau* 3AI, and fragments of 4–10 kb were purified and ligated to *Bam*HI-cut YCp50 (Rose *et al.*, 1987). The resulting plasmids were amplified in Electromax DH10B cells (Invitrogen, Carlsbad, CA) and transformed into A3224 cells carrying HSP104(G217S/T499I)-b/Leu, and $\sim 10^5$ colonies were screened for suppression of the $Hsp104p^{G217S/T499I}$ growth phenotype.

Thermotolerance and ATPase Assays

Heat-stress treatments were conducted as described previously (Schirmer *et al.*, 1994). Briefly, mutant and wild-type proteins encoded in the pGALSc104 plasmid were induced in strain SL304A for 4 h in mid-log phase, and cells were then heat shocked at 50°C with or without a preconditioning treatment at 37°C, fivefold serially diluted, and spotted onto SD plates at 25°C to repress further expression of the mutant Hsp104p. Induced cells that did not receive a heat stress were plated onto SD and SG to confirm the growth-inhibition phenotype of the plasmid they contained. Experiments were repeated at least three times with strains isolated from independent transformations, yielding similar results in all cases.

Proteins for ATPase assays were purified from *E. coli* as described by Schirmer and Lindquist (1998) by using the pET28a expression vector (Novagen, Madison, WI), and the assays were performed as described by Schirmer *et al.* (1998).

Microscopy

SL304A cells carrying pGALSc104, its mutant variants, or the parent vector were induced from mid-log phase for 7–13 h in SG. Cells were fixed for 1 h in 3.7% formaldehyde and permeabilized after cell wall removal with Triton X-100, as described by Pringle *et al.* (1991). Next, the cells were incubated with anti-Hsp104p antibody 8–1 (polyclonal to the C-terminal 15 residues; Parsell and Lindquist, unpublished data), 4G-10 (monoclonal to the MR; Jison, Ramakrishnan, and Lindquist, unpublished data), or anti-septin antibodies (affinity purified, polyclonal) specific for Cdc11p (Ford and Pringle, 1991) or Cdc3p (Kim *et al.*, 1991). After incubation with the appropriate secondary antibodies (Organon-Teknika, Durham, NC), images were obtained either on an Olympus epifluorescence microscope and scanned from 35-mm slides, or on an Axioplan 2 microscope interfaced with a LSM410 confocal module (Carl Zeiss, Thornwood, NY) microscope with Openlab 2.25 software.

The same strain and plasmids used for the light/fluorescence microscopy were similarly induced before fixation for electron microscopy. The electron microscopy was carried out as described previously (Parsell *et al.*, 1994).

Wild-type (A3224) and suppressor (A3685, A3686) strains carrying the GFP-CDC3 and HSP104(G217S/T499I)-b/Leu plasmids were analyzed live after a 12-h induction by using the Lab-Tek II chambered #1.5 German coverglass system (Nalge Nunc International, Naperville, IL). Images were obtained using an Axiovert S100TV microscope (Carl Zeiss) interfaced to a Bio-Rad MRC1024 confocal system. All images were processed using Photoshop 6.

RESULTS

Screen for Mutations That Perturb the Regulated Function of Hsp104p

Our approach was based on the observation that although Hsp104p has powerful protein-remodeling functions, it can be overexpressed at high levels without affecting growth (Lindquist and Kim, 1996); thus, its broad capacity to interact with varied substrates and remodel them must normally be tightly controlled. Plasmids carrying *HSP104* were randomly mutagenized to determine whether Hsp104p is under tight regulation, and if so, to identify critical regulatory regions and residues. The mutants were screened for the capacity to inhibit growth at normal temperatures. Such a phenotype would reflect disruption of the normally tight controls on Hsp104p activities.

Pools of plasmid carrying *HSP104* under the control of a galactose-inducible promoter (*GAL1*) were chemically mutagenized to various extents (see MATERIALS AND METHODS). To favor the recovery of single point mutations, the frequency of mutations in a marker on the same plasmid was used to select a pool with a relatively low mutation rate. Wild-type cells transformed with these plasmids were screened for the ability to grow on glucose (which represses *GAL1* expression) but not on galactose (which induces expression). We used a diploid strain to reduce the recovery of extraneous mutations that simply perturb galactose metabolism; most such mutations are recessive. Of ~3100 transformants screened, 35 had strong growth-inhibition phenotypes on galactose. Each was put through several secondary screens to eliminate false positives and to eliminate mutations that had complex interactions with spontaneous genomic mutations (see MATERIALS AND METHODS for details). Of the 22 mutants passing these secondary screens, five were inhibited for growth on galactose at either 25 or 37°C, whereas 17 were inhibited for growth at 37°C but not at 25°C.

Positions of Mutations

Unexpectedly, although wild-type *HSP104* plasmids are readily recovered from *E. coli* (Schirmer *et al.*, 1994), the mutant plasmids were not. Further experiments indicated that this was due to toxicity of the mutant proteins in *E. coli.* The *GAL1* promoter is generally silent in this organism, but we found that sequences in the leader region of *HSP104* promoted transcription and, hence, protein expression (Schirmer and Lindquist, unpublished data). This was innocuous with the wild-type protein, but lethal with the mutants. To circumvent this problem, the mutant genes were recovered directly from yeast (where tight regulation of the galactose promoter kept them silent) by PCR (see MATERIALS AND METHODS). We focused on analysis of six mutants, representing the two growth phenotypes (five that inhibited growth at 25 or 37°C and one that inhibited growth at 37°C but not 25°C). These six mutants contained a total of 30 nucleotide changes, 22 of which created amino acid substitutions (Figure 1). Among them, substitutions at three residues in the 908-amino acid protein were recovered more than once, suggesting that they are particularly important in Hsp104p function. Notably, despite the low rate of mutagenesis observed within the marker gene in the same plasmid, every sequenced Hsp104p mutant contained multiple substitutions. This suggested that more than one mutation might generally be required to produce a dominant growth-inhibition phenotype for Hsp104p, a suggestion confirmed by further analysis (see below).

