Molecular Chaperone Hsp104 Can Promote Yeast Prion Generation

Dmitry S. Kryndushkin, Abbi Engel, Herman Edskes and Reed B. Wickner1

Laboratory of Biochemistry and Genetics, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892-0830

Manuscript received February 14, 2011
Accepted for publication April 1, 2011

ABSTRACT

[URE3] is an amyloid-based prion of Ure2p, a regulator of nitrogen catabolism in Saccharomyces cerevisiae. The Ure2p of the human pathogen Candida albicans can also be a prion in S. cerevisiae. We find that overproduction of the disaggregating chaperone, Hsp104, increases the frequency of de novo [URE3] prion formation by the Ure2p of S. cerevisiae and that of C. albicans. This stimulation is strongly dependent on the presence of the [PIN+] prion, known from previous work to enhance [URE3] prion generation. Our data suggest that transient Hsp104 overproduction enhances prion generation through persistent effects on Rnq1 amyloid, as well as during overproduction by disassembly of amorphous Ure2 aggregates (generated during Ure2p overproduction), driving the aggregation toward the amyloid pathway. Overproduction of other major cytosolic chaperones of the Hsp70 and Hsp40 families (Ssa1p, Sse1p, and Ydj1p) inhibit prion formation, whereas another yeast Hsp40, Sis1p, modulates the effects of Hsp104p on both prion induction and prion curing in a prion-specific manner. The same factor may both enhance de novo prion generation and destabilize existing prion variants, suggesting that prion variants may be selected by changes in the chaperone network.

The [URE3] prion is a self-propagating amyloid (a β sheet-rich filament) of the Saccharomyces cerevisiae Ure2 protein that largely prevents Ure2p’s repression of catabolism of poor nitrogen sources, leaving it stuck in the “on” position (LaGroute 1971; Wickner 1994; Taylor et al. 1999; Brachmann et al. 2005). [PSI+] is an amyloid prion of Sup35p, abrogating Sup35p’s essential translation termination function enough to allow some read-through of termination codons (Cox 1965; Wickner 1994; Glover et al. 1997; King et al. 1997; Paushkin et al. 1997; King and Diaz-Avalos 2004; Tanaka et al. 2004). The [PIN+] prion was discovered on the basis of its requirement for efficient generation of the [PSI+] prion by overproduction of Sup35p (Derkatch et al. 1997). It was later shown to be an amyloid of Rnq1p (Sondheimer and Lindquist 2000; Derkatch et al. 2001) and to promote de novo formation of [URE3] as well (Bradley et al. 2002). Full-length recombinant Sup35p, Ure2p, and Rnq1p form amyloid fibers spontaneously at neutral pH, and these fibers can infect yeast cells with the corresponding prion (King and Diaz-Avalos 2004; Tanaka et al. 2004; Brachmann et al. 2005; Patel and Liebman 2007). These and other yeast and fungal prions (Saape 2007; Wickner et al. 2010) have been useful models of the mammalian prion diseases, the transmissible spongiform encephalopathies (TSEs) (Collinge and Clarke 2007; Aguzzi et al. 2008; Caughhey et al. 2009).

A single prion protein sequence can form any of several prion variants, with distinct biological properties, due to differences in amyloid structure, a phenomenon first demonstrated in mammalian TSEs (Bessen and Marsin 1992; Caughhey et al. 1998; Bruce 2003) and now known in several yeast prion systems as well (Derkatch et al. 1996; King 2001; Schlimpberger et al. 2001; Bradley et al. 2002; King and Diaz-Avalos 2004; Tanaka et al. 2004; Brachmann et al. 2005; Toyama et al. 2007). Yeast prion variants may be distinguished biologically by the intensity of the prion phenotype, the stability of prion propagation, the sensitivity to deficiency or overproduction of various chaperones, or the ease of transmission to other species (Derkatch et al. 1996; Newnam et al. 1999; Kushnirov et al. 2000; Borchsenius et al. 2006; Edskes et al. 2009).

[PSI+], [URE3], and [PIN+] all require the disaggregating chaperone Hsp104 for their propagation (Chernoff et al. 1995; Derkatch et al. 1997; Moriyama et al. 2000). Hsp104, along with other cooperating chaperones, breaks long amyloid filaments into shorter ones, thereby creating new prion seeds (reviewed by Romanova and Chernoff 2009; Haslberger et al. 2010). In addition, overproduction of Hsp104 efficiently cures [PSI+], but not [URE3] or [PIN+] (Chernoff et al. 1995; Derkatch et al. 1997; Moriyama et al. 2000).
Hsp70 and Hsp40 chaperones also play a prominent role in prion propagation, with considerable specificity in which chaperone helps or hinders which prion (reviewed by Sharma and Masison 2009). Mutants of the cytoplasmic Hsp70 ssa1 lose [PSF+], but not [URE3], while mutants of ssa2 lose [URE3] but not [PSF+] (Jung et al. 2000; Roberts et al. 2004). Overproduction of the Hsp40 family chaperone Ydj1p cures [URE3] via an interaction with Hsp70s (Moriyama et al. 2000; Sharma et al. 2009), but does not cure [PSF+] (Kuminrov et al. 2000). Sis1p, another Hsp40, is necessary for the propagation of [URE3], [PSF+], and [PIN+] (Sondheimer et al. 2001; Higurashi et al. 2008). The 34-residue repeat (“tetraicopeptide”)—containing cochaperones, Sti1p and Cpr7p, that mediate Hsp70—Hsp90 interactions have also been found to be involved in prion propagation (Jones et al. 2004; Reidy and Masison 2010), as have nucleotide exchange factors for Hsp70s, namely Sse1p and Fes1p (Fan et al. 2007; Kryndushkin and Wickner 2007). In addition to molecular chaperones, cytoskeletal proteins, elements of the ubiquitin system, and other factors modulate prion propagation in S. cerevisiae (Bailleul et al. 1999; Bailleul-Winslett et al. 2000; Allen et al. 2007; Kryndushkin et al. 2008).

