hsp110 Protects Heat-denatured Proteins and Confers Cellular Thermoresistance*

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The 110-kDa heat shock protein (hsp110) has long been recognized as one of the primary heat shock proteins in mammalian cells. It belongs to a recently described protein family that is a significantly diverged subgroup of the hsp70 family and has been found in organisms as diverse as yeast and mammals. We describe here the first analysis of the ability of hsp110 to protect cellular and molecular targets from heat damage. It was observed that the overexpression in vivo of hsp110 conferred substantial heat resistance to both Rat-1 and HeLa cells. In vitro heat denaturation and refolding assays demonstrate that hsp110 is highly efficient in selectively recognizing denatured proteins and maintaining them in a soluble, folding-competent state and is significantly more efficient in performing this function than is hsc70. hsp110-bound proteins can then be refolded by the addition of rabbit reticulocyte lysate or hsc70 and Hdj-1, whereas Hdj-1 does not itself function as a co-chaperone in folding with hsp110. hsp110 is one of the principal molecular chaperones of mammalian cells and represents a newly identified component of the primary protection/repair pathway for denatured proteins and thermotolerance expression in vivo.

It has been long recognized that the major heat shock proteins (hsp)s of mammalian cells are observed at 28, 70, 90, and 110 kDa (1–3) and other hsp families, e.g., hsp60 and hsp40, have been subsequently identified. All of these stress protein groups have been intensively studied, excluding the hsp110 species. The cloning of hsp110 from hamster, mouse, yeast, arabadopsis, and a variety of other species has been recently described (4–11). These studies indicate that hsp110 is a significantly enlarged and diverged relative of the hsp70 family of proteins but also includes unique sequence components. The notable constitutive expression and stress inducibility of hsp110 is highly suggestive of a major role in unstressed cells as well as in the heat shock response and the expression of thermotolerance (1, 2). A description of the heat shock response in eucaryotes is not possible without an understanding of the roles played by this major stress protein. We describe here an analysis of the characteristics of hsp110, both in vivo and in vitro.

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the luciferase solution, and the buffer was adjusted to radioummune precipitation buffer (phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing 1 mg/ml ovalbumin and incubated for 1 h at 4 °C followed by incubation with protein A-Sepha-
rose for 1 h at 4 °C. The protein A-Sepharose pellet was collected and washed six times, and the pellet was resuspended in SDS-polyacrylamide gel electrophoresis and subjected to Western analysis with anti-hsp110 or anti-luciferase antibody.

Luciferase Reactivation Experiments—Luciferase (150 nM) was incu-
bated with ovalbumin, hsp110, or hsc70 at 43 °C for 30 min and diluted to 15 nM into 60% rabbit reticulocyte lysate (RRL; Promega) or hsc70 bated with ovalbumin, hsp110, or hsc70 at 43 °C for 30 min and diluted 5 mM MgCl₂, 2 mM dithiothreitol, and 2 mM ATP (total, 50 mM)sodium deoxycholate, 0.1% SDS) containing 1 mg/ml ovalbumin and
precipitation buffer (phosphate-buffered saline, 1% Nonidet P-40, 0.5%
The measurement of luciferase activity, the reactivation solution was 5-fold-diluted in 25 mM Heps, pH 7.5, and 10 μl was added to luciferase assay solution (Promega); the activity was measured with
Lumat LB 9501 (Berthold).

Survival Assay—Hamster hsp110-transfected Rat-1-R12 cells (described above) were grown in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum and were induced to overexpress hsp110 by removal of 2 ng/ml doxycycline. Cells were cultured in the absence of
doxyccycline for 2 days with fresh changes of doxyccycline-free media twice a day to remove residual drug. After indicated heat exposures, cells were counted and plated at different dilutions. After 10–14 days, colonies were stained with methylene blue and counted. The uninduced
cells were treated identically, except doxycycline was maintained in the
culture media. The presence or absence of doxyccycline had no effect on
the heat sensitivity of the parental Rat-1 cells. HeLa tetracycline-de-
pendent transactivator 1 cells (HtTA-1, described above) were cultured and treated as described for Rat-1 cells.