Ten of the 22 amino acid altering mutations occurred in the first nucleotide-binding domain (NBD1); four of these were in or very near the Walker A and B consensus sequences of the highly conserved phosphate-binding loop (P-loop; Walker *et al.*, 1982; Saraste *et al.*, 1990; Leipe *et al.*, 2002). Two occurred in the putative sensor 2 consensus of NBD1, which is thought to be located after the MR (Neuwald *et al.*, 1999). Unexpectedly, seven of the remaining 12 substitutions clustered in the highly variable MR. In fact, every mutant recovered in the screen for disruption of Hsp104p regulation had at least one MR mutation.

Although the amino acid sequence of the MR is highly variable, one small segment of 11 residues (Figure 2) exhibits moderate conservation among those members of the HSP100 family that function in thermotolerance (including plant, bacterial, and fungal members; Gottesman *et al.*, 1990; Schirmer *et al.*, 1996). Five of the seven MR mutations recovered in our screen were located in this small conserved segment (Figures 1, open box under MR; and 2).

All mutant proteins that affected growth at both temperatures carried at least one NBD mutation in addition to the MR mutation. The mutant that affected growth only at 37°C did not contain an NBD substitution. We chose one mutant from each of the two growth-inhibition categories for further detailed analysis. G217S/T499I was selected because it was independently recovered twice in the screen (the second time with an additional mutation; Figure 1) and had a relatively small number of substitutions. A503V/A509D was selected because its ability to block growth was temperature dependent.

General Characterization of Mutants

We first confirmed that the Hsp104p mutations were solely responsible for the selected phenotypes by recreating the mutant alleles through directed mutagenesis in fresh plasmids and fresh strains (our unpublished data). Next, we asked whether the growth-inhibition phenotype was dependent on either the high levels of expression characteristic of the *GAL1* promoter or some feature of galactose metabolism or galactose-regulated gene expression. The mutants were transferred to galactose expression vectors with different selectable markers (plasmids pGALSc104, 104b-U1, and HSP104-b/Leu; Table 2). A hormone-responsive promoter (Louvion *et al.*, 1993) and a methionine-regulated promoter (Korch *et al.*, 1991) were tested as alternative induction systems (see MATERIALS AND METHODS). The phenotypes of the two mutants were reproduced with all induction

B subfamily	consensus	hXKAAELRYGX
Saccharomyces cerevisiae Hsp104p		TATAADLRYFA
Mutations (this study)		I V
		I V D
Leishmania major		METAADLKYRV
Leishmania donovani		METAADLKYNV
Arabidopsis thaliana		LARAADLRYGA
Glycine max		LARAADLRYGA
Nicotinia tabacum		LARAADLRYGA
Triticum aestivum		LARVADLKYGA
Chlamydia trachomatis		YNRVAELRYSL
Rickettsia prowazekii		LAKASELKYGI
Dichelobacter nodosus		FARASEIQYGL
Saccharomyces cerevisiae Hsp78p		YTKASELRYSR
Escherichia coli		LARMSELQYGK
Haemophilus influenzae		LAKMSELQYGR
Synechocystis sp PCC6803 1		YNKAAELQYGK
Synechococcus sp PCC7942		LNKAAELKYGK
Corynebacter glutamicum		YGRVAELRYGR
Mycobacterium tuberculosis		LAKAAELRYGR
Treponema pallidum		LNKAAELRYGK
Bacillus anthracis		LNRAAELRHGK
Mycobacterium leprae		LAKAAELRYGR
Mycoplasma genitalium		YESASKILYSD
Synechocystis sp PCC6803 2		WEKAAKIKYGE

C subfamily	consensus	FEKAAXLRDRE
Bacillus subtilis		FEKAASLRDTE
Mycobacterium leprae		FEKAASLRDRE
Mycobacterium tuberculosis		FEKAASLRDRE
Bacillus anthracis		FEKAASLRDME
Serpulina hyodysenteriae		FEDAAKIRDEI
Lycopersicon esculentes		FEKAGELRDRE
Pisum sativum		FEKAGELRDKE
Brassica napus		FEKAGTLRDRE
Arabidopsis thaliana		FEMAGTLRDRE
Synechococcus sp PCC7942		FDKAGELRDRE
Synechocystis sp PCC6803		FEQAGELKDRE
Odontella sinensis		FDIAKQLVDHE
Heterosigma carterae		FELAMEIRDAE

Figure 2. Mutations in the conserved sequence element within the MR. The core of this element, KAAXLR, occurs in both the B and C subfamilies of HSP100/Clp proteins; however, each has its own variation. h, a hydrophobic residue.

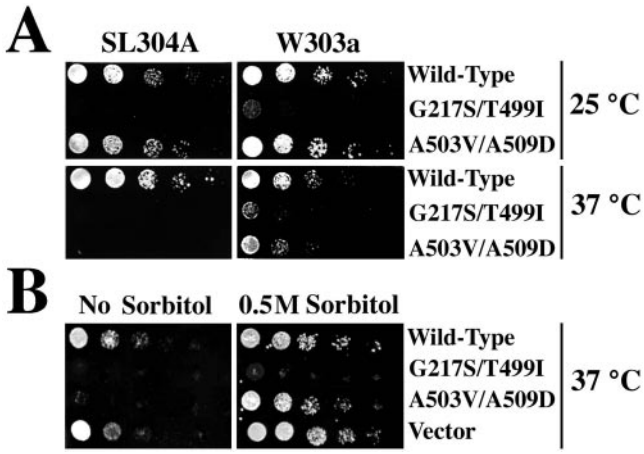

Figure 3. Dominant lethal phenotypes of mutants. (A) *HSP104* mutants inhibited growth. Wild-type (W303a) and Δ*hsp104* mutant cells (SL304A) carrying pGALSc104, its mutant variants, or the pLA1 vector control plasmid were first grown in noninducing liquid medium. Equal numbers of cells were then spotted onto galactose plates in fivefold serial dilutions (shown) to induce expression of the mutant proteins, or onto glucose plates to repress synthesis of the mutant proteins. No inhibition of growth was seen on the glucose plates (not shown). (B) Sorbitol suppressed the Hsp104p$^{A503V/A509D}$ mutant but not the Hsp104p$^{G217S/T499I}$ mutant. SL304A (Δ*hsp104*) cells carrying the same plasmids and grown as in A were plated on galactose medium with or without sorbitol.

systems, which varied >20-fold in the levels of protein they produced (our unpublished data), and with different selectable markers. Thus, the mutant phenotypes do not require high levels of protein expression and are not specific to any particular nutritional state.