Compared to prion propagation, much less is known about cellular proteins that can influence yeast prion generation. It was shown that either [PIN+] or overproduction of one of the several polyglutamine-rich proteins, including Rnp1p, is necessary for efficient [PSF+] de novo induction (Derratch et al. 2001; Osherovich and Weissman 2001). Chaperone Ssa1p, a member of the Hsp70 family, was reported to enhance [PSF+] generation (Allen et al. 2005). In addition, deficiency of the ribosome-associated Hsp70s, called Ssb1/2, results in increased spontaneous or induced [PSF+] formation (Chernoff et al. 1999). Since cellular chaperones actively interact with misfolded protein aggregates, it is reasonable to suggest that other chaperones may influence prion generation in vivo. Consistent with this hypothesis, several chaperones influence the rate of spontaneous assembly of both Sup35p and Ure2p into amyloid-like fibers in vitro. Members of Hsp70 and Hsp40 families were reported to reduce the polymerization rate for both proteins (Krzewska and Melki 2006; Savistchenko et al. 2008; Shorter and Lindquist 2008). In contrast, Hsp104p was the only factor reported to promote amyloid fibrilization in vitro of both Sup35p and Ure2p (Krzewska and Melki 2006; Shorter and Lindquist 2006). Hsp104’s fiber-severing action may be speeding the gross appearance of amyloids, not by forming the very first seeds, a largely undetectable event, but by accelerating the amplification process, and so appear to be stimulating initiation.

In this study, we show that overproduction of chaperone Hsp104p can enhance [URE3] prion generation induced by excess of Ure2p, whereas other chaperones act in an opposite direction, inhibiting prion generation. A similar, but much stronger effect was obtained with a prion, [URE3alb], formed by Ure2p from Candida albicans (Ure2ab) in S. cerevisiae. Analysis of prion-specific chaperone action profiles reveals that the same factor may both increase de novo prion generation and destabilize existing prions.

**MATERIALS AND METHODS**

**Strains, media, and plasmids:** Strains BY241 (MATa ura3 leu2 trpl 1 PIN/DAL5-ADE2 P/DAL5-CANI karl [URE3] or [ure-o] [PIN+]) and BY251 (MATa leu2 trpl 1 his3 P/DAL5-ADE2 P/DAL5-CAN1 karl [URE3] or [ure-o] [PIN+]) were described previously (Brachmann et al. 2005). DK174 (MATa ura3 leu2 trpl 1 his3 P/DAL5-ADE2 P/DAL5-CAN1 karl [ure-o] [PIN+]) was made by mating between BY241 and BY251. Strain 1075 (MATa ura3 leu2 trpl 1 his3 P/DAL5-ADE2 karl [URE3] or [ure-o] [PIN+]) was a gift of Dr. Daniel Masison (Sharma and Masison 2008). BY92 has the URE2 cerevisiae open reading frame (ORF) replaced with the C. albicans URE2 ORF, but driven by the constitutive cerevisiae URE2 promoter (Edskes et al. 2011). All of the above strains contain the ADE2 gene under control of the DAL5 promoter, allowing detection of the prion state of Ure2p (or Ure2pab) and even different [URE3] prion variants by colony color (Brachmann et al. 2005). The [ure-o] [pin+] derivatives were obtained by growth on media with 5 mM GuHCl. For [PSF+] experiments, strain 74-D694 (MATa ade1-14 ura3 leu2 trpl 1 his3 [PIN+] [psi+]) (Chernoff et al. 1995) was used.

Standard yeast media and cultivation procedures were used (Sherman 1991). To obtain better red color development, 1/2 YPD medium, containing half the normal amount of yeast extract, was used instead of YPD. For prion selection, –Adex medium consists of standard synthetic defined (SD) medium supplemented only with standard amounts of substances required for growth (0.002% of tryptophan, histidine, uracil, and 0.01% leucine). During prion induction, raffinose medium contains 2% raffinose as a carbon source, whereas galactose–raffinose medium contains 2% galactose and 1% raffinose instead of dextrose as a carbon source; no additional amino acids except those required for growth were added.

Plasmids used are listed in Table 1. Plasmid construction is described in supporting information.

**Prion curing and induction experiments:** To measure [URE3] or [URE3alb] loss under chaperone overproduction, ~20 random yeast colonies from a transformation plate were inoculated in liquid YPD media and grown overnight to allow plasmid loss (which was ~70–90% complete according to further analysis); then 5000 cells were spread on a 1/2 YPD plate. The ratio of red to white colonies was scored.

To perform prion induction, the nonprion strain containing either the empty vector or a high-copy plasmid with a chaperone gene of interest was transformed with the corresponding overexpression plasmid (a prion gene under control of a galactose-inducible promoter). Transformants were grown overnight in liquid raffinose medium and then were shifted to galactose–raffinose medium for 2 days. Finally, for selection of prion-containing cells, aliquots were plated on –Adex medium, where the Pgal promoter is repressed. After 5 days of growth at 30°C, medium and large Ade+ colonies were counted. The high-copy plasmid with a chaperone gene was maintained during the induction in galactose–raffinose medium, but was not selected during growth on –Adex plates; since overproduction of some chaperones can cure [URE3]...
or [URE3alb], we did not want to unnecessarily destabilize the prions that did arise.

Induction of [URE3] is particularly sensitive to the composition of selective media (−Ade). Absolute numbers for induction are greatly dependent on the presence of particular amino acids and their amounts in the medium (see also Brachmann et al. 2006). To get consistent results, it is very important to follow the same recipe for all experiments. We chose to use minimal SD media with only four supplements (Leu, Trp, His, and Ura). This recipe ensured the most reproducible results for all strains tested, including BY302.

