The Chaperoning Activity of hsp110

IDENTIFICATION OF FUNCTIONAL DOMAINS BY USE OF TARGETED DELETIONS*

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hsp110 is one of major heat shock proteins of eukaryotic cells and is a diverged relative of the hsp70 family. It has been previously shown that hsp110 maintains heat-denatured luciferase in a soluble, folding competent state and also confers cellular heat resistance in vivo. In the present study the functional domains of hsp110 that are responsible for its chaperoning activity are identified by targeted deletion mutagenesis using the DNAK structure as the model. The chaperoning activity of mutants is assessed based on their ability to solubilize heat-denatured luciferase as well as to refold luciferase in the presence of rabbit reticulocyte lysate. It is shown that these functions require only an internal region of hsp110 that includes the predicted peptide binding domain and two immediately adjacent C-terminal domains. It is also shown that although hsp110 binds ATP, binding can be blocked by its C-terminal region.

hsp70 is perhaps the best studied of the major heat shock proteins. hsp70 family members have been shown to play essential roles in a variety of cellular activities, for example, folding of nascent polypeptides, protein translocation across intracellular membranes, and regulation of the activities of steroid hormone receptors and kinases (1-3). The unifying mechanism for their action is based on their chaperoning activity, i.e., their ability to recognize and bind to peptide segments that are not normally exposed to the aqueous environment because they are normally buried in the interior of the protein or are hidden by interactions with other proteins (4-6). Multiple members of hsp70 family occur within individual organisms, and it is believed that these different hsp70s perform differing roles. In Saccharomyces cerevisiae, it has been shown that different hsp70s perform specific functions, e.g., transport across membranes or translation, and that one member may not be interchangeable for another (7-9). This division of labor occurs despite the very high degree of amino acid sequence identity between these hsp70 family members, usually better than 60%.

Based on strong inducibility, quantity, and presence in many cell types, hsp110 has also been recognized for the last two decades as a major heat shock protein, specifically in mammalian cells (10-13). hsp110 has been recently cloned from a variety of organisms as diverse as yeast and man (14-23). The hsp110s of S. cerevisiae have been termed the Stress Seventy E (SSE) family (23). However, the hsp110 family is a distinct subset of the hsp70 family which, in addition to their uniqueness, is divergent compared with the hsp70s, differ in their significant sequence divergence from the archetypal hsp70s (14). In light of the differential functions of S. cerevisiae hsp70s, the appearance of the highly diverged hsp110 family, which exists in parallel with the hsp70s in the cytoplasm and nucleus in diverse organisms, argues strongly for related but differential functions and properties for these two major stress protein groups.

Initial studies have shown that hsp110 has certain functional properties that are shared with hsp70, i.e., i) the overexpression of hsp110 has been shown to confer cellular heat resistance, and ii) hsp110 has been shown to have the ability to prevent protein aggregation and keep denatured protein in a folding-competent state; however, with apparently greater capacity compared with hsc70 (24). To further analyze the functional properties of hsp110 and how it may differ from the hsp70 family, a mutational investigation of hsp110 has been undertaken. By sequence alignment and prediction of secondary structure, we have constructed several targeted deletion mutants. We describe here the properties of these mutants and test their ability to exhibit chaperoning functions.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—All plasmid constructs were made to produce 6-histidine-tagged proteins. For the polymerase chain reaction (PCR), the following primers were synthesized. Each primer sequence contained proper restriction enzyme recognition sequences, which are underlined and whose name is indicated in parenthesis. Primers, A, B, and C are 5' (or forward primers for PCR) and primers, i, ii, iii, iv, v, and vi are 3' (or backward primers for PCR). Primer A (BanHI), 5'-GCTAGAAGATCCTGTTGCAGTGTTCAATATTGA-3'; primer B (BanHI), 5'-GACAGAAGATCCTGTTGCAGTGTTCAATATTGA-3'; primer C (XbaI), 5'-CGCGCCCTCGAATGTCGGTGTTGGGGACTAGAGTAGC-3'; primer D (SalI), 5'-GACAGAAGATCCTGTTGCAGTGTTCAATATTGA-3'.