Third, we addressed the possibility that the phenotypes were not due to the activities of the mutant proteins themselves, but rather to their ability to form mixed complexes with wild-type Hsp104p and perturb its function. Because Hsp104p functions in a hexameric complex, and the strain used in the screen expressed wild-type Hsp104p, mixed hexamers were probably formed. We compared plasmid-dependent phenotypes in isogenic wild-type and *hsp104* deletion strains (Figure 3A). Each of the phenotypes was recapitulated in both genetic backgrounds: Hsp104p$^{G217S/T499I}$ inhibited growth at 25 or 37°C, and Hsp104p$^{A503V/A509D}$ inhibited growth only at 37°C. However, both growth defects were more severe in the Δ*hsp104* background (Figure 3A, 37°C; our unpublished data). We conclude that the loss-of-growth phenotypes were not dependent upon interaction with wild-type Hsp104p, and, indeed, were partially attenuated by its presence.

Because the mutant proteins produce dominant gain-of-function phenotypes, we asked whether their effects were due to an alteration in their interaction with Sup35p, the only known essential substrate of Hsp104p. Sup35p, a translation-termination factor, is the protein determinant of the yeast prion [*PSI*$^+$] (reviewed in Serio and Lindquist, 2000). The C-terminal region of Sup35p contains the essential translation activity (Ter-Avanesyan *et al.*, 1993), and the N-terminal region confers upon Sup35p the capacity to assume distinct prion and nonprion conformations. The change between these states is regulated by interactions between the N-terminal domain and Hsp104p (Patino *et al.*, 1996; Schirmer and Lindquist, 1997; Cashikar *et al.*, 2002). When Sup35p prion conversion is too efficient it can be toxic (Ter-Avanesyan *et al.*, 1993; Derkatch *et al.*, 1996; Li and Lindquist, 2000), because the essential translation-termination activity of the C-terminal domain is inhibited when the protein is in the [*PSI*$^+$] state. Thus, it is possible that the gain-of-function mutant *HSP104* phenotypes were due to excess conversion of Sup35p to the prion state. To examine this possibility, we introduced a high-copy plasmid expressing the Sup35p C-terminal domain (Table 2), which does not enter the prion state, into the mutant cells. Expression of the C terminus alone did not mitigate the phenotype of either Hsp104p mutant (our unpublished data). Therefore, the growth inhibition caused by the Hsp104p mutants is not due to an enhancement of Sup35p prion conversion.

Finally, we explored the basis of the temperature sensitivity of the A503V/A509D allele. When Δ*hsp104* cells carrying the A503V/A509D mutant plasmid were grown at 37°C on medium containing 0.5 M sorbitol, growth was restored

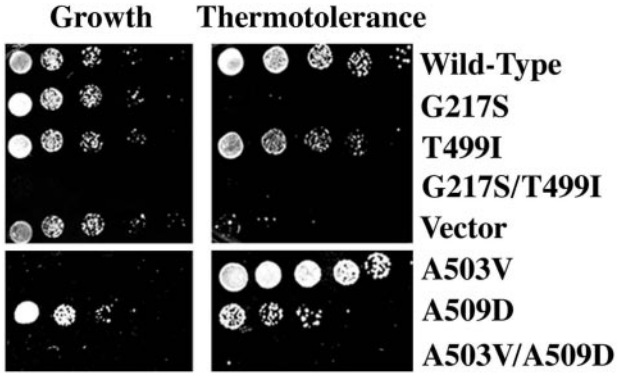

Figure 4. Phenotypes of individual mutations. Left, lethal phenotypes. pGALSc104 plasmids carrying the individual or double substitutions were tested in strain SL304A (Δ*hsp104*) on inducing medium. Cells were grown at 25°C (top) or 37°C (bottom). Right, thermotolerance phenotypes. Using the same strain and plasmids as for the growth assessments, mutant proteins were first induced for 4 h. Cells were then pretreated at 37°C for 30 min (to induce other tolerance factors) before an extreme heat stress of 50°C for 30 min. To determine survival rates, cells were plated at 25°C on glucose medium (which repressed further synthesis of the mutant protein). All were plated in fivefold serial dilutions.

(Figure 3B). Because sorbitol stabilizes protein structure (Gekko and Ito, 1990), this suggested that the mutant phenotype was caused by an effect of temperature on the structural state of the protein. Sorbitol can also stabilize cells against cell lysis defects, but mutant cells had no such defects. Further suggesting that the temperature sensitivity of the Hsp104p$^{A503V/A509D}$ phenotype involves a protein-folding problem, it was partially suppressed by overexpression of the chaperones Hsp90p and Sis1p (an HSP40 member; our unpublished data). In contrast, neither sorbitol nor the chaperone proteins alleviated the non-temperature-sensitive phenotype of Hsp104p$^{G217S/T499I}$.

Effects of Individual Substitutions on Growth

The fact that the mutants recovered in our original screen contained more substitutions than expected (see MATERIALS AND METHODS) suggested that more than one mutation may generally be required to produce a dominant growth-inhibiting form of Hsp104p. To investigate this, we recreated the individual amino acid substitutions in two plasmids, pGALSc104 and 104b-U1 and compared the phenotypes they produced with those of the original double mutants in both wild-type (our unpublished data) and Δ*hsp104* (Figure 4) strain backgrounds.

Neither G217S nor T499I alone produced a growth inhibition at either 25°C (Figure 4, top left) or at 37°C (our unpublished data). The single mutation A503V was sufficient to recapitulate the growth-inhibition phenotype at 37°C. A509D alone produced no significant growth defect (Figure 4, bottom left). In some experiments, with longer incubations or higher cell densities, the A509D mutation enhanced the severe growth defect caused by A503V (our unpublished data).