Plasmid loss in Figure 3 was performed by growing yeast in YPD and streaking to single colonies on synthetic complete plates (to keep the plasmid). Colonies were tested for the presence of the HSP104 plasmid by replica plating on the SC −Leu. A colony that lost the HSP104 plasmid was grown further and used in induction experiments. The result was confirmed for three independent colonies that lost the HSP104 plasmid.

RESULTS

Hsp104p dramatically promotes the prion conversion of Ure2p from C. albicans: We used the cerevisiae strain BY302 having the C. albicans URE2 open reading frame in place of that of cerevisiae, but driven by the cerevisiae URE2 promoter (Edskes et al. 2011). Among the genes derepressed on inactivation of Ure2p by prion formation is DAL5, encoding the allantoate permease (Turossky and Cooper 1987). BY302 also carries Ade + clones (Brachmann et al. 2005). The C. albicans Ure2 protein (Ure2palb) can form the [URE3alb] prion in S. cerevisiae (Edskes et al. 2011) and, as with other prions (Wickner 1994), the frequency of prion formation is dramatically increased when this protein is overexpressed (Edskes et al. 2011). BY302 also carries the [PIN+] prion that enhances [URE3] generation in cells initially lacking the prion ([ure-0]) (Bradley et al. 2002). Using two stable [URE3alb] isolates, we examined the effects of overexpression of cellular chaperones that were shown to influence the stability of other yeast prions. These include Ssa1p (a member of the yeast Hsp70 family), Sse1p (a nucleotide-exchange factor for yeast Hsp70s), and Ydj1p and Sis1p (different members of the yeast Hsp40 family). We found that the curing profile of [URE3alb] was similar to that observed for conventional [URE3] (Table 2) (Moriyama et al. 2000; Schwimmer and Manson 2002; Kryndushkin and Wickner 2007). Ydj1p efficiently cured [URE3alb], while Sse1p and Ssa1p showed moderate curing. No curing was observed for Hsp104p or Sis1p. Overexpression of chaperones was confirmed by Western blotting (Figure S1).

Further, we asked how the chaperones affect de novo induction of [URE3alb] prions in S. cerevisiae. The same set of chaperones was tested. Since the frequency of spontaneous prion induction is low (about 1 per million cells) and is difficult to estimate due to chromosomal mutations arising, we used transient overproduction of

<table>
<thead>
<tr>
<th>Yeast strains</th>
<th>Control</th>
<th>Ssa1</th>
<th>Ydj1</th>
<th>Sse1</th>
<th>Sis1</th>
<th>Hsp104</th>
</tr>
</thead>
<tbody>
<tr>
<td>BY302 [URE3alb]</td>
<td>1 ± 0.2</td>
<td>4 ± 0.8</td>
<td>60 ± 10</td>
<td>10 ± 1</td>
<td>1 ± 0.2</td>
<td>1 ± 0.2</td>
</tr>
<tr>
<td>BY241 [URE3]</td>
<td>0 ± 0</td>
<td>20 ± 2</td>
<td>55 ± 10</td>
<td>85 ± 10</td>
<td>0 ± 0</td>
<td>8 ± 1</td>
</tr>
</tbody>
</table>

[URE3alb] and [URE3] cells were transformed with chaperone overexpression plasmids. After overnight growth in rich medium, the percentage of cells that lost prion was scored. Overproduction of the indicated chaperone proteins is documented in Figure S1.

Data shown are the average of three experiments.

TABLE 1

<table>
<thead>
<tr>
<th>Plasmid name</th>
<th>Plasmid description</th>
<th>Promoter</th>
<th>Marker</th>
<th>Copy number</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>DK30 pRS425–Ssa1</td>
<td>ADH1</td>
<td>Leu2</td>
<td>High copy</td>
<td>Kryndushkin and Wickner (2007)</td>
<td></td>
</tr>
<tr>
<td>DK26 pRS425–Sse1</td>
<td>SS1</td>
<td>Leu2</td>
<td>High copy</td>
<td>Kryndushkin and Wickner (2007)</td>
<td></td>
</tr>
<tr>
<td>DK5 pRS425–Ydj1</td>
<td>TEF1</td>
<td>Leu2</td>
<td>High copy</td>
<td>Kryndushkin and Wickner (2007)</td>
<td></td>
</tr>
<tr>
<td>DK8 Yep181–Hsp104</td>
<td>HSP104</td>
<td>Leu2</td>
<td>High copy</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>DK117 Yep181–Sis1</td>
<td>SIS1</td>
<td>85 %</td>
<td>High copy</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>DK68 P GAL pRS425</td>
<td>native</td>
<td>Leu2</td>
<td>High copy</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>DK69 pGAL–URE2</td>
<td>GAL1</td>
<td>Ura3</td>
<td>High copy</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>DK69 pH729</td>
<td>GAL1</td>
<td>Ura3</td>
<td>High copy</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>pH729</td>
<td>HSP104</td>
<td>Trp1</td>
<td>High copy</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>pH729</td>
<td>URE2alb</td>
<td>Ura2</td>
<td>Centromeric</td>
<td>Edskes et al. (1999)</td>
<td></td>
</tr>
<tr>
<td>pVTG12</td>
<td>Ure2N–GFP</td>
<td>URE2</td>
<td>Leu2</td>
<td>Centromeric</td>
<td>Nakayashiki et al. (2005)</td>
</tr>
<tr>
<td>RNQ1–GFP</td>
<td>RNQ1–GFP</td>
<td>ADH1</td>
<td>Leu2</td>
<td>Centromeric</td>
<td></td>
</tr>
</tbody>
</table>