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The abbreviations used are: hsp, heat shock protein; hsc, constitutive heat shock protein; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; CHO, Chinese hamster ovary.

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Functional Domains for Chaperoning Activity of hsp110

PieriSETC-hsp110 plasmid that was described previously (24) was digested with SstI and HinIII, and fragments containing the N-terminal portion of hsp110 sequence was subcloned and ligated with the PCR fragments. The results of these procedures is that constructs BLH, LH, AB, and ABL contain N-terminal fusion of six histidines, enterokinase recognition sequences, and additional Asp-Arg-Trp-Gly-Ser for BLH and BLH', Asp-Arg-Trp for LH, Asp-Arg-Trp-Ile-Arg-Pro-Arg-Asp-Leu-Gln-Pro-Ala for AB and ABL. The construct ΔL contains N-terminal fusion of six histidines and Gin-Met-Ser.

Expression and Purification—The constructs were transformed into Escherichia coli JM109 (DE3) cells. E. coli transformed with the constructs BLH and LH were cultured at 37 °C until the A_{600} reached 0.06, 1 mM isopropyl-1-thio-β-D-galactopyranoside was added, and E. coli were further cultured for 5 h. Cells containing all other constructs were cultured in a same way, except that the expression was induced with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside and at 30 °C. Cells were lysed by lysozyme treatment and sonication in 20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 20 mM imidazole, 0.5% Nonidet P-40, and protease inhibitor mixture (Roche Molecular Biochemicals). The lysate was centrifuged at 42,000 rpm for 2 h, and soluble fraction was used for purification. The proteins were purified using nickel nitrilotriacetic acid agarose columns (Qiaogen, Inc.) following the manufacturer’s instructions. The column matrices were extensively washed with wash buffer containing 20 mM Tris-HCl, pH 7.8, 0.5 mM NaCl, 60 mM imidazole, and 10% glycerol, and recombinant proteins were eluted with wash buffer containing 500 mM imidazole. The eluted proteins were dialyzed for 48 h against 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 10 mM β-mercaptoethanol. Proteins were concentrated using Slide-A-Lyzer concentrator (Pierce). Proteins were quantified using the Bio-Rad pro-teinassay kit (Bio-Rad) with bovine serum albumin as a standard. Each mutant was visibly pure as indicated by Coomassie-stained gels, except ABL, which contained a small amount of a truncated form of this mutant, as shown by Western blot analysis. This mutant was not further purified.

Luciferase Aggregation and Refolding Assay—0.15 μM luciferase (Sigma) and various test proteins were incubated in 25 mM Hepes, pH 7.9, 5 mM magnesium acetate, 50 mM KCl, 5 mM β-mercaptoethanol at 43 °C for 30 min, and the protein aggregation was monitored by the increase of optical density at 320 nm. At the end of the heating, the solution was centrifuged at 16,000 × g for 15 min, and soluble and pellet fractions were separated, run on SDS-PAGE, and subjected to Western analysis with anti-luciferase antibody (Promega). For luciferase refolding assay, luciferase together with various test proteins were heated in refolding buffer (25 mM Hepes, pH 7.6, 5 mM MgCl{\textsubscript{2}}, 2 mM dithiothreitol, and 2 mM ATP) at 43 °C for 30 min. The heated luciferase was diluted 100-fold into 60% rabbit reticulocyte lysate (Promega) containing refolding buffer and incubated at 30 °C for 2 h. For the measurement of activity, luciferase solution was further diluted 5-fold in 25 mM Hepes, pH 7.6, 5 mM GTP, 0.5 mM bovine serum albumin, and 10 μl was added to 100 μl of luciferase assay solution (Promega); the activity was measured with Lumat LB9501 (Berthold).