We also tested individual substitutions from other mutants recovered in the screen (A392T, A551T, P679L, and T726I). None produced a growth-inhibition phenotype on its own at either 25 or 37°C (our unpublished data). This suggests that with rare exceptions, such as the A503V mutation at high temperatures, gain-of-function growth-inhibiting phenotypes require more than one mutation in Hsp104p.

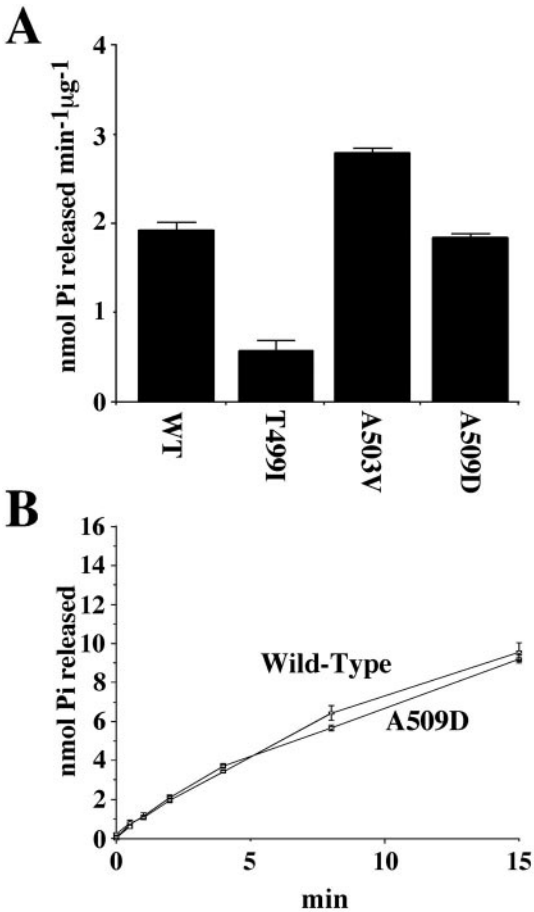

Figure 5. ATPase activities of proteins carrying single substitutions. Wild-type Hsp104p, Hsp104p^{T499I}, Hsp104p^{A503V}, and Hsp104p^{A509D} proteins were purified from *E. coli*. (A) Basal ATP hydrolysis rates of each protein were assayed in a physiological salt buffer by release of Pi from ATP. (B) To establish that the similarity in ATP hydrolysis rates between Hsp104p^{A509D} and wild-type Hsp104p is not restricted to early time points, they were compared over a range of time.

Effects of Mutations on Thermotolerance Function

Next, we asked what effects the mutations had on Hsp104p's ability to perform its normal functions. Preliminary results indicated that the mutations affected propagation of the prion [*PSI*$^+$] (Chernoff, Schirmer, and Lindquist, unpublished data). However, defining the molecular nature of these effects was complicated by the fact that prion maintenance is disrupted by either too much or too little Hsp104p function. The relationship between Hsp104p and thermotolerance is simpler: the higher the concentration of Hsp104p, the greater the thermotolerance (Lindquist and Kim, 1996).

Thermotolerance assays for Hsp104 function were possible with the mutant proteins because such assays use short-term inductions, whereas growth-inhibition phenotypes require many hours of induction. Accordingly, mutant proteins were induced with galactose to a level comparable with that obtained with wild-type Hsp104p after a strong tolerance-inducing preheat treatment. Cells were then exposed to a severe heat shock at 50°C and plated on glucose medium at normal temperatures to repress further expression of the mutant proteins and to determine whether they had been able to increase the number of cells surviving the heat shock.

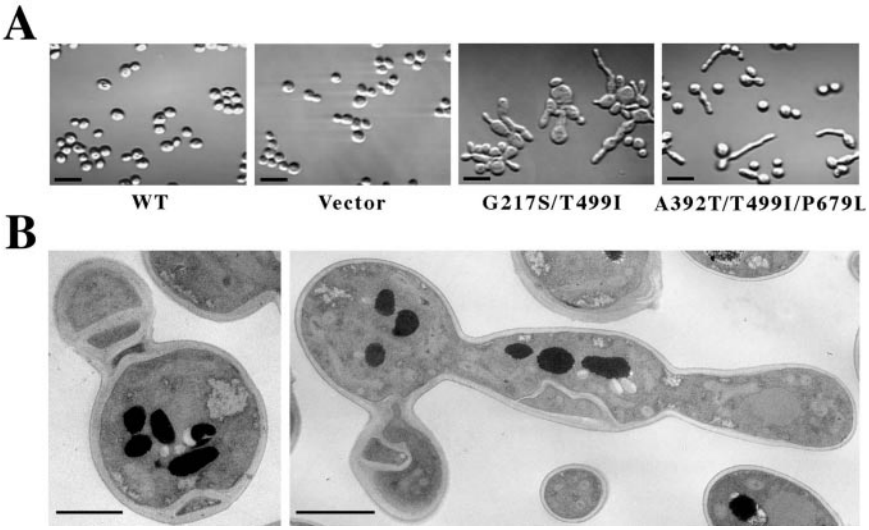

Figure 6. Cells that express proteins carrying the T499I substitution in combination with NBD substitutions exhibit elongated buds with misplaced cell-wall deposition. (A) Differential interference contrast micrographs of Δ*hsp104* cells (strain SL304A) carrying the parent vector or expressing Hsp104p, Hsp104p$^{G217S/T499I}$, or Hsp104p$^{A392T/T499I/P679L}$. Elongated buds began to occur after 5–6 h of mutant protein expression. Cells were fixed at ~10 h. Bars, 10 μm. (B) Electron microscopy of the *hsp104*$^{G217S/T499I}$ mutant cells, grown as described in A. Material resembling cell wall was deposited in strands or sheets within the cell and between the mother and daughter cells. Bars, 2 μm.