TABLE 2

[URE3alb] and [URE3] prion curing after chaperone overproduction
Ure2p to induce [URE3alb] in strain BY302. BY302, containing either the empty vector or a high copy plasmid with a chaperone gene, was transformed with the P<sub>GAL</sub>–Ure2alb plasmid. For Ure2p overproduction, transformants were grown overnight in liquid raffinose medium and then were shifted to galactose–raffinose medium for 2 days. Finally, for [URE3alb] detection, cells were plated on dextrose–Ade medium, where the P<sub>GAL</sub> promoter is repressed. Induction of [URE3alb] inactivates Ure2p, allowing ADE2 transcription. Thus, the number of colonies on –Ade medium reflects [URE3alb] induction frequency. Remarkably, we found that overproduction of Hsp104p dramatically increased (~70-fold) the induction of [URE3alb] (Figure 1). In addition, overproduction of Sse1p or Ydj1p also had a moderate stimulatory effect on [URE3alb] formation (~8-fold), whereas excess of either Ssa1p or Sis1p had no effect (Figure 1). We confirmed that the vast majority of Ade<sup>+</sup> colonies were indeed [URE3alb] by growth on medium containing GuHCl, an agent eliminating all known [URE3]s (Edskes et al. 2009). The result with Sse1p and Ydj1p is particularly surprising since these chaperones can cure an established [URE3alb]. It suggests that cellular chaperones can remodel amyloid aggregates, resulting either in generation of a new prion variant or in prion loss.

**Effects of Hsp104p and other chaperones on [URE3] prion generation:** To determine the specificity of the observed effect for Hsp104p, we tested whether the same set of chaperones can also influence the formation of the *cerevisiae* [URE3]. The basic setup of the experiments was the same as was used for [URE3alb]: strain BY241 [ure-o] [PIN<sup>+</sup>], containing either empty vector or a high-copy plasmid with a chaperone gene, was transformed with the P<sub>GAL</sub>–URE2 plasmid, and prion induction experiments were performed. We could still detect a stimulatory effect of overproduced Hsp104p on [URE3] de novo prion formation, although much smaller (~3-fold; Figure 2A; Table 3) compared to the effect on [URE3alb] (~70-fold; Figure 1). In contrast, overproduction of Ssa1p, Sse1p, and Ydj1p reproducibly showed a mild inhibitory effect on [URE3] formation (Table 3), consistent with their [URE3] curing effects (Table 2). Since the absolute effects of chaperones on [URE3] induction were relatively small, we reproduced our findings in two other [ure-o] [PIN<sup>+</sup>] yeast strains: DK174 (with related background) and 1075 (Sharma and Masison 2008) (with unrelated background). In each case we found an ~3-fold increase of [URE3] prion formation when an elevated level of Hsp104p was present in cells, whereas an excess of Ssa1p, Sse1p, or Ydj1p repressed [URE3] induction (Table 3). Again, the majority of colonies on –Ade plates were indeed [URE3], as confirmed by GuHCl curing. Different numbers of colonies on –Ade plates were not related to any kind of toxicity, because simultaneous plating of cells on rich medium resulted in equal colony number with and without overproduced Hsp104p (data not shown). However, the effect of overproduced Sis1p was dependent on genetic background. Elevated levels of Sis1p enhanced [URE3] de novo prion
induction in BY241 and the related strain DK174, while reducing [URE3] induction in 1075.

To investigate the effect of Sis1p further, we constructed a high-copy plasmid that contained both SIS1 and HSP104 genes. Simultaneous overproduction of Sis1p and Hsp104p resulted in an additive increase of [URE3] induction in strains BY241 and DK174 (Table 3). The observed effect is consistent with the proposed cooperation between Sis1p and Hsp104p during prion propagation (Tipton et al. 2008). While Hsp104 is believed to fragment prion fibers, Sis1p is involved in the delivery of prion substrates to Hsp104 (Tipton et al. 2008).

Hsp104p was shown in vitro to be able to eliminate the lag phase of recombinant Sup35NM amyloid assembly, a result interpreted to mean that Hsp104 catalyzes the formation of prion nuclei (Shorter and Lindquist 2004). If this were the mechanism of Hsp104 stimulation of [URE3] and [URE3alb] formation, then the stimulatory effect on prion induction should be observed even in the absence of the seeding factor, [PIN+] . To test this hypothesis, we repeated the induction experiments described above, using the isogenic strains BY241 [ure-α] [pin-] or BY302 [pin-], obtained by eliminating the [PIN+] determinant by growth on rich YPD medium supplemented with 5 mM GuHCl. We confirmed the absence of [PIN+] by introducing the centromeric plasmid pH 126 RNQ1–GFP and subsequent fluorescent microscopy analysis that showed diffuse cytoplasmic staining of RNQ1–GFP. Unlike the stimulation seen with a [PIN+] strain, we saw no stimulation of [URE3] or [URE3alb] generation in the [pin-] cells by overproduction of Hsp104p (Table 4). This result argues that in vivo Hsp104p can not efficiently produce initial amyloid seeds; rather, it makes the Rnq1p amyloid a better primer for Ure2p and/or accelerates the amyloid formation after seeds are formed.

Sis1p modifies the Hsp104p curing of yeast prions: Having shown a potential cooperation between Sis1p and Hsp104p during induction of [URE3], we turned to the question of whether Sis1p can modify Hsp104p-mediated curing of yeast prions. This effect is highly prion specific, with [PSI+] much more sensitive to Hsp104p overproduction compared to [URE3] and [PIN+]. We tested two different strains of both [PSI+] and [URE3] and compared the efficiency of prion curing by high-copy plasmids expressing either Hsp104p alone or Hsp104p together with Sis1p. Surprisingly, we found that Sis1p modifies the Hsp104p curing effect dependent on the prion tested: Sis1p enhanced curing of [PSI+] by Hsp104p, but protected [URE3] from curing by Hsp104p (Table 5). This effect was specific for Sis1 since overproduction of another Hsp40 member, Ydj1p, did not show an additive effect in [PSI+] curing (Table 5). Currently it is not clear why Sis1p acts differently on these two yeast prions. It might reflect the difference in the substrate specificity for Sis1p or may be a consequence of different actions of Hsp104p on yeast prions. Certainly the two amyloids are quite different, with Ure2p amyloid being extremely stable, while that of Sup35p is more easily dissociated into monomers (Baxa et al. 2004; Kryndushkin and Wickner 2007; Toyama et al. 2007).