ATP Binding Assay—ATP binding assay was essentially the same as previously reported, with minor modification (25). ATP-agarose (Sigma) was washed and stored in buffer B (20 mM Tris-HCl, 20 mM NaCl, 0.1 mM EDTA, 2 mM dithiothreitol) as a 1:1 slurry. One 10-cm dish of confluent CHO cells are lysed in 600 μl of phosphate-buffered saline, 1% Triton X-100, and protease inhibitors, and soluble proteins are collected after centrifugation at 16,000 × g for 5 min. 120 μl of lysate was incubated with 200 μl of ATP-agarose beads for 16 h at 4 °C. For ATP competition, 10 μl ATP was added to the mixture of lysate and ATP-agarose. The ATP-agarose beads were washed 2 times with buffer B containing 500 mM NaCl and 3 mM MgCl{\textsubscript{2}}, 2 times with buffer B containing 5 mM GTP, and 2 times with buffer B. ATP-agarose-bound proteins were eluted by incubation in buffer B containing 10 mM ATP and 3 mM MgCl{\textsubscript{2}}, for 4 h at 4 °C. The eluted proteins were boiled in SDS-gel-loading buffer (1% SDS, 125 mM Tris-HCl, pH 6.8, 10% glycerol, 1% β-mercaptoethanol, 1 mM EDTA, and bromphenol blue) for 10 min. The samples were run on 10% SDS-PAGE, transferred to nitrocellulose membrane (Bio-Rad), and probed with anti-hsp110 or anti-hsp70 antibody (StressGen). In case of E. coli expressing recombinant hsp110 or ATP binding domain, the cells were cultured, and recombinant proteins are expressed in the same way as for protein purification. The cells were lysed by sonication in buffer B containing protease mixture and centrifuged at 42,000 rpm for 2 h, and soluble fractions were used for ATP-agarose binding assay. 500 μg of total proteins was used for this assay. ATP-agarose was washed 5 times with buffer B containing 500 mM NaCl and 3 mM MgCl{\textsubscript{2}}, and 2 times with buffer B. ATP-agarose-bound proteins were eluted and analyzed as described above.

RESULTS

Predicted Structure of hsp110 and the Purification of Targeted Mutants—In order that we might construct targeted mutations of the hsp 110 cDNA, we built a model of hsp110 based upon its sequence similarity with the DnaK family of stress proteins. The model (Fig. 1) is shown diagrammatically and is compared with a similar diagram of DnaK, based upon its x-ray structure determination (26). Residues 1–394 of hsp110 (designated as domain A in Fig. 2) show 34% identity in amino acid sequence to the same region of DnaK. This region in DnaK is responsible for ATP binding. From amino acid 394 to amino acid 509, hsp110 is predicted to exhibit seven β strands (the β sheet domain, or B in Fig. 2). This domain demonstrates some sequence similarity to, and structurally aligns with, the corresponding region of the DnaK. It is this region of DnaK that represents its “peptide binding domain” and consists of eight major β strands arranged as a β sandwich. The following 98 amino acids of hsp110 are composed of a number of negatively charged residues that computer analysis fails to predict as having any obvious secondary structure. This region is therefore referred to as the loop domain (residues 510–608, or domain L). Finally, distal to the loop domain, the C-terminal residues of hsp110 are predicted to form a series of α helices (residues 608–858, or domain H). Although there is little sequence identity in this region between hsp110 and DnaK (or hsc70), DnaK shows three large helices, which make up a helix-turn-helix structure. Fig. 1 correspondingly arranges the α helices of hsp110. It is in this region that the hsp110 family members exhibit a high degree of sequence homology among themselves, which is an identifying feature for this family (14).

However, a small degree of sequence similarity can be found between hsp110 and DnaK families in certain of these helices, specifically αB and αC. In DnaK, the β sandwich serves as the peptide binding site, whereas the helix-turn-helix structure, poised above the β sandwich, is thought to regulate entry and/or exit of the peptide substrate (26).