Neither mutant protein had any effect on the thermotolerance function of the endogenous Hsp104p (our unpublished data). In a Δ*hsp104* background, neither Hsp104p$^{G217S/T499I}$ nor Hsp104p$^{A503V/A509D}$ was capable of providing thermotolerance (Figure 4, right). Surprisingly, the individual constituent mutations had very different effects (Figure 4, right). Protein containing only the T499I mutation had thermotolerance function, but protein containing only the G217S mutation did not. Protein containing only the A503V substitution, which severely inhibits growth when continuously expressed at 37°C (Figure 4, left), conferred wild-type levels, or greater, of thermotolerance (Figure 4, right). In contrast, protein containing only the A509D substitution, which did not inhibit growth, had a greater than 50-fold reduction in thermotolerance function relative to wild-type Hsp104p. Similar results were obtained with (Figure 4, right) and without (our unpublished data) a preconditioning treatment at 37°C, that is, with or without other heat-inducible factors.

MR Mutations Alter ATP Hydrolysis

Because Hsp104p function requires ATP hydrolysis (Parsell *et al*., 1991; Schirmer *et al*., 1998), we examined the effects of the mutations on ATPase activity. Due to difficulties in recovering the G217S/T499I double-mutant protein from bacteria and yeast cells, we confined the analysis to the individual mutations.

The G217S substitution drastically lowered activity (our unpublished data); this was not unexpected because a G217V substitution (produced by site-directed mutagenesis, Schirmer *et al*., 1998) lowers the ATPase activity of Hsp104p to undetectable levels. Hsp104p^{T499I} exhibited reduced ATP hydrolysis (less than one-half that of wild-type; Figure 5A), whereas Hsp104p^{A503V} exhibited significantly elevated basal ATPase activity at 37°C (Figure 5A; Cashikar *et al*., 2002). Hsp104p^{A509D} hydrolysis rates were indistinguishable from those of wild-type protein (Figure 5, A and B). Thus, the distinct phenotypes of the MR substitutions are associated with distinct biochemical characteristics.

Aberrant Morphology Produced by Hsp104p$^{G217S/T499I}$ but Not by Hsp104p$^{A503V/A509D}$

Hsp104p$^{G217S/T499I}$ produced a striking morphological abnormality: cells arrested with elongated, often grossly distended buds in both wild-type and Δ*hsp104* backgrounds (Figure 6A; our unpublished data). Electron microscopy revealed abundant, abnormally placed cell wall material (Figure 6B) similar to that observed in *cdc3* mutants (Slater *et al*., 1985). In contrast, Hsp104p$^{A503V/A509D}$ produced no morphological changes that were obvious, by either light or electron microscopy (our unpublished data).

The similarity of the Hsp104p$^{G217S/T499I}$ phenotype to that produced by mutant septin proteins (Hartwell, 1971; Slater *et al*., 1985; Longtine *et al*., 1996; Gladfelter *et al*., 2001) led us to investigate the effect of the mutant Hsp104p on septin localization. To do so, we used antibodies specific to two primary structural components of the septin ring, Cdc11p (Figure 7) and Cdc3p (our unpublished data) (Ford and Pringle, 1991; Kim *et al*., 1991). In cells arrested by Hsp104p$^{G217S/T499I}$, the septins often failed to localize to the bud neck and sometimes formed linear strands or rings in aberrant locations rather than normal rings at the neck (Figure 7, A and B).

To determine whether Hsp104p$^{G217S/T499I}$ colocalizes with the misplaced septins, cells were stained with antibodies specific to either the C terminus or the middle region of Hsp104p (Cashikar *et al*., 2002). Both antibodies gave the same pattern: cells expressing Hsp104p$^{G217S/T499I}$ often had strong staining of aberrant ring structures superimposed on diffuse general staining (Figure 7, A and B). These rings resembled (Figure 7A) and seemed to colocalize with (Figure 7B) the aberrant septin rings found in the same cells. In contrast, cells expressing either Hsp104p or Hsp104p$^{A503V/A509D}$ exhibited only diffuse Hsp104p staining (Figure 7; our unpublished data). The diffuse staining of wild-type Hsp104p was absent at the septin rings (Figure 7B, arrowheads). However, overexpression of wild-type Hsp104p did cause septin mislocalization in some (<5%) cells (Figure 7B, insets), suggesting that Hsp104p does have a very weak intrinsic potential to interact with the septin ring.

Mutations in CDC12 Partially Suppress the G217S/T499I Phenotype

To investigate the nature of the interactions of Hsp104p$^{G217S/T499I}$ with the septins, we conducted two types of screens for suppressors of the Hsp104p$^{G217S/T499I}$-induced growth inhibition. First, we screened for proteins that would suppress the mutant phenotype when overex-