**TABLE 3**

<table>
<thead>
<tr>
<th>Yeast strains</th>
<th>Control</th>
<th>Ssa1</th>
<th>Sse1</th>
<th>Ydj1</th>
<th>Sis1</th>
<th>Hsp104</th>
<th>Sis1 + Hsp104</th>
</tr>
</thead>
<tbody>
<tr>
<td>BY241</td>
<td>56 ± 4</td>
<td>21 ± 4</td>
<td>36 ± 6</td>
<td>32 ± 5</td>
<td>91 ± 10</td>
<td>146 ± 19</td>
<td>165 ± 22</td>
</tr>
<tr>
<td>DK174</td>
<td>21 ± 3</td>
<td>10 ± 2</td>
<td>12 ± 1</td>
<td>12 ± 3</td>
<td>55 ± 18</td>
<td>66 ± 12</td>
<td>153 ± 13</td>
</tr>
<tr>
<td>1075</td>
<td>58 ± 3</td>
<td>45 ± 4</td>
<td>42 ± 4</td>
<td>40 ± 5</td>
<td>25 ± 6</td>
<td>182 ± 10</td>
<td>38 ± 7</td>
</tr>
</tbody>
</table>

Three different yeast strains were transformed with both a chaperone overexpression plasmid and the P$_{GAL}$–URE2 plasmid. A total of 10^6 cells were spread on –Ade plates to detect and compare [URE3] induction frequencies. Numbers are averages of three independent experiments.

**TABLE 4**

<table>
<thead>
<tr>
<th>Yeast strains</th>
<th>Ade+ colonies/10^6 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>URE2^rev+</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>URE2^rev+</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>URE2^all+</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>URE2^all+</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>URE2^rev+</td>
<td>56 ± 4</td>
</tr>
</tbody>
</table>

Standard induction procedure was performed for [pin-] strains. A total of 10^6 cells were spread on –Ade plates. Three independent experiments were performed. hc, high copy. The URE2^all+ strain is BY241 and the URE2^all+ strain is BY302. The [PIN+] control data are from Table 3.
The ade1-14 allele in 74-D694 allows selection of [PSI⁺]-containing cells on –Ade plates due to functional inactivation of Sup35p. Although overproduction of Hsp104p efficiently cures [PSI⁺] (Chernoff et al. 1995), we detected de novo generated [PSI⁺] clones (Figure 2B, Table 6), as did others (Borchsenius et al. 2006). As in [URE3] induction, we did not select for plasmid retention during selection on –Ade medium, so cells can lose the HSP104 expression plasmid during growth on –Ade plates. In fact most Adecells had lost this plasmid during colony formation (data not shown). We assume that some [PSI⁺] prion variants are more resistant than others to the curing action of Hsp104p. For example, “stronger” prion variants recognized by greater Sup35p functional inactivation are less sensitive in general to Hsp104p overproduction compared to “weaker” variants (Cox et al. 2007). Indeed, despite an overall decrease in numbers of generated [PSI⁺] clones, the fraction of strong [PSI⁺] variants was substantially increased (Table 6).

Hsp104p has little effect on spontaneous prion formation: Spontaneous prion formation is a rare and poorly understood event. Little is known about modulators of this process. We tested whether Hsp104p can promote spontaneous prion formation in the same way as it does for prion formation induced by excess of prion protein. BY241, DK174, and BY302 containing either empty vector or high-copy plasmid with HSP104 were grown overnight in liquid minimal medium selective for the presence of plasmids. About 5 × 10⁷ cells from each culture were spread on –Ade plates to select for spontaneously induced [URE3] or [URE3]ab. Surprisingly, almost no difference in the numbers of Ade⁺ colonies was observed (Table 7), indicating that Hsp104p has no stimulatory role on spontaneous [URE3] or [URE3ab] induction. The result with [URE3]ab is especially striking, considering the very high stimulatory effect of Hsp104p on induced [URE3]ab prion formation (Figure 1). Since the prion induction frequencies were low and the detection of true prion isolates was challenging due to the intrinsic instability of newly arising prions and the high proportion of Ade⁺ mutants, we introduced into strain BY241 the additional centromeric plasmid pVTG12, expressing a Ure2N–GFP fusion under control of the native URE2 promoter. Expression of Ure2N–GFP at low levels does not induce or cure [URE3] (Edske et al. 1999), but enables testing for the prion by fluorescent microscopy analysis: Ure2N–GFP shows diffuse cytoplasmic staining in nonprion cells and one or two foci in prion-containing cells due to co-aggregation with prion aggregates. Using this additional test, we confirmed that most of the medium or large size colonies on –Ade plates were indeed [URE3] and an excess of Hsp104p did not significantly affect spontaneous [URE3] formation. In addition, we show for the first time that spontaneous [URE3] formation is clearly [PIN⁺] dependent, similar to the induced [URE3] formation by an excess of Ure2p (Table S1).

Persistent epigenetic effect of overproduced Hsp104p on [URE3]ab prion induction: The ability of Hsp104p to stimulate prion formation only in the presence of preformed amyloid seeds ([PIN⁺] factor) indicates that Hsp104p may work directly on the Rnq1p aggregates of the [PIN⁺] prion, making them more suited for priming Ure2p amyloid formation. To check

### Table 5
Sis1p modifies the Hsp104p curing of yeast prions

<table>
<thead>
<tr>
<th>Yeast strains</th>
<th>Control</th>
<th>Sis1</th>
<th>Hsp104</th>
<th>Sis1 + Hsp104</th>
<th>Ydj1 + Hsp104</th>
</tr>
</thead>
<tbody>
<tr>
<td>BY241[URE3]</td>
<td>0</td>
<td>0</td>
<td>8 ± 1</td>
<td>2.0 ± 0.6</td>
<td>ND⁺</td>
</tr>
<tr>
<td>1075 [URE3]</td>
<td>0</td>
<td>0</td>
<td>1.0 ± 0.2</td>
<td>0.2 ± 0.1</td>
<td>ND⁺</td>
</tr>
<tr>
<td>74D-694 [PSI⁺]</td>
<td>0</td>
<td>0</td>
<td>15 ± 4</td>
<td>44 ± 5</td>
<td>15 ± 4</td>
</tr>
<tr>
<td>779-6A [PSI⁺]</td>
<td>0</td>
<td>0</td>
<td>84 ± 6</td>
<td>94 ± 3</td>
<td>85 ± 7</td>
</tr>
</tbody>
</table>

The curing assay was performed as described in Table 2. Numbers are averages of three independent curing experiments. 