RESULTS

To assess the role of hsp110 in vivo, we determined the effect of
its overexpression on the long-recognized phenomenon of
thermotolerance. For this purpose, we established two tissue
culture cell lines (HeLa and Rat-1) in which the expression of
hsp110 can be selectively controlled by a tetracycline-regulated
expression system ("tet-off") in which removal of tetracycline
induces its gene expression (12). Fig. 1 shows the expression of
hamster hsp110 (induced), with a Myc-tag added to the
carboxyl end in the control and heated Rat-1 cells using this
system. In the top panel, an antibody reactive with both ham-
ster and rat hsp110 demonstrates the expression of these
hsp110 proteins in control and heated Rat-1 cells, whereas in
the bottom panel, an anti-Myc antibody demonstrates the expres-
sion of hamster hsp110 in control cells. In these in vivo
expression studies, the inducible level of hamster plus rat
hsp110 in control cells was approximately comparable to the
level of induction of rat hsp110 alone after heat exposure (i.e. is
physiological). The expression data from the HeLa line were
comparable. Hamster hsp110-induced cells were then chal-
enged with potentially lethal heat doses, following which, the
number of surviving cells was determined by clonogenicity. The
effect of overexpression of hsp110 on the resistance of Rat-1
cells and HeLa cells is presented in Fig. 1, B and C, respec-
tively. It is evident that cells containing exogenous hsp110
were significantly more resistant to heat killing. For com-
parative purposes, a survival analysis of fully thermotolerant cells
arising from a conventional pre-heat treatment is also pre-
seated. Overexpression of hsp110 alone is capable of achieving
approximately 25–33% full thermotolerant effect (the survival
for uninduced Rat-1 cells at the later time point fell below the
level of detection). This indicates that in the absence of over-
expression of other hsps, hsp110 is still effective in protecting
cells from potentially lethal heat exposures.

To better understand how hsp110 may protect cells from
thermal shock in vivo, we determined the characteristics of this
protein in vitro by utilizing previously applied assays for the
analysis of other heat shock proteins and molecular chaper-
ones. For this purpose, we purified histidine-tagged hsp110
from E. coli to homogeneity. Since hsp110 shares sequence

similarities with the hsp70 family and since hsp70 proteins
have been studied for their abilities to inhibit protein aggrega-
tion and promote protein folding (13–15), we similarly exam-
ined the ability of hsp110 to perform these chaperoning func-
tions. For aggregation studies, we used luciferase and citrate
synthase as model proteins. It is seen in Fig. 2a that hsp110 is
efficient in inhibiting the heat-induced aggregation of lucifer-
ase in vitro. Most notably, hsp110 was nearly totally effective
in inhibiting aggregation as assayed by light scattering when
present in a 1:1 molar ratio. This suggests that the interaction

FIG. 1. The effect of hsp110 overexpression on thermo-resistance. Hamster hsp110 was placed on a tet-off-inducible promoter. The expression of hsp110 in Rat-1 cells was then assessed as indicated in panel A using an antibody reactive with both exogenous and endoge-


ous hsp110 (anti-hsp110, top) and with Myc-tagged hamster hsp110
only. The effect of control (C) and heat shock (H) on uninduced (no expression of hamster hsp110) is shown together with the expression


expression of hsp110 plus endogenous hsp110 without heat. Cell cultures
were then heated as indicated, and the percentage of surviving cells was
determined by colony survival. The overexpression of hsp110 (induced)
confers substantial thermostolerance to both Rat-1 (panel B) and HeLa (panel C) cells. In the case of Rat-1 cells, the longest time point yielded
no viable colonies and was not plotted. The preheated curve indicates a
standard thermostolerance curve for these cell lines resulting from a
45 °C, 15 min heat shock delivered 20 h before the challenge exposure.
of one hsp110 to one denatured luciferase protein was sufficient to maintain solubility as measured in this way. For comparative purposes, the efficiency of hsc70 in this process was examined in parallel. Consistent with some earlier studies (13) but not others (15), hsc70 was also capable of inhibiting the protein aggregation. However, in this case, total suppression of luciferase aggregation requires the association of 4 hsc70 proteins to 1 luciferase protein compared with the 1:1 ratio obtained with hsp110. Heat-induced aggregation studies using citrate synthase (shown in Fig. 2

The effect of hsp110 on luciferase aggregation as presented in Fig. 2A was also examined by Western blotting analysis using an antibody against luciferase. Fig. 2C demonstrates that whereas some freshly prepared luciferase is insoluble (pellet) under control conditions, most of the enzyme remains in the supernatant (Spt). However, when heated in the presence of bovine serum albumin, most luciferase is seen to become insoluble (-hsp110). If hsp110 is present during heating at a 1:1 molar ratio with luciferase (+hsp110), it is clear that the enzyme remains maximally soluble. In these studies, the presence or absence of ATP had no effect on the outcome, despite the fact that hsp110 contains the consensus sequences for ATP binding characteristic of this family of ATP binding proteins (4, 16).