Based on these similarities between the predicted structural elements of hsp110 and the actual structure of DnaK, we have constructed various deletion mutants. These mutants were constructed to include the specific amino acids residues defined above and to also include a small number of additional amino acids on either side of the domain to both increase the likelihood of correct folding of the domain and account for small errors in the computer predictions. Fig. 2A shows a schematic diagram of these deletion mutants. The mutants were expressed and purified from E. coli, and their mobilities were measured by SDS-PAGE (Fig. 2B). When the recombinant full-length hsp110 is run on SDS-PAGE, its mobility (from which the N-terminal fusion peptide is subtracted) is essentially identical to that of native hsp110, determined by Western analysis of CHO lysate using an anti-hsp110 antibody. However, the calculated molecular mass of hsp110, based on the amino acid composition, is more than 10 kDa smaller than the molecular mass determined from SDS-PAGE mobility (Table 1). This sort of discrepancy is unusual although not rare. To determine whether the mobility retardation is caused by overall sequence or by specific region(s) of hsp110, calculated molecular weights and mobilities of the different deletion mutants were compared. It is apparent from Fig. 2B that the acidic loop region, which was also noted for its unusual amino acid composition, predicted flexibility, and lack of secondary structure, is solely responsible for this retardation. This also explains why mutant
ABL (614 amino acids) exhibited slower mobility than mutant ΔL (757 amino acids), i.e., full-length hsp110, which lacks the loop domain.

Identification of Domains Required for the Chaperoning Activity of hsp110—Two assays were employed to identify the functional domains of hsp110 required for chaperoning activity. These are 1) the ability of the mutants to prevent protein aggregation induced by heat treatment, which is assessed by the suppression of the increase in light scattering obtained upon heat treatment as well as the solubility of the reporter protein, firefly luciferase, and 2) the ability to sustain denatured luciferase in a folding competent state, which is assessed by measuring the ability of the denatured luciferase to refold (i.e., recover activity) upon the addition of folding chaperones present in rabbit reticulocyte lysate.

First, as seen in Fig. 3, the ability of the different mutants to prevent heat-induced aggregation is presented. As shown in Fig. 3, A and B, mutant BLH (i.e., containing only the β sheet peptide binding domain (B), loop (L), and α helical domain (H)) is able to prevent the aggregation of heat-denatured luciferase as equally well as does wild type hsp110. However, mutant LH does not prevent luciferase aggregation. This indicates that domain B containing amino acids from 375 to 507 (β-sheet domain) contains the substrate binding domain, in agreement with the predictions of the sequence alignment and secondary structure. To address whether β-sheet domain alone is sufficient for this chaperoning activity, mutant AB (ATP binding and β sheet-peptide binding domains) was examined. In the presence of mutant AB, heat denaturation of luciferase resulted in a change in optical density that was 3-fold greater than that observed with luciferase alone (i.e., 300%, not plotted in Fig. 3A), in which case luciferase is found almost entirely in the pellet fraction (Fig. 3B). This indicates that mutant AB is not capable of preventing aggregation and, further, that it itself aggregates. Although it was determined above that BLH is totally effective in “holding” denatured luciferase and that B is required, the additional involvement of L and/or H was still a possibility. We therefore examined mutants ABL and BLH′ (H′ containing only the first major α helix of the complete H domain). Mutant ABL is inactive (even at a 2× molar concentra-
tion), but mutant BLH' is active, even though its efficiency is 50% that of wild type hsp110 or mutant BLH. Thus, the C-terminal boundary of an “effective” peptide binding domain resides between amino acids 614 and 655, which defines the first part of the “cap” domain. This data further indicates that the complete holding ability of the protein requires additional C-terminal sequence components in the cap. Finally, to examine the role of the loop domain in this holding activity, mutant D_L was next examined. It is observed that D_L (containing full-length hsp110, excluding L) was also capable of inhibiting the aggregation of heat-denatured luciferase. In addition to luciferase, we have also tested the ability of BLH, LH, and BLH' to inhibit the heat-induced aggregation of citrate synthase using Coomassie Blue staining to measure the amount of citrate synthase in the supernatant and pellet. These results were essentially identical to those observed using luciferase. Taken together, the predicted β-sheet domain and α-helical cap domain are necessary and sufficient for the total ability of