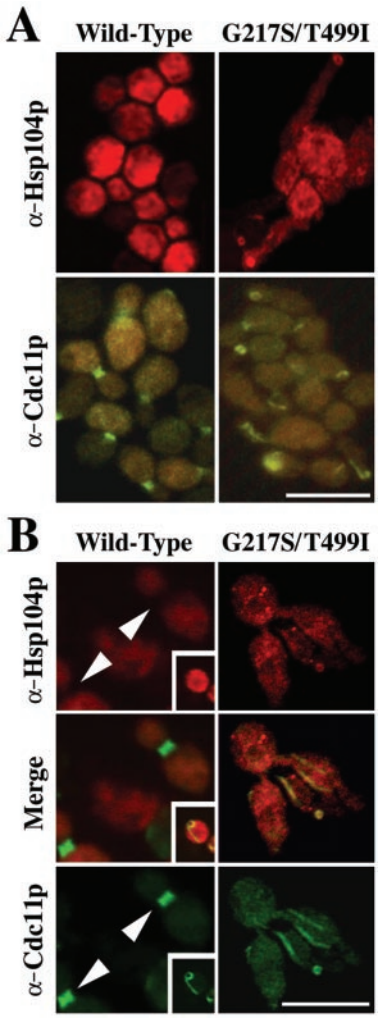

Figure 7. Abnormal septin organization and colocalization of Hsp104p$^{G217S/T499I}$ with the septins in cells expressing the mutant, but not the wild-type, Hsp104p. SL304A (Δ*hsp104*) cells expressing wild-type Hsp104p (left) or Hsp104p$^{G217S/T499I}$ (right) were examined. (A) Cells were stained with polyclonal antibodies that recognize either the C terminus of Hsp104p (top) or the Cdc11p septin (bottom). Cells were fixed after ~9 h of expression of wild-type or mutant protein. No difference was observed between cells processed with formaldehyde/Triton or with MeOH/acetone. Images were obtained with an Olympus epifluorescence microscope. (B) Cells were stained with an anti-Hsp104p monoclonal antibody against the Hsp104p MR region and the anti-Cdc11p affinity-purified polyclonal antibody. Arrowheads, regions of septin rings, showing little staining for Hsp104p. Inset, example of rare cells with mislocalized septins in a cell overexpressing wild-type Hsp104p. Cells were fixed after ~12 h of expression. Photomicrographs are confocal sections. Bars, 10 μm.

pressed. In a screen of approximately 1 million transformants from a galactose-inducible genomic expression library, no suppressors were isolated (see MATERIALS AND METHODS). Second, using a mini-transposon insertion library, we searched for proteins whose deletion or truncation would suppress the mutant phenotype. Screening of ~10^5 transformants yielded two strains in which growth was restored (see MATERIALS AND METHODS).

In both cases, suppression of the Hsp104p$^{G217S/T499I}$-induced growth defect was dominant when the haploid suppressor strain was mated to a wild-type strain, and each diploid yielded 2:2 segregation of the suppressor in all six tetrads dissected. Surprisingly, suppression was unlinked to the transposon insertion. Thus, the suppression must have been due to spontaneous genomic mutations that were unrelated to the insertional mutagenesis. When the two suppressor strains were mated and sporulated, all progeny exhibited the suppressor phenotype, suggesting that the two independently isolated suppressors mapped to the same locus.

To identify this locus, we constructed a genomic library from one of the suppressor strains and transformed it into cells expressing Hsp104p$^{G217S/T499I}$ (see MATERIALS AND METHODS). Transformants were screened for suppression of the Hsp104p$^{G217S/T499I}$ loss-of-growth phenotype on galactose medium. Three plasmids that conferred suppression were sequenced. All carried a mutant allele of the septin gene *CDC12* (K351N) in which the lysine was changed to asparagine at residue 351, adjacent to the predicted coiled-coil domain of Cdc12p (amino acids 356–407 as defined by BioKnowledge Library [Incyte Palo Alto, CA]; amino acids 370–407, as defined using the algorithm of Wolf *et al.*, 1997). Sequencing the *CDC12* locus of the second independently obtained suppressor strain revealed a mutation in the same region, E368Q, that changed glutamate to glutamine at position 368. Notably, Cdc12p is a component of the septin ring at the mother-bud neck (Haarer and Pringle, 1987).

The suppressor strains expressing Cdc12p^{K351N} or Cdc12p^{E368Q} grew well in the presence of Hsp104p$^{G217S/T499I}$ (Figure 8A). Although some cells still exhibited the elongated bud morphology, this was much less frequent and less severe than in strains expressing Hsp104p$^{G217S/T499I}$ without the mutant Cdc12p (Figures 6A and 8B). The ability of each *CDC12* mutant to suppress the Hsp104p$^{G217S/T499I}$ phenotype was confirmed by subcloning the mutant *CDC12* open reading frames into plasmids and retesting them for suppression. Each mutant allele, but not the wild-type *CDC12* allele, suppressed the growth defect of Hsp104p$^{G217S/T499I}$ when expressed using either a galactose-inducible promoter or the upstream region of *CDC12* itself (our unpublished data).

To determine whether the suppressors restored normal septin localization in cells expressing Hsp104p$^{G217S/T499I}$, we used GFP fused at or near the N terminus of either wild-type Cdc3p (Figure 8B) or wild-type Cdc12p (our unpublished data). Both proteins localized normally in wild-type and *CDC12* suppressor strain backgrounds in either the presence or absence of wild-type Hsp104p (our unpublished data). However, in cells expressing Hsp104p$^{G217S/T499I}$, the GFP-septins were mislocalized in virtually all cells expressing wild-type Cdc12p, but in only a fraction of those expressing the suppressor mutants Cdc12p^{K351N} or Cdc12p^{E368Q} (Figure 8B).

The identification of mutant septin alleles as efficient suppressors of Hsp104p$^{G217S/T499I}$ indicates that the toxic effect of Hsp104p$^{G217S/T499I}$ is directly linked to the septin defects observed after its induction. The lack of a visible septin defect in strains expressing Hsp104p$^{A503V/A509D}$ suggested that this mutant protein has a different mode of toxicity. Indeed, the Cdc12p mutations (K351N and E368Q) did not suppress the loss-of-growth phenotype of Hsp104p$^{A503V/A509D}$ (our unpublished data).

Specificity of the Cell Morphology Phenotype

Neither of the *CDC12* mutations produced a mutant phenotype on its own. Either in the presence of wild-type Hsp104p or in its absence (a Δ*hsp104* strain), the cells grew at normal