To verify that this protective effect of hsp110 was due to its direct interaction with luciferase, we precipitated hsp110 and examined the coimmunoprecipitation with luciferase. As seen in Fig. 2D, luciferase coprecipitates with hsp110 when it was incubated with pre-heated hsp110 at room temperature (data not shown). Therefore, these experiments demonstrate that hsp110 selectively recognizes denatured proteins and prevents their aggregation during heat shock in vitro. That these proteins are heat-denatured is indicated by their loss of enzymatic activity. Additionally, whereas hsp110 inhibited aggregation, it had no effect on the rate of loss of this enzyme activity, nor was activity
regained by further incubations of the hsp110-luciferase complex at room temperature (data not shown). Thus, hsp110 is a potent chaperone in inhibiting aggregation but is incapable of refolding of heat-denatured proteins on its own.

This suggests that hsp110 would require the cooperation of other hsps and/or chaperones to refold denatured substrate proteins. To initially address this question, luciferase was heated in the presence of ovalbumin, hsc70, or hsp110 and then added to 60% RRL that has been shown to be an optimal refolding medium (17, 18). As seen in Fig. 3A, luciferase heated in the presence of hsp110 regained 70% original activity, whereas luciferase heated with hsc70 regained only 20% original activity (at the same molar ratio, 20 × hsp110 or hsc70 to 1 × luciferase). It is clear from this data that optimal folding requires significant additional hsp110 or hsc70 compared with optimal maintenance of solubility (Fig. 2). This demonstrates that when bound to hsp110, denatured luciferase is maintained in a folding competent state.

Since hsc70 and Hdj-1 alone have been shown to be a folding ensemble, we repeated the above RRL refolding assay using only these specific molecular chaperones. Although less efficient than RRL, Fig. 3B demonstrates that hsp110 functionally interacts with hsc70-Hdj-1 to refold heat-denatured luciferase to 25% original activity. Whereas ATP is not required for inhibition of luciferase aggregation, it is necessary for the refolding functions of RRL or hsc70-Hdj-1 as has been demonstrated previously (13–15). Lastly, when the refolding step employs hsp110 (and not hsc70) and Hdj1, no recovery of luciferase activity is obtained when the holding step also uses hsp110 (Fig. 3B). However, when the holding step (i.e. during heating) employs hsc70, a small amount of refolding is achieved by adding hsp110 and Hdj-1, probably reflective of the interaction of hsc70 (already present) and Hdj-1. This indicates that (i) hsp110/hsc70-Hdj-1 is a relevant protein folding machine, (ii) that based on Figs. 2, A and B, and Fig. 3A, hsp110 is notably more efficient than is hsc70 in protecting the denatured protein during the initial heating phase for subsequent refolding and iii), that Hdj-1 does not function as a folding co-chaperone with hsp110.

**DISCUSSION**

hsp110 was one of the earliest heat shock proteins described in mammalian cells and has been noted in numerous studies (e.g. Refs. 1–3). In Chinese hamster cells, hsp110 accounts for 0.7% total cell protein after heat shock compared with 3.2% for hsc70 + hsp70 and 1.2% for hsp90 (1). Its expression levels in the Rat-1 and HeLa cells are less distinctive than in Chinese hamster cells; however it remains as one of the major heat-inducible proteins in these cell lines. Its constitutive expression in different murine tissues varies widely, with highly significant levels of expression in liver and brain (4, 6). Indeed, the expression of hsp110 in brain is comparable to heat-shocked Chinese hamster cells (i.e. making it approximately 0.7% total brain mass). Additionally, like the hsp70 family, the hsp110 family possesses at least three distinct members, each of which is approximately 60% identical in amino acid sequence to the other. These are hsp110, apg-1 (osp94), and apg-2 (9–11). The cell and tissue expression of apg-1 and apg-2 is less well characterized than hsp110, although in testis and in renal medullary duct cells, apg-1/osp94 has been shown to be highly expressed (9, 11). The level of expression of hsp110 and its family members in different cell lines and in murine tissues speaks to a significant role for this class of stress proteins in both the stress response and in the normal functioning of the non-stressed cell.