|                | By amino acid composition | Estimate on SDS-PAGE | Difference |
|----------------|--------------------------|----------------------|------------|
| ABLH (wild type) | 101                      | 112                  | 11         |
| BLH             | 59                       | 75                   | 16         |
| LH              | 44                       | 62                   | 18         |
| AB              | 61                       | 57                   | 4          |
| ABL             | 87                       | 82                   | 5          |
| AΔL             | 72                       | 85                   | 13         |
| ABLH'           | 35                       | 50                   | 15         |
| A              | 47                       | 41                   | 6          |

FIG. 2. Construction and purification of deletion mutants. A, schematic diagram of deletion mutants. The predicted domains are denoted as the following: A, ATP binding domain; B, β-sheet domain; L, acidic loop; H, α-helices; H', first two α-helices. ND, not determined. B, SDS-PAGE of purified mutant proteins. About 1 μg of each protein is run on SDS-PAGE and stained with Coomassie Blue. WT, wild type.

Functional Domains for Chaperoning Activity of hsp110

Identification of domains for the in vitro chaperoning activity of hsp110. A, examination of the ability to prevent protein aggregation by light scattering. 0.15 μM of luciferase was incubated with 0.15 μM or 0.3 μM mutant hsp110, depending on the mutant, at 43 °C for 30 min. Aggregation was monitored by measuring the increase of optical density at 320 nm. The molar ratio of test protein:luciferase is indicated in each case. The optical density of the luciferase heated alone was set to 100% OVA, ovalbumin. B, Western blot analysis of luciferase in the supernatant (Spt) and pellet of control (25 °C) and heat-shocked (43 °C) solutions in the presence of wild type (ABLH) or the various mutants designated in Fig. 2. C, folding competency of luciferase bound to various deletion mutants. The heat-denatured luciferase was refolded by rabbit reticulocyte lysate. 1.5 μM wild type (WT) or mutant hsp110 was used. In case of AΔL and BLH', two concentration, 1.5 μM and 3.0 μM, were used. The activity of luciferase obtained with 1.5 μM wild type hsp110 was set to 100%.
hsp110 to prevent aggregation and, further, that the significant component of this holding activity requires only the first major α helix of cap. The loop domain and ATP binding domain are dispensable for this function.

Second, we examined the ability of the various mutants to provide a folding competent substrate. To examine this question, luciferase was incubated with each mutant at 43 °C for 30 min, following which rabbit reticulocyte lysate was added. The solution was further incubated at 30 °C for 2 h, a sufficient time to enable maximal refolding with wild type hsp110 (approximately 70% of the activity of unheated luciferase, designated 100% in Fig. 3C for the comparison of mutants). As shown in Fig. 3C, it is seen that when the luciferase is sustained by the mutant in a soluble form in aggregation assays in Figs. 3, A and B, it was found to be refoldable (i.e. recover activity). It is also seen in this figure that the mutants ΔL and BLH, although holding luciferase in a folding-competent state, do so less efficiently than does wild type hsp110 (about one-half as efficient at equal concentration). Doubling the concentration of ΔL and BLH improved the recovery of luciferase activity. In case of BLH, efficiency was increased to a level equivalent to that of the wild type protein. However, in case of ΔL, recovery of activity was still significantly less than wild type hsp110 or BLH. Last, it is also seen that the ATP binding domain (A) is neither required for folding or holding of luciferase in these studies, as discussed above. Thus, the β-sheet domain, loop domain, and parts of the α-helical domain (specifically αB) are the minimal components required for hsp110 to function as a molecular chaperone that can hold substrate in a folding-competent state.