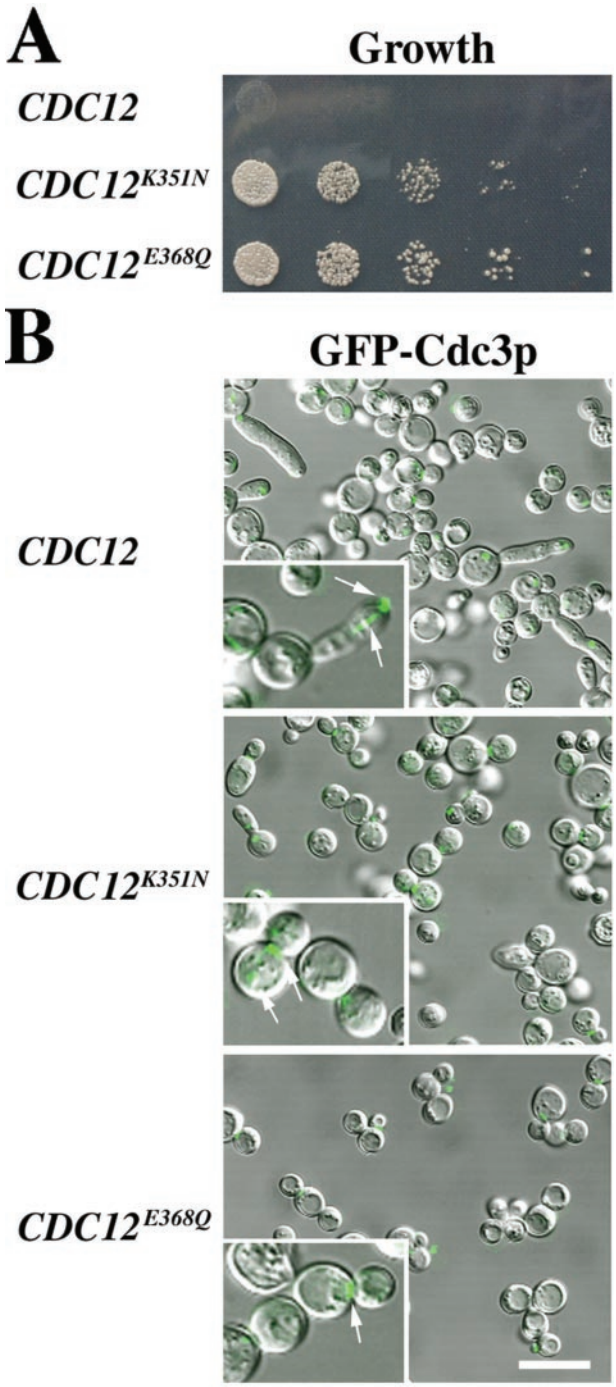

Figure 8. Suppression of the G217S/T499I mutant by mutations in *CDC12*. (A) *hsp104*Δ strains A3224 (*CDC12*), A3685 (*CDC12*^{K351N}), and A3686 (*CDC12*^{E368Q}) carrying plasmid HSP104(G217S/T499I)-b/Leu were grown to mid-log phase in SD and plated on SG inducing medium at 30°C in fivefold serial dilutions. (B) Strains used in A were transformed with the additional plasmid GFP-CDC3, grown to late log phase in SD, washed, and induced in SG for ~13 h. Cells were viewed live by confocal microscopy. Pictures are merged differential interference contrast and GFP images. Bar, 10 μm. Insets, cells at higher magnification with arrows denoting Cdc3p septin fluorescence. Linear septin strands were only observed in the presence of wild-type (WT) Cdc12p; mislocalized septin rings were observed with WT and occasionally with the mutant forms of Cdc12p.

rates and seemed normal by light microscopy and septin staining (our unpublished data). Thus, the Cdc12p mutations did not perturb septin function. Rather, they specifically and strongly suppressed the ability of Hsp104p$^{G217S/T499I}$ to interact with the septin complex and perturb its function.

To determine whether the specificity of the bud morphology defect observed with Hsp104p$^{G217S/T499I}$ was due to the MR substitution (T499I) or to the NBD substitution (G217S), we asked whether T499I produced a similar defect when combined with other NBD mutations. The substitutions A392T and P679L (originally isolated in combination with other mutations; Figure 1) were chosen because they were located in different structural elements than G217S (found in the Walker A sequence of NBD1); A392 is located in box VII″ of NBD1 (a highly conserved sequence motif; Neuwald *et al.*, 1999), whereas P679 occurs at the edge of the Walker B sequence of NBD2. Neither A392T nor P679L produced a thermotolerance defect on its own. In combination with each other, they produced only a partial defect (our unpublished data). More importantly, expression of these NBD mutants, like that of the G217S mutant, produced no growth defects, either alone or in combination (our unpublished data).

When the individual NBD mutations A392T or P679L were combined with the T499I substitution (Hsp104p$^{A392T/T499I}$ or Hsp104p$^{T499I/P679L}$), no inhibition of growth was observed. However, when both mutations were combined with T499I (Hsp104p$^{A392T/T499I/P679L}$), the triple mutant had the same phenotype as Hsp104p$^{G217S/T499I}$: growth inhibition was observed that was not suppressed by sorbitol, elongated buds were produced with misplaced septin rings, the mutant Hsp104p colocalized with the septins, and the phenotype was suppressed by both Cdc12p^{K351N} and Cdc12p^{E368Q} (Figure 6A, right; our unpublished data). Thus, the specificity of this septin-assembly phenotype depended upon the MR substitution, but its manifestation also required a perturbation of NBD function.

DISCUSSION

Critical Role of the Middle Region

Using random mutagenesis, we have conducted a screen for mutations that disrupt the normally tight regulation of Hsp104p activity. Specifically, we looked for mutations that created a dominant gain-of-function effect that blocks cell growth. To our knowledge, this is the first such screen conducted with any member of the large superfamily of AAA$^+$ proteins. It has provided important and unexpected insights, suggesting a promising strategy for study of other members of this broad family. Every mutant we isolated depended upon a substitution in the MR of Hsp104p to exert its effect. This unequivocally demonstrates the importance of the MR, a region little studied and, until recently, thought to be of little significance.

Beyond establishing the MR as an important region, our work pinpoints a small, moderately conserved sequence of 11 amino acids (residues 499–509) as being particularly critical for Hsp104p function. Remarkably, three single amino acid substitutions found in this region caused extremely diverse alterations of Hsp104p function. Hsp104p^{A503V} strongly inhibited growth at 37°C, yet it could promote survival after extreme stress as effectively as wild-type Hsp104p. Conversely, the MR mutant Hsp104p^{A509D} had no deleterious effect on growth but strongly impaired thermotolerance function. (It is the first Hsp104p mutation to impair thermotolerance without also reducing basal ATP hydrolysis.) The third MR mutant, Hsp104p^{T499I}, had reduced ATP

hydrolysis but was able to confer thermotolerance and did not affect growth. Strikingly, the combination of T499I with very different mutations in the NBDs of Hsp104p (G217S or A392T plus P679L) caused mislocalization of septins and associated defects in bud morphogenesis and cell wall deposition.