*Experiments were not performed, because Ydj1p cures [URE3] efficiently, making a comparison with Sis1p senseless.

### Table 6
Induction of [PSI⁺] by excess Sup35p

<table>
<thead>
<tr>
<th>74D-694 [PIN⁺]</th>
<th>Strong Ade⁺</th>
<th>Weak Ade⁺</th>
<th>% of strong variants</th>
</tr>
</thead>
<tbody>
<tr>
<td>P_GAL–Sup35 + control</td>
<td>21 ± 8</td>
<td>294 ± 31</td>
<td>7</td>
</tr>
<tr>
<td>P_GAL–Sup35 + hcHSP104</td>
<td>40 ± 14</td>
<td>142 ± 41</td>
<td>22</td>
</tr>
</tbody>
</table>

The [psi–][PIN⁺] strain was transformed with both the HSP104 overexpression plasmid and the P_GAL–SUP35 plasmid. A total of 10⁶ cells were spread on –Ade plates to detect and compare [PSI⁺] induction frequencies. Averages of three independent induction experiments are shown.
Spontaneous induction of [URE3] and [URE3alb]

<table>
<thead>
<tr>
<th>Yeast strains</th>
<th>Ade(^+) colonies/10(^7) cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>BY241 [PIN(^+)] + control</td>
<td>22 ± 8</td>
</tr>
<tr>
<td>BY241 [PIN(^+)] + hcHSP104</td>
<td>27 ± 5</td>
</tr>
<tr>
<td>DK174 [PIN(^+)] + control</td>
<td>19 ± 2</td>
</tr>
<tr>
<td>DK174 [PIN(^+)] + hcHSP104</td>
<td>20 ± 5</td>
</tr>
<tr>
<td>BY302 [PIN(^+)] + control</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>BY302 [PIN(^+)] + hcHSP104</td>
<td>3 ± 2</td>
</tr>
</tbody>
</table>

Yeast strains were transformed with either the control vector or the HSP104 overexpression plasmid. A total of 10\(^7\) cells were spread on –Ade plates to detect and compare prion induction frequencies. Numbers are averages of three independent induction experiments.

This hypothesis, we introduced the high-copy plasmid with HSP104 into yeast strain BY302, bearing the P\(_\text{GAL}–\text{Ure2}\) plasmid. After growth for 30 generations, the plasmid with HSP104 was lost (see MATERIALS AND METHODS) and cells were grown further for 20 generations to ensure that the level of Hsp104p returned to normal (Figure 3, A and B). Then the culture was split; one portion of cells was washed with galactose–raffinose medium and [URE3alb] was induced as usual (2 days on galactose–raffinose medium followed by spreading on –Ade plates), whereas another part was grown for 20 more generations in dextrose before induction. For controls, the same strain that either had the HSP104 overexpression plasmid or never received the HSP104 plasmid were used for [URE3alb] induction without the 20- or 40-generation delay. Finally, cells that were grown for 20 + 20 generations after the HSP104 plasmid was lost were also subjected to [URE3alb] induction. The results of all induction experiments were analyzed and compared (Figure 3C). Remarkably, the presence of overproduced Hsp104p for a limited time creates long-lasting conditions for increased prion generation, indicating that preincubation with an excess of Hsp104p may modify the intrinsic [PIN\(^+\)] factor. The full stimulatory effect of Hsp104p requires the presence of an excess of Hsp104p during prion induction. Note that a similar experiment on the S. cerevisiae [URE3] would be very challenging since the stimulatory effect of Hsp104p on [URE3] is much smaller.

**DISCUSSION**

The primary physiological function of Hsp104 is to disaggregate proteins inactivated by heat or other stresses; Hsp104 is a true “heat shock protein” essential for induced resistance to heat shock but otherwise dispensable (Sánchez and Lindquist 1990; Parsell et al. 1994). There is conflicting evidence about whether Hsp104p by itself can interact with and fragment yeast prion aggregates in vitro (Inoue et al. 2004; Shorter and Lindquist 2004, 2006; Krzewska et al. 2007), but little doubt that, in concert with Hsp70s, Hsp40s and possibly other components, this is its major role in vivo (Paushkin et al. 1996; Ness et al. 2002; Kryndushkin et al. 2003; Satpute-Krishnan et al. 2007). Here, we show for the first time that Hsp104p can increase de novo [URE3] and [URE3alb] prion generation. Moreover, we tested other chaperones that were known to interact with prions (including members of Hsp70 and Hsp40 members and their cofactors) in both induction and curing assays. Interestingly, the effects on the [URE3alb] prion were particularly striking. In the past, similar prions formed by homologous proteins from different species were used

![Diagram](image-url)
successively to reveal additional chaperone players involved in prion propagation (Kryndushkin et al. 2002). Such genetic systems can be attractive models for studying cellular interactions of prions when they are more sensitive to perturbations in the cellular environment compared to conventional prions.

Chaperones are the main system for correcting protein folding, and they are induced by many stressful conditions, including the presence of the [URE3] and [PSI+] prions (Jung et al. 2000; Schwimmer and Masison 2002). Nonetheless, prions can propagate stably during cell growth. Both induction and propagation of prions are tightly dependent on cellular chaperones. By analogy with viruses, whose characteristics are shaped by their hosts, yeast prions are shaped and selected by the cellular chaperone environment.