**hsp110 Has a Functional ATP Binding Domain—** Sequence analysis has indicated that hsp110 exhibits an ATP binding domain, and moreover, the domain shares significant sequence homology to hsp70. Because hsp70 binds ATP and ATP is required for its chaperoning function, the ATP binding ability of hsp110 was examined using ATP-agarose. CHO cell lysates were incubated with ATP-agarose beads and bound proteins eluted with free ATP. The eluted proteins were then run on SDS-PAGE and subjected to Western analysis with anti-hsp110 or anti-hsp70 antibody (Fig. 4A). It is seen that hsp70 is a very good ATP-agarose binder (as has been long recognized), but that hsp110 is not. Free ATP competition during the ATPagarose incubation shows the specificity of this assay. Because hsp110 has all the known consensus sequences for ATP binding, the possibility of the structural blocking of the ATP binding site in hsp110 was examined by using the mutant containing only the ATP binding domain (A). The E. coli lysates expressing recombinant full-length hsp110 or the ATP binding domain alone were subjected to the same assay as the CHO cell lysate just described. The proteins bound to ATP-agarose were then analyzed with anti-oligohistidine antibody on a Western blot. The recombinant, full-length hsp110 still did not bind ATP-agarose. However, it is seen that the ATP binding domain (A) mutant was able to bind ATP-agarose. This indicates that the ATP binding domain of hsp110 is functional, but that its ability to bind ATP is masked by some part of its more C-terminal regions.

**DISCUSSION**

It has been long recognized that the major heat shock proteins (hsp) of mammalian cells are observed at 28, 70, 90, and 110 kDa (10, 12, 27–29), and other hsp families, e.g. hsp60 (GroEL) and hsp40 (DnaJ), were later identified. All of these hsp except hsp110 have been intensively studied, and their interactions in numerous cellular processes are today broadly recognized. The situation with hsp110 is somewhat curious. Studies from the beginning of the 1980s from this laboratory as well as virtually every other laboratory that examined the induction of heat shock proteins described a major hsp of 100 to 112 kDa size (Refs. 10, 12, 27–29, among many references). These studies demonstrated that hsp110 was inducible by a broad variety of stresses that induced other hsps and that it was a major hsp whose expression strongly correlated with the expression of thermotolerance (10, 12, 28).

The cloning of hsp110 from hamster, mouse, yeast, arabidopsis, sea urchin, and a variety of other species has been described in the last few years (15–23, 30). Surprisingly, although hsp110 appears as a conserved and distinctive protein species in countless heat shock studies, its sequence shows that it is a significantly enlarged and divergent relative of the hsp70 family of proteins. Therefore, questions arise as to the differences and similarities between the hsp70 and hsp110 families and purpose for having these two major subgroups expressed in parallel in organisms as diverse as yeast and man. The present study continues our investigation of this question and identifies the functional domains of hsp110 in comparison with the available knowledge of hsp70 organization and function. It is shown in the present study that 1) the ATP binding domain of hsp110 is functional, but appears to be normally masked, 2) the ATP binding domain is not required for the activity of hsp110.
as a holding chaperone, 3) the β-sheet domain and C-terminal α-helical cap appear to compose the substrate binding pocket.

hsp110 has been previously shown to be an efficient holding chaperone, a characteristic that includes most identified molecular chaperones. Moreover, we have shown previously that this holding function is independent of ATP (24). Thus, it is not surprising to find here that the ATP binding domain is not required for the holding activity of hsp110. A somewhat similar situation is seen with other holding chaperones such as hsp90 and hsp25 (31–33). This may also be true for hsp70 because recombinant hsc70, without its ATP binding domain, is capable of binding reduced carboxymethylated lactalbumin (34). Moreover, it has been shown that an hsp70 mutant, which lacks its ATP binding domain, was able to confer thermotolerance in tissue culture cells as well as does wild type hsp70 (35). Taken together with the in vitro substrate binding studies, this suggests that the ATP domain of hsp70 has a dispensable role in holding substrates in a folding-competent state and that the chaperoning function of the constructs without this domain is sufficient to protect cells from stress. This is analogous to the present results obtained with hsp110. Therefore, hsp70 and hsp110 may be similar with respect to the lack of involvement of their ATP binding domains in their basic chaperoning/holding functions. However, wild type hsp70 is also capable of folding substrate in the presence of DnaJ co-chaperones (albeit, to a limited degree only (24)). However, this does require ATP and, logically, the hsp70 ATP binding domain. Although a folding function for hsp110 has not been identified to date, there is still the possibility of hsp110 being a folding chaperone in conjunction with other co-chaperone(s) that have yet to be identified. It would then be conceivable that its ATP binding domain could play an essential role.

Analysis of crystal structure of DnaK, the E. coli homologue of hsp70, revealed the substrate binding pocket of DnaK, and sequence alignment of DnaK, bovine hsp70, and BiP predicts that these hsp70 family members have a common substrate binding pocket (26). This is supported by several deletion mutation studies of hsp70. Boice and Hightower (36) reported a mutational analysis of hsc70 guided by secondary structure predictions showing that hsp70 and DnaK have very similar peptide binding domains. Although this domain represents the peptide binding cleft, the precise boundaries within hsp70 required for peptide binding remain uncertain. Earlier studies demonstrated that an 18-kDa fragment of hsp70 immediately after the ATP binding domain has essentially the same affinity for synthetic peptide as does wild type hsc70 (37). This result is analogous to the results presented here for hsp110. It has also been shown that a 60-kDa fragment of bovine hsc70, which excludes the extreme C-terminal 10-kDa domain, can bind the clathrin cage but cannot act in its disassembly (38). This latter result suggests that hsp70 substrate binding activity does not involve the C-terminal 10-kDa region, but its regulation does. Yet, a third study has shown that the highly conserved C-terminal EEVD motif at the extreme C-terminal of hsp70 is required for binding to reduced carboxymethylated lactalbumin (34). Thus the actual roles of the C-terminal regions of hsp70 in regulation of its chaperoning functions is complex. Indeed, different substrates, e.g. reduced carboxymethylated lactalbumin, synthetic peptide, and clathrin, may contribute to the differing conclusions regarding the varying requirements for parts of this region of hsp70 in its function.

The results obtained in the present study on hsp110 indicate that it has a similar substrate binding pocket to DnaK. The loop domain and the extension of the expanded C-terminal α-helical cap domain are not absolutely required for the chaperoning activity of hsp110 as defined here. However, they are both required for hsp110 to be fully efficient in its activity. There are at least two possibilities for the role of these two domains. 1) These two domains may be required for maintaining structural stability of hsp110, and/or 2) they may be required for hsp110 to bind and release substrates efficiently. Curiously, the loop domain in hsp110/SSE family members is essentially absent in hsp70 family members. We have shown previously that hsp110 is more efficient in solubilizing luciferase than is hsc70 at an equal molar concentration. The deletion of the loop would make hsp110 more similar in its holding capacity to hsc70. The loop and the expansion of the C-terminal α-helical domain could alter the peptide binding characteristics of hsp110 relative to hsp70 in ways that have yet to be clearly defined.

We have shown here that hsp110 does not bind ATP in vitro, despite the conservation of its ATP binding domain, whereas its ATP binding domain mutant (A) does. The inability to detect ATP binding activity experimentally seems to be due to the structural locking of the ATP binding site by more C-terminal domains. Nonetheless, the ATP binding ability of the ATP binding domain mutant and the high degree of conservation of ATP binding motif in hsp110 argues strongly that hsp110 does in fact exhibit ATP binding properties in vivo. If so, then the binding and putative hydrolysis of ATP could be expected to have regulatory implications in hsp110 holding and (possibly) folding functions and/or interactions with co-chaperones. Moreover, the apparent constriction on ATP binding in hsp110 would indicate an additional regulatory feature of this chaperone that would appear to differ from the present models for hsp70.