The importance of the MR in Hsp104p function was unexpected due to its extreme variability: it is the most diverse region among class 1 HSP100/Clp proteins, in both length and sequence (Gottesman *et al.*, 1990; Schirmer *et al.*, 1996). In fact, it has previously been termed, very naturally, the "spacer" region (Gottesman *et al.*, 1990). It is also highly variable even among members of the B-type HSP100 proteins from plants, bacteria, and fungi (Schirmer *et al.*, 1996), all of which function in stress tolerance (Figure 2). While this manuscript was in preparation, a complete deletion of the MR in the *E. coli* HSP100 protein ClpB was found to block the protein's chaperone activity without affecting its stability or expression levels (Mogk *et al.*, 2003). This strongly suggests that despite variation in its sequence, the MR will prove to have important functions in all members of this large family of protein-remodeling factors.

Further biochemical analysis of the A503V substitution in our laboratory (Cashikar *et al.*, 2002) has suggested that it disrupts the mechanism of communication between Hsp104p's two NBDs. When poly-lysine binds the C-terminal region of wild-type Hsp104p, it triggers nucleotide hydrolysis in NBD2, which in turn causes a conformational change in the MR and stimulates hydrolysis at NBD1. Protein carrying the A503V substitution binds poly-lysine but does not respond with elevated hydrolysis. The unpublished crystal structure of the *Thermus thermophilus* ClpB protein indicates that the MR forms a large coiled-coil domain in a position where it might influence communication between the two NBDs (Lee and Tsai, personal communication). The 11-amino acid sequence that we have identified by genetic analysis as critical for Hsp104p function lies at one apex of the coiled-coil domain. Together, genetic, structural, and biochemical data from several laboratories are beginning to produce a picture of how interdomain communication might be involved in driving the complex conformational changes that are the heart of Hsp104p function.

Molecular Explanation for a Gain-of-Function Phenotype

In addition to establishing that changes in the MR can produce dominant gain-of-function mutations in Hsp104p, we have used cell biological and genetic methods to provide a specific molecular explanation for one of the phenotypes. Hsp104p$^{G217S/T499I}$ affects cell morphology by interacting with the septins. This effect on morphology was unexpected for several reasons: 1) no other Hsp104 mutant analyzed to date (in this or several other studies) affects morphology; 2) deletion of *HSP104* has no effect on septin localization or cell morphology, either at 25°C or at higher temperatures; and 3) even very strong overexpression of wild-type Hsp104p causes septin mislocalization in only a few cells. Either wild-type Hsp104p does not interact with septins, or Hsp104p has a weak ability to interact with septins, but this interaction is transient and unimportant under laboratory conditions. It might facilitate assembly or disassembly of the septin ring in a manner that matters under some circumstances in the wild. Regardless, the phenotype is a novel gain of function, in which a potentially very weak intrinsic capacity to interact with the septins is strongly enhanced by the T499I mutation in the MR.

An extensive search for suppressors of the Hsp104p$^{G217S/T499I}$ growth defect failed to uncover any high-copy suppressors or deletion mutant suppressors. Instead, two spontaneous genomic suppressor mutations were found, each residing in *CDC12*, which encodes a component of the septin ring (Haarer and Pringle, 1987; Longtine *et al.*, 1996) and of the septin complex that can assemble into filaments in vitro (Frazier *et al.*, 1998). Interestingly, Cdc12p resembles the best characterized substrate of Hsp104p, the NM domain of the Sup35p prion protein, which forms filaments and has a domain enriched in lysines (Glover *et al.*, 1997) that influences its interaction with Hsp104p (Liu *et al.*, 2002). The two independently isolated Cdc12p suppressor mutants, Cdc12p^{K351N} and Cdc12p^{E368Q}, each contained a substitution in a lysine-rich region.

Although specificity of the Hsp104p$^{G217S/T499I}$ mutation for interaction with the septins is dictated by the T499I substitution, the associated phenotype requires an additional impairment of Hsp104p NBD function. The fact that perturbation of septin structure by either Hsp104p$^{G217S/T499I}$ or Hsp104p$^{A392T/T499I/P679L}$ is accompanied by colocalization of the protein with the septins suggests that impairment of ATPase activity inhibits the release of binding with Cdc12p. Thus, the Hsp104p$^{G217S/T499I}$:Cdc12p interaction may represent a snapshot of the normally very transient interaction between this AAA$^+$ protein and its substrates, which has been difficult to capture with wild-type protein. In conjunction with the Cdc12p suppressors, the Hsp104p$^{G217S/T499I}$ mutant could serve as a valuable tool for further exploration of the determinants of Hsp104p substrate recognition.

The Evolution of AAA$^+$ Protein Functions

A common theme in molecular evolution is the radiation of proteins with broad functions into a class of proteins that fill specific niches through alterations in their regulation and substrate selection. In some cases, such as the acquisition of a new domain, the process leading to the acquisition of a novel function is clearly defined (Patthy, 2003). It remains a major puzzle, however, for the broad superfamily of AAA$^+$ proteins, which couple the function of their ATP-hydrolysis domains to the remodeling of a bewildering variety of substrates. Our gain-of-function mutants provide an enticing picture of how such radiations might occur. Single amino acid changes in one small region of the protein can create a variety of functionally distinct mutants. Thus, it becomes possible to imagine how subtle changes in Hsp104p MR sequence and NBD function could serve to modulate substrate recognition and the duration of substrate interactions, as we have observed in our Hsp104p$^{G217S/T499I}$ mutant. Hence, the apparent paradox we have uncovered, that the most variable region in the broad family of proteins to which Hsp104p belongs plays such a critical role in Hsp104p function, can be resolved. Some MR residues may vary due to low sequence constraints, but others may vary precisely because they are so important that they provide an opportunity to rapidly modulate the function and specificity of the protein during evolution.

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