Remarkably, in some cases we observed that the same factor may both enhance de novo prion generation and destabilize existing prion variants. For example, many variants of [URE3] are mildly destabilized by Hsp104p overproduction and at the same time we found that overproduction of Hsp104p caused overall increase in [URE3] induction levels. Similarly, existing [URE3-alb] variants can be destabilized by overexpression of either Ydj1p or Sse1p; however, the same overexpression resulted in an overall increase in [URE3-alb] colonies. This observation can be explained by the dual role of chaperones in the replication cycle of amyloid-based prions. Stable prion propagation requires mechanisms for fragmentation of prion fibers, which is mediated by cellular chaperones. Insufficient fragmentation will cause the formation of large prion polymers with limited seeding capacity and impaired ability to transmit to daughter yeast cells. We assume that overproduction of Hsp104p causes the remodeling of amyloid aggregates generated during prion induction. It eliminates conformations that are especially sensitive to the chaperone network and accumulate variants that can be fragmented but not destroyed, thus resulting in an overall increase in the numbers of prion-positive yeast colonies. Chaperones of the Hsp70 and Hsp40 families work together with Hsp104p and may cause similar effects. One prediction from the above explanation is that the spectrum of arising prion variants will be narrower when Hsp104p is overproduced during prion induction. However, it is extremely difficult to prove this hypothesis experimentally. The majority of de novo arising variants are extremely unstable and there are no methods available to measure the distinctions among them. Our observation of selective decrease of weak [PSI+] variants generated during excess of Hsp104p is consistent with this idea.

Since several members of the Hsp70 and Hsp40 families can cooperate with Hsp104p, it is important to understand the substrate specificity of its action. Recent studies have proposed that the yeast prion propagation cycle involves a Sis1p-dependent delivery of prion substrates to Hsp104p (Higurashi et al. 2008; Tipton et al. 2008). Our results support the hypothesis of cooperation between Sis1p and Hsp104p during yeast prion maintenance. Sis1p can modulate the effects of Hsp104p on both prion induction and prion curing in a prion-specific manner. In contrast, another major yeast Hsp40 member, Ydj1p, acts differently, indicating the specificity of Sis1p action.

What is the mechanism of Hsp104p’s stimulatory effect on prion induction? On the basis of its known cellular function, Hsp104p may act in several different ways. It may induce the initial nucleation event assembling Ure2p monomers or small oligomers into amyloid seeds; a similar mechanism was proposed on the basis of in vitro studies (Shorter and Lindquist 2004, 2006). Alternatively, Hsp104p may act on other stages of the prion assembly process by remodeling existing prion aggregates or by preventing off-pathway aggregation of overproduced prion protein. We tried to distinguish between these possibilities using a genetic approach. First, we can detect a stimulatory effect of Hsp104p only in [PIN+] strains, indicating that preformed amyloid seeds are required (Tables 3 and 4). Second, we showed that the presence of overproduced Hsp104p for a limited time period generates persistent conditions for increased prion induction (Figure 3). Presumably, Hsp104p modifies the [PIN+] variant by fragmenting and/or reshaping Rnq1 amyloid seeds, making them more suited for priming Ure2p amyloid formation. Finally, the excess of Hsp104p influences only prion formation induced by overproduction of the prion protein, but has no effect on prions generated from endogenous prion protein (Table 7), suggesting the involvement of Hsp104p in disassembly of nonprion aggregates of the overproduced prion protein that drives the aggregation toward the amyloid pathway (Figure 4).
The efficient disaggregation activity of Hsp104p raises hopes that Hsp104p might hold therapeutic potential for antagonizing several devastating neurodegenerative amyloid disorders, including Alzheimer, Huntington, and Parkinson diseases. Surprisingly, no metazoan homolog or analog of Hsp104p has been identified; such unique disaggregation activity is restricted to bacteria, fungi, and plants. Several attempts were made to introduce the well-characterized yeast HSP104 gene in rodent models of neurodegenerative disorders (Vacher et al. 2005; Perrin et al. 2007; Arimon et al. 2008; Lo Bianco et al. 2008). These reports showed that Hsp104 was able to reduce aggregation rates for various amyloids and even prolong the lifespan of a Huntington disease mouse model by ~20% (Vacher et al. 2005). Transgenic mice expressing HSP104 appear to be normal, indicating that Hsp104p does not interfere with mammalian development (Vacher et al. 2005). However, our study reveals an additional potential complication for this approach, namely that overproduction of Hsp104 may generate new prion variants or that partial disassembly of amyloid aggregates by Hsp104p could generate new amyloid seeds that might be self-propagating. Remodeling of amyloid aggregates by Hsp104p might make them more transmissible to the nearest tissue and result in faster spread of a disease state. Further studies are required to assess potential dangers of Hsp104p expression in the mammalian brain. It would be of interest to test whether overproduced Hsp104p can increase the production of self-propagating species made from disease-associated proteins in cell culture models of neurodegenerative disorders.

We thank D. Masison [National Institutes of Health (NIH), Bethesda, MD] and M. D. Ter-Avanesyan (Cardiology Research Center, Moscow, Russia) for plasmids and strains; D. M. Cyr (University of North Carolina, Chapel Hill, NC) and P. Needham (University of Pittsburgh, Pittsburgh, PA) for kindly sharing antibodies; K. O’Connell (NIH) for help with microscopy; and members of our lab for a critical reading of the paper. This research was supported by the Intramural Research Program of the NIH, National Institute of Diabetes and Digestive and Kidney Diseases.

LITERATURE CITED


Molecular Chaperone Hsp104 Can Promote Yeast Prion Generation

Dmitry S. Kryndushkin, Abbi Engel, Herman Edskes and Reed B. Wickner

Copyright © 2011 by the Genetics Society of America
DOI: 10.1534/genetics.111.127779
Supporting Methods

**Plasmid information**

High copy plasmids for overexpression of chaperones pRS425-Ssa1, pRS425-Sse1, pRS425-Ydj1 were described in (KRYNDUSHKIN and WICKNER 2007). To make Yeplac181-Hsp104 overexpression plasmid used in all induction studies, 

HSP104 was amplified from yeast genomic DNA using primers 5'-aatttgatccgacgacaagttggcctc-3' and 5'-ggatgtacatagcccttattaacaatgg-3'. The fragment was cut with BamHI and BssGI restriction enzymes (New England Biolabs) and ligated with Yeplac181 high copy vector linearized by BamH1 and Asp718 enzymes. To make Yeplac181-Sis1 and Yeplac181-Hsp104-Sis1 overexpression plasmids, SIS1 was amplified from yeast genomic DNA using primers 5'-aggtggatccgcagatactaagataacagac-3' and 5'-caacctgcagctaaatacatctcgtgttatg-3'. The fragment was cut with BamHI and PstI restriction enzymes and cloned into either Yeplac181 or Yeplac181-Hsp104 plasmid linearized by BamH1 and PstI enzymes.

To obtain P\(^{\text{GAL}}\)-URE2 overexpression plasmid, the plasmid pH376 containing URE2 under control of the GAL1 promoter (Bradley et al. 2002) was cut with Nhel and XhoI. The fragment containing the galactose-induced URE2 gene was placed into pH396 (high copy, URA3, pRS426 based) linearized by Nhel and XhoI, which resulted in the P\(^{\text{GAL}}\)-URE2 high copy plasmid. For SUP35 overexpression, the SUP35 open reading frame was amplified with primers 5'-aatttgatccatgtcggattcaaaccaag-3' and 5'-aagctcgagtttactcggcaattttaacaat-3', cut with BamH1 and XhoI and cloned into BamH1 and XhoI linearized P\(^{\text{GAL}}\)-URE2 plasmid instead of the URE2 gene, which resulted in the P\(^{\text{GAL}}\)-SUP35 high copy plasmid. To obtain P\(^{\text{GAL}}\)-URE2alb overexpression plasmid, the Nhel/BamH1 bordered ADH1 promoter of pH401 (EDSKES and WICKNER 2002) was replaced with the similarly bordered GAL1 promoter from pH250 (EDSKES and WICKNER 2002), resulting in pH610 vector (TRP1, high copy, promoter-GAL1). C. albicans URE2 was transferred as a BamH1/XhoI fragment from pH563 (EDSKES and WICKNER 2002) into the same window of pH610, generating P\(^{\text{GAL}}\)-URE2alb.

For detection of prion aggregates in vivo, centromeric plasmid pVTG12, expressing a Ure2N-GFP fusion under control of the native URE2 promoter (EDSKES et al. 1999) and pH126 Rnq1-GFP (NAKAYASHIKI et al. 2005), were used. All constructs were verified by sequencing.

**Microscopy**

Spinning disc confocal imaging of live yeast cells expressing the appropriate GFP-tagged fusion proteins was performed on a Nikon Eclipse E800 microscope with a Perkin-Elmer Ultraview LC1 CSU10 scanning unit and an argon/krypton ion laser (Melles Griot, Carlsbad, CA), and an ORCA ER cooled CCD camera (Hamamatsu, Japan). Image acquisition and analysis were
carried out with Openlab 3 software (Improvision, Lexington, MA).

**Analysis of protein expression in yeast cell lysates**

Yeast strains bearing corresponding chaperone expression plasmids were grown in liquid SD medium supplemented with amino acids required for growth. At OD$_{600}$ of 1.5, yeast cells were harvested, washed in buffer A (25mM Tris–HCl, pH 7.4, 150mM NaCl, 1mM dithiothreitol and complete protease inhibitor cocktail [Roche Applied Science, Indianapolis, IN]) and lysed by glass beads in the same buffer. Cell debris was removed by centrifugation at 10000 g for 10 min. Protein levels were measured with BCA reagent (Pierce, Rockford, IL) and equalized. Cell lysates were then analyzed by standard SDS-PAGE and immunoblotting procedures. Antibodies used for protein detection were against Hsp104p (Stressgen, Victoria, BC), Ssa1p and Sis1p (a kind gift of DM Cyr), Sse1p (a kind gift of P Needham), Ydj1p (Santa Cruz Biotechnology, Santa Cruz, CA) and Act1p (GeneTex, San Antonio, TX).
FIGURE S1.—Overproduction of chaperone proteins in yeast cells was confirmed by Western blotting analysis. Strain BY241 [ure-o], bearing either control vector or high copy plasmid with a chaperone gene, was grown in liquid SD supplemented with required amino acids. Yeast cells were collected and lysates were prepared and equalized by the protein amount. Levels of Ssa1p, Ydj1p, Sse1p, Sis1p and Hsp104p were compared by immunoblotting with corresponding antibodies. The level of actin served as a loading control. Antibody to Ssa's detects Ssa1p, Ssa2p, Ssa3p and Ssa4p, with Ssa2p being the major cytoplasmic Hsp70 in unstressed cells. Thus Ssa1p is actually elevated more than one would judge by the small increase in the intensity of the band in the upper left panel.
TABLE S1

**Spontaneous [URE3] formation is [PIN⁺] dependent**

<table>
<thead>
<tr>
<th>Yeast strains</th>
<th>Number of [URE3] colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>BY241 [pin⁻] + cenUreN-GFP</td>
<td>0</td>
</tr>
<tr>
<td>BY241 [PIN⁺] + cenUreN-GFP</td>
<td>35</td>
</tr>
</tbody>
</table>

Yeast strains were grown overnight in liquid SD medium supplemented with required amino acids. 2x10⁷ cells were spread on –Ade-Leu plates and grown for 5 days. [URE3] formation was confirmed by fluorescence microscopy.
REFERENCES