As with the hsp70 family, multiple members of hsp110 family have been identified in individual organisms with three members having been identified in mice (15–17, 21, 22). Although hsp110 appears to be the principal family member expressed in most murine tissues, apg-1 (another family member) is also abundantly expressed in testes and heart. That such a multiplicity of genes exist for each family, hsp70 and hsp110, further argues for important differences in the general functions of each. Indeed, a different requirement for hsp110 and hsc70 in cells is suggested by the fact that they are differentially expressed in different tissues and different regions of the same tissue (e.g. brain) (14, 15). Last, in addition to hsp110 and its family members, there is a second large relative of the hsp70 family that resides in the endoplasmic reticulum, known as grp170 (39–41). Although grp170 does contain an hsp70/ hsp110-like ATPase domain and a large hsp110-like loop domain, it and its family are as diverged from the hsp110 family as it is from the hsp70 family (i.e. hsp70, hsp110 and grp170 segregate into three major subfamilies on a phylogram) (41, 42). Further studies of hsp110 and grp170 will provide additional insight into the purpose for these unusual and unexpected relatives of the hsp70 family and how they interact and cooperate with each other and hsp70 in cellular function.

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REFERENCES
1. Glick, B. S. (1995) Cell 80, 11–14
2. Craig, E. A., Gambill, B. D., and Nelson, R. J. (1993) Microbiol. Rev. 57, 402–414
3. Rutherford, S. L., and Zuker, C. S. (1994) Cell 79, 1129–1132
4. Bukau, B., and Horwich, A. L. (1998) Cell 92, 351–366
5. Hartl, F. U. (1996) Nature 381, 571–579
6. Johnson, J. L., and Craig, E. A. (1997) Cell 90, 201–204
7. Craig, E. A., and Jacobsen, K (1985) Mol. Cell. Biol. 5, 3517–3524
8. Brodsky, J. L., Hamamoto, S., Feldheim, D., and Schechter, R. (1993) J. Cell

2 B. Hylander, J. H. Oh, P. Graf, and J. Subjeck, unpublished data.
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Biol. 120, 95–102
9. Gao, B. C., Biosca, J., Craig, E. A., Greene, L. E., and Eisenberg, E. (1991) J. Biol. Chem. 266, 19565–19571
10. Subjeck, J. R., and Sciandra, J. J. (1982) in Heat Shock: From Bacteria to Man (Schlesinger, M., Ashburner, M., and Tissieres, A., eds) pp. 405–411, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
11. Levinson, W., Oppermann, H., and Jackson, J. (1980) Biochim. Biophys. Acta 606, 170–180
12. Landry, J., Bernier, D., Chretien, P., Nicole, L. M., Tanguay, R. M., and Marceau, N. (1982) Cancer Res. 42, 2457–2461
13. Hightower, L. E. (1980) J. Cell. Physiol. 102, 407–427
14. Lee-Yoon, D., Easton, D., Murawski, M., Burd, R., and Subjeck, J. R. (1995) J. Biol. Chem. 270, 15725–15733
15. Kaneko, Y., Nishiyama, H., Nonoguchi, K., Higashitsuji, H., Kishishita, M., and Fujita, J. (1997) J. Biol. Chem. 272, 2640–2645
16. Kaneko, Y., Kimura, T., Kishishita, M., Noda, Y., and Fujita, J. (1997) Gene 189, 19–24
17. Morozov, A., Subjeck, J., and Raychaudhuri, P. (1995) FEBS Lett. 371, 214–218
18. Maaz, R., Jaworski, D., Kamei, N., and Glabe, C. G. (1997) Dev. Biol. 184, 31–37
19. Storozenko, S., De Pauw, P., Kushnir, S., Van Montagu, M., and Inze, D. (1996) FEBS Lett. 390, 113–118
20. Foltz, K. R., Partin, J. S., and Lennarz, W. J. (1993) Science 259, 1421–1425
21. Yasuda, K., Nakai, A., Hatayama, T., and Nagata, K. (1995) J. Biol. Chem. 270, 29718–29723
22. Kojima, R., Randall, J., Brenner, B. M., and Gullans, S. R. (1996) J. Biol. Chem. 271, 12327–12332
23. Mukai, