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Guanidine Hydrochloride Inhibits Hsp104 Activity In Vivo: A Possible Explanation for Its Effect in Curing Yeast Prions

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Abstract. The presence of millimolar concentrations of guanidine hydrochloride (Gdn-HCl) in growth media causes efficient loss of the normally stable [PSI^+] element from yeast cells. Although it has become common practice to include 5 mM Gdn-HCl in growth media to cure [PSI^+] and other prions of yeast, the biochemical mechanism by which it cures is unknown. We find that 5 mM Gdn-HCl significantly reduces Hsp104-mediated basal and acquired thermotolerance. Gdn-HCl also reduced the ability of Hsp104 to restore activity of thermally denatured luciferase in vivo. The abundance of Hsp104 was not reduced in cells grown in the presence of Gdn-HCl, ruling out negative effects on expression or stability of Hsp104. We therefore conclude that Gdn-HCl inhibits Hsp104 activity in vivo. Since replication of yeast prions is dependent on Hsp104, our results suggest that Gdn-HCl cures prions by inhibiting Hsp104 activity.

Unlike the Hsp70 class of protein chaperones, Hsp104 does not prevent aggregation of unfolded proteins but aids in reactivating heat-denatured proteins by solubilizing protein aggregates [7, 13]. This activity is critical for recovery of viability when yeast cells have accumulated protein damage after exposure to elevated temperature.

The level of active Hsp104 in the cell is also critical for propagation of the yeast [PSI^+] element, an aggregated prion form of the essential polypeptide release factor Sup35p [3, 15, 19]. Both deletion of *HSP104* and overexpression of *HSP104* at normal growth temperature cures cells of [PSI^+] [2]. The mechanism of Hsp104's essential role in prion replication remains speculative. Hsp104 may be required to maintain Sup35p in a state soluble enough to be efficiently transmitted to daughter cells [15], or to generate folding intermediates required for efficient conversion of Sup35p into the prion form [14].

Recently it was shown that Gdn-HCl cures [PSI^+] by blocking a critical step in prion replication rather than by inducing an active process that eliminates [PSI^+] [5]. [PSI^+] is then lost by dilution of preexisting inheritable [PSI^+] "seeds" among progeny during cell division, eventually leading to [psi^-] cells. Although the biochemical mechanism underlying this effect remains unknown, inhibition of Hsp104 activity was proposed as being consistent with this curative effect [5].

Unlike [PSI^+], the [URE3], [PIN^+], and hybrid [PSI^+_{PS}] prions of yeast are not eliminated by Hsp104 overexpression [4, 9, 12]. Like [PSI^+], however, these elements require Hsp104 for propagation and are readily cured by 5 mM Gdn-HCl. Here we demonstrate that 5 mM Gdn-HCl in the growth medium significantly inhibits Hsp104 activity in vivo. Although cells grown in the presence of Gdn-HCl have no less Hsp104, these cells display significant reductions in thermotolerance and in reactivation of heat-denatured luciferase. Our results suggest that impairment of Hsp104 activity by Gdn-HCl may underlie its ability to cure yeast prions.

Materials and Methods

Yeast strains, media, and plasmids. Strains 779-6A (*MAT*α *kar*1 *ade*2.1 *SUQ*5 *his*3Δ202 *leu*2Δ1 *trp*1Δ63 *ura*3.52 [psi^-]) and J104NC were used. J104NC is 779-6A that has the coding region of *HSP104* precisely replaced with that of the Kanamycin gene, obtained by transformation with a PCR-amplified Kanamycin gene [19]. The primers used were (1): 5′-AAAGAAATCAACTACACGTACCATAAAATATACAGAATATAGATTGTACTGAGAGTGCAC-3′ and (2): 5′-ATTCTTGTTCGAAAGTTTTTAAAAATCACACTATATTAAACT-

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GTGCGGTATTTCACACCG-3′. These have 5′ ends with 40 bases homologous to *HSP104* non-coding DNA for targeted integration of the PCR product [1]. Complex medium contains 1% yeast extract, 2% peptone, 400 mg/L adenine, and 2% dextrose (YPAD), and defined medium is as described [8]. Gdn-HCl was added to liquid cultures to a final concentration of 5 mM by dilution of a 1 M stock made in YPAD. The doubling time of both 779-6A and J104NC at 30°C in YPAD was 94 ± 2 min, and in YPAD containing 5 mM Gdn-HCl it was 109 ± 4 min. Plasmid pGPD-luxAB(HIS), from S. Lindquist, is a high-copy plasmid carrying a gene encoding a thermosensitive luciferase and has been described [13].

Thermotolerance assays. Mid-log phase cultures in YPAD were diluted to OD_{600} = 0.05–0.07 and grown at 30° to OD_{600} = 0.3–0.5. For basal thermotolerance, 800 μl was transferred to a 13 × 100 mm borosilicate test tube, and after transfer of 100 μl of this to a prechilled tube on ice for a zero time point, the test tube was put into a circulating 52°C water bath. At indicated times, 100-μl aliquots were similarly transferred to ice. For acquired thermotolerance, 1 ml of the original culture was first transferred to a prewarmed tube and incubated at 39°C for 30 min before collection of samples. Collected samples were titered by dilution in water and plating on YPAD. Colonies were counted after incubating for 2 days at 30°C. Where indicated, titers were done on YPAD plates containing a final concentration of 3 mM Gdn-HCl, another concentration used to cure [PSI^+]. Plating efficiency of untreated cells was reduced on 5 mM Gdn-HCl but not on 3 mM Gdn-HCl.

Results

The ability of yeast to survive exposure to lethal heat (52°C) is lower in cells lacking Hsp104, and over-expressing Hsp104 from a plasmid is sufficient to confer elevated thermotolerance [10, 16]. Similarly, brief exposure of cells to elevated non-lethal temperature (37°–44°C), which induces expression of Hsp104, significantly elevates thermotolerance. This acquired thermotolerance is not seen in cells that lack Hsp104. We assayed basal and acquired thermotolerance of cells grown in the presence of Gdn-HCl as a measure of its effects on Hsp104 activity.

Growing cells in the presence of 5 mM Gdn-HCl reduced basal thermotolerance of our wild-type strain by about 30-fold (after 8 min of exposure to 52°C, Fig 1). This lower level of tolerance was similar to that of an isogenic strain lacking Hsp104 grown without Gdn-HCl. Gdn-HCl had little effect on basal thermotolerance of the *hsp*104$^-$ mutant. Acquired thermotolerance of wild-type cells grown in 5 mM Gdn-HCl was 50-fold lower than that of the same cells grown in the absence of Gdn-HCl (after 15 min at 52°C; Fig. 1). In *hsp*104$^-$ cells, acquired thermotolerance was threefold less than this and was essentially unaffected by Gdn-HCl. Thus, the presence of 5 mM Gdn-HCl in the growth medium significantly reduced both basal and acquired thermotolerance of our wild-type strain, but had little effect on cells lacking Hsp104. This suggests that the effects of Gdn-HCl on thermotolerance are due to inhibition of Hsp104 function.

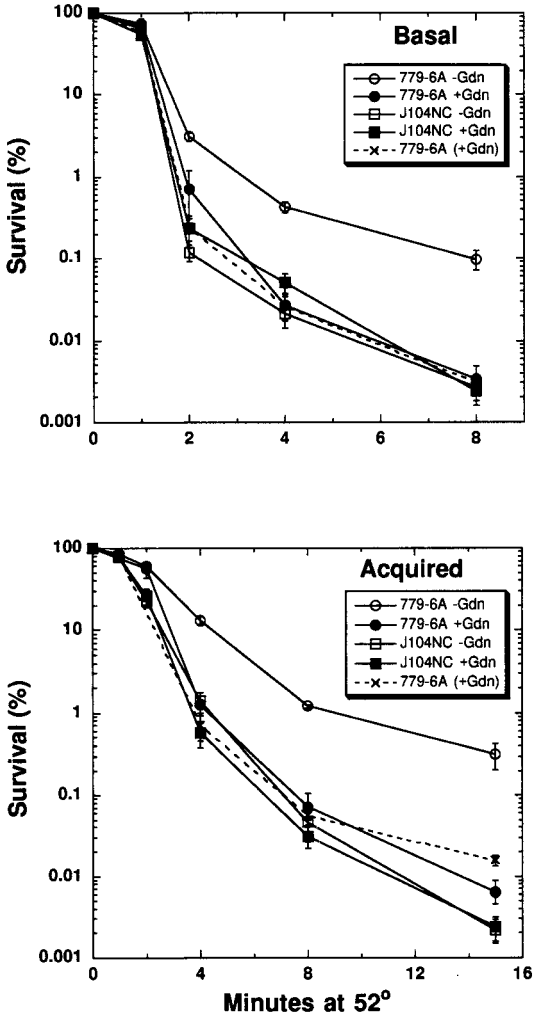

Fig. 1. Gdn-HCl reduces basal and acquired thermotolerance. Survival of cells exposed to 52°C was assayed on YPAD plates incubated at 30°C. The dashed line shows survival of the same heat-shocked, wild-type cells grown without Gdn-HCl, but which recovered on plates containing 3 mM Gdn-HCl. Error bars indicate the range of values from at least two experiments. For the wild-type strain grown with Gdn-HCl, cells were maintained in log phase from 1 to 19.5 h (0.5–11 cell doublings) after the addition of Gdn-HCl. Plots in both panels represent the average of five assays done at different times spanning this period.

Because Hsp104 does not prevent heat damage to proteins but resolubilizes proteins that have aggregated [7, 13], we expected that if Gdn-HCl was inhibiting Hsp104, then it would exert its effects if added after the lethal heat treatment. Indeed, when cells grown in the absence of Gdn-HCl were obliged to recover from exposure to 52°C on plates containing 3 mM Gdn-HCl, basal thermotolerance was reduced 30-fold (Fig. 1, dashed line). This was very similar to that of cells grown in 5 mM Gdn-HCl, which recovered in the absence of Gdn-HCl, and of cells lacking Hsp104. Acquired ther-

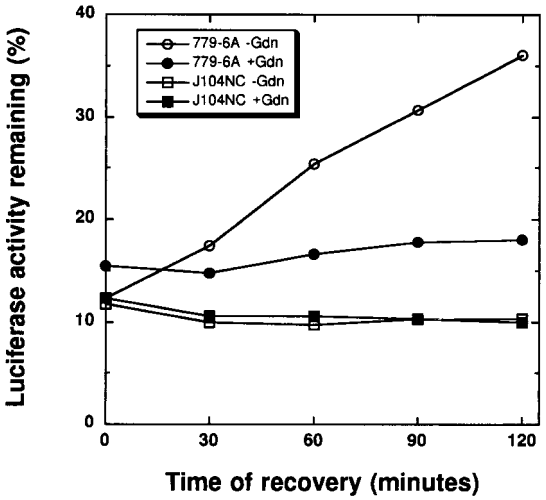

Fig. 2. Gdn-HCl reduces reactivation of heat-denatured luciferase. Luciferase activity during recovery from a 1-h exposure to 44°C was assayed as described [13] and when Gdn-HCl was added to a concentration of 5 mM at the start of the recovery period. Activity is presented as a percentage of that measured immediately before exposure to 44°C. Cycloheximide was added to the cultures 10 min before removing them from 44°C to prevent luciferase synthesis during the recovery period.

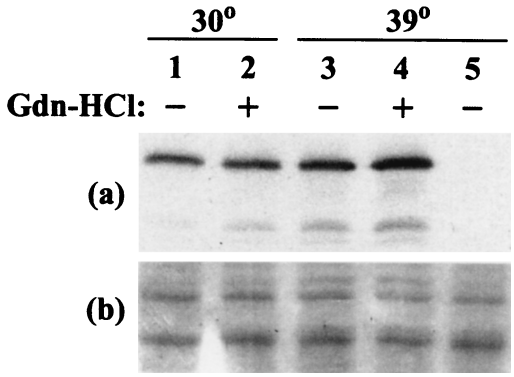

Fig. 3. Hsp104 abundance is modestly elevated in cells grown in the presence of Gdn-HCl. Immunoblot of proteins from cell lysates of wild-type (779-6A, lanes 1–4) and *hsp*104$^-$ (J104NC lane 5) cells, probed with antiserum to Hsp104, was done as described [8]. Cells used in lanes 3, 4, and 5 were collected after shifting a portion of the 30°C cultures to 39°C for 30 min. The lower panel shows a Coomassie-stained loading control of the same region of an identical gel.

motolerance was reduced 20-fold when cells grown without Gdn-HCl recovered on plates containing 3 mM Gdn-HCl. Thus, the presence of Gdn-HCl does not make cells more sensitive to direct effects of exposure to lethal heat, but rather inhibits their recovery from such treatment.

It was shown previously that reactivation of a heat-denatured thermosensitive form of luciferase [6] is dependent primarily on Hsp104 [13]. We measured this reactivation as an additional assay for effects of Gdn-HCl on Hsp104 activity. After cells expressing luciferase were shifted from 30° to 37°C for half an hour to induce Hsp104 expression, they were subsequently shifted to 44°C for an hour to inactivate luciferase and then allowed to recover at 25°C. Consistent with previously reported results, we saw no reactivation of luciferase in *hsp*104$^-$ cells (Fig. 2). Gdn-HCl had no effect on the residual luciferase activity in this strain, indicating that it was not affecting activity or stability of the active form of luciferase. In wild-type cells, heat-denatured luciferase was reactivated at a constant rate throughout the duration of the recovery period. In the same cultures which were adjusted to contain 5 mM Gdn-HCl at the start of the recovery period, there was only a slight, transient restoration of luciferase activity. Thus, Gdn-HCl significantly impaired the ability of Hsp104 to reactivate heat-denatured luciferase in vivo.

To determine whether a reduction in Hsp104 abundance was contributing to the observed effects, we compared the amount of Hsp104 in cells from cultures grown with and without Gdn-HCl, collected before and after the 39°C pretreatment. We saw a modest elevation in both basal and induced levels of Hsp104 in cells grown in the presence of Gdn-HCl (Fig 3). This is consistent with previous observations that Gdn-HCl induces Hsp104 expression [2, 11] and may reflect reduced negative feedback of expression owing to a reduced level of Hsp104 activity. Thus, Gdn-HCl did not reduce overall abundance of Hsp104 under optimal or inducing growth conditions, further suggesting that the effects of Gdn-HCl are on Hsp104 activity.

Discussion

We show for the first time that Hsp104 activity in yeast cells is significantly reduced by millimolar amounts of Gdn-HCl in growth media. Since yeast prions are dependent upon Hsp104 for their replication and are curable by growth in the presence of 5 mM Gdn-HCl, our results suggest that inhibition of Hsp104 activity underlies the ability of Gdn-HCl to cure prions. Our results are consistent with an earlier observation that the ATPase activity of Hsp104 is inhibited by low concentrations of guanidine *in vitro* [7]. Also, it has been shown that mutations that disrupt ATPase activity of Hsp104 adversely affect both [PSI^+] propagation and reactivation of heat-denatured luciferase [2, 13].

One role of Hsp104 in [PSI^+] propagation may be in aiding the conversion of the normal form of Sup35p into the prion form. Through specific direct interactions, Hsp104 may establish or maintain partially unfolded forms of Sup35p, thus overcoming thermodynamic bar-

riers to changes in protein conformation [2, 14, 17]. Alternatively, the disaggregating activity of Hsp104 may be required simply to keep the essential Sup35p soluble enough for cells to grow, or to keep the Sup35p aggregates in pieces small enough for [*PSI*$^+$] to be efficiently transmitted during cell division [15]. These roles are not exclusive, and Hsp104 may act in any or all of them.

Gdn-HCl does not appear to affect the nature of transmissible [*PSI*$^+$] particles directly, but rather blocks a critical step in [*PSI*$^+$] replication. The non-replicating particles are then randomly distributed among dividing cells until they are diluted to the point where [*PSI*$^+$] is lost [5]. Through its ability to inhibit Hsp104 activity, Gdn-HCl could cure [*PSI*$^+$] in this manner by preventing Hsp104 from acting in any of its suggested roles in [*PSI*$^+$] propagation. An alternative explanation would be that Gdn-HCl is causing the accumulation of aggregated protein that sequesters Hsp104 activity. However, it is unlikely that 3–5 mM Gdn-HCl significantly alters protein conformation, and conditions of stress that induce Hsp104 expression and cause accumulation of Hsp104 substrates weaken [*PSI*$^+$] but do not reduce the normal number of [*PSI*$^+$] particles per cell [5].

Overexpressing Hsp104 at optimal temperature has been suggested to cure [*PSI*$^+$] by leading to complete solubilization of Sup35p [2]. While the increase in Hsp104 by Gdn-HCl in our strains does not appear to be significant enough to have such an effect, induction of Hsp104 expression by Gdn-HCl has previously been suggested to cure [*PSI*$^+$] this way [2, 11]. A recent analysis of Gdn-HCl curing of [*PSI*$^+$] found that curing is not accelerated when done under conditions of stress that induce Hsp104, also indicating that this may not be the case [5]. The opposite suggestion, that Gdn-HCl inactivates Hsp104, was offered as a plausible explanation for the ability of Gdn-HCl to block [*PSI*$^+$] replication. Our data provide clear evidence in support of this latter hypothesis.

Literature Cited

1. Baudin A, Ozier-Kalogeropoulos O, Denouel A, Lacroute F, Cullin C (1993) A simple and efficient method for direct gene deletion in *Saccharomyces cerevisiae*. Nucleic Acids Res 21:3329–3330
2. Chernoff YO, Lindquist SL, Ono B, Inge-Vechtomov SG, Liebman SW (1995) Role of the chaperone protein Hsp104 in propagation of the yeast prion-like factor [psi+]. Science 268:880–884
3. Cox BS (1965) "Ψ" a cytoplasmic suppressor of super-suppressor in yeast. Heredity 20:505–521
4. Derkatch IL, Bradley ME, Zhou P, Chernoff YO, Liebman SW (1997) Genetic and environmental factors affecting the *de-novo* appearance of the [*PSI*$^+$] prion in *Saccharomyces cerevisiae*. Genetics 147:509–519
5. Eaglestone SS, Ruddock LW, Cox BS, Tuite MF (2000) Guanidine hydrochloride blocks a critical step in the propagation of the prion-like determinant [*PSI*$^+$] of *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 97:240–244
6. Escher A, O'Kane DJ, Lee J, Szalay AA (1989) Bacterial luciferase alpha beta fusion protein is fully active as a monomer and highly sensitive in vivo to elevated temperature. Proc Natl Acad Sci USA 86:6528–6532
7. Glover JR, Lindquist SL (1998) Hsp104, Hsp70, and Hsp40: a novel chaperone system that rescues previously aggregated proteins. Cell 94:73–82
8. Jung G, Jones G, Wegrzyn R, Masison D (2000) A role for cytosolic Hsp70 in yeast [*PSI*$^+$] prion propagation and [*PSI*$^+$] as a cellular stress. Genetics 156:559–570
9. Kushnirov VV, Kochneva-Pervukhova NV, Chechenova MB, Frolova NS, Ter-Avanesyan MD (2000) Prion properties of the Sup35 protein of yeast Pichia methanolica. EMBO J 19:324–331
10. Lindquist S, Kim G (1996) Heat-shock protein 104 expression is sufficient for thermotolerance in yeast. Proc Natl Acad Sci USA 93:5301–5306
11. Lindquist S, Patino MM, Chernoff YO, Kowal AS, Singer MA, Liebman SW, Lee K-H, Blake T (1995) The role of Hsp104 in stress tolerance and [PSI+] propagation in *Saccharomyces cerevisiae*. Cold Spring Harbor Symp. Quant. Biol. 60:451–460
12. Moriyama H, Edskes H, Wickner R (2000) [URE3] prion propagation in *Saccharomyces cerevisiae*: requirement for chaperone Hsp104 and curing by overexpressed chaperone Ydj1p. Mol Cell Biol 20:8916–8922
13. Parsell DA, Kowal AS, Singer MA, Lindquist S (1994) Protein disaggregation mediated by heat-shock protein Hsp104. Nature 372:475–478
14. Patino MM, Liu J-J, Glover JR, Lindquist S (1996) Support for the prion hypothesis for inheritance of a phenotypic trait in yeast. Science 273:622–626
15. Paushkin SV, Kushnirov VV, Smirnov VN, Ter-Avanesyan MD (1996) Propagation of the yeast prion-like [psi+] determinant is mediated by oligomerization of the *SUP35*-encoded polypeptide chain release factor. EMBO J 15:3127–3134
16. Sanchez Y, Lindquist SL (1990) *HSP104* required for induced thermotolerance. Science 248:1112–1115
17. Schirmer EC, Lindquist S (1997) Interactions of the chaperone Hsp104 with yeast Sup35 and mammalian PrP. Proc Natl Acad Sci USA 94:13932–13937
18. Wach A, Brachat A, Pohlmann R, Philippsen P (1994) New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. Yeast 10:1793–1808
19. Wickner RB (1994) Evidence for a prion analog in *S. cerevisiae*: the [URE3] non-Mendelian genetic element as an altered *URE2* protein. Science 264:566–569