The recent cloning of the cDNA for this protein from a variety of organisms as diverse as yeast and mammals has shown it to be a large and highly diverged relative of the hsp70 family (4–11, 29, 30). Secondary structure analysis of hsp110 demonstrates that it exhibits significant similarity to the secondary structure of hsp70 and DnaK, whose structures are well studied by several methods including crystallography. hsp70/DnaK is composed of 1) an amino-terminal ATP binding domain followed by 2) a 100-amino acid β-sheet region that has been identified as the peptide binding domain and 3) a carboxyl-terminal α-helical region that is involved in regulation of hsp70/DnaK function and appears as a lid covering the peptide binding domain (19). Analysis of the sequence and secondary structure of hsp110 demonstrates that it appears to have the same basic 1) ATP binding domain, 2) β-sheet (peptide binding) domain, and 3) carboxyl end α-helical region. However, the ATP binding domain of hsp110 binds and hydrolyzes ATP poorly relative to hsc70 in vitro (6, 16). The β-sheet configuration (i.e. the predicted peptide binding region of hsp110) shows some
sequence homology to corresponding regions in the structure of DnaK and appears to be of similar overall size and organization. 3 The regulatory, α-helical “lid” region of hsp110 is similar to that of DnaK but is larger than the corresponding region in DnaK. The predominant structural difference between hsp110 and DnaK is that the α-helical lid of hsp110 is connected by a 100-amino acid “loop” to the β-sheet peptide binding domain, a structure that is virtually absent in hsp70 (4) and that is responsible for much of the increased size of hsp110. If the α-helical lid plays an important role in peptide binding as suggested (cf. Ref. 19), the interposition of this 100-amino acid loop between it and the peptide binding domain may have significant functional implications that could be related to the differences between hsp110 and hsc70 described here. It is also in the predicted α-helical lid region of hsp110 that the conserved sequences, which act as a signature for members of the hsp110/SSE family, reside (4).

The data presented here demonstrate that hsp110 must be added to the list of identified molecular chaperones that can inhibit the aggregation of denatured cellular proteins, a list that includes the small heat shock proteins, hsp90, the immunophilins, p23, as well as members of the hsp70 family (13, 20–24). However, it is evident from both the aggregation studies of luciferase and citrate synthase (Fig. 2) and from the refolding studies of luciferase with reticulocyte lysate (Fig. 3) that hsp110 is significantly more efficient in maintaining heat-damaged proteins in a soluble, refoldable state than is its evolutionary relative, hsc70. Interestingly, earlier reports are contradictory concerning the ability of mammalian hsp70 to hold denatured protein in vitro. Minami et al. (15) report that hsc70 alone does not inhibit aggregation of luciferase, whereas hsc70 plus Hdj-1 does. Freeman and Morimoto (13) show that hsp70 and hsc70 maintain the solubility of β-galactosidase as indicated by native gel electrophoresis, an observation in agreement with the present report. It is also shown here that hsp110 cannot refold luciferase on its own or with the addition of Hdj-1, a well known co-chaperone for folding with hsc70. However, the refolding obtained with rabbit reticulocyte lysate was much faster and higher than by hsc70 and Hdj-1, suggesting that other components in this lysate are required for efficient refolding of the denatured proteins. It is likely that other chaperones and co-chaperones exist, one or more of which could interact in protein folding with hsp110, possibly including a DnaJ homolog other than Hdj-1. Whereas the structural and sequence similarities between hsp110 and hsc70 indicate a similarity in function between these proteins, hsp110 could also play a primary role in the folding pathway without being directly involved in the final folding step. Furthermore, a molecular interaction between hsc70 and hsp110 has been observed in all cell lines and mouse tissues examined as indicated by coimmunoprecipitation studies and can be reproduced in vitro. 4 This suggests that in many instances at least, hsp110 may not function independently of hsc70 (and vice versa) and that the true in vivo physiologic function of these chaperones requires a cooperative interaction with substrate proteins, perhaps utilizing the different peptide binding capacities and ATP binding and hydrolysis characteristics of each. Indeed, since hsp110 is a prominent mammalian hsp (as it also appears to be in other organisms), it may play a primary role for holding the unfolded peptide chain, with hsc70 playing a secondary function in this initial chaperoning step.

That these in vitro chaperoning functions have a physiologic role in vivo is indicated by the thermoresistance/thermotoler-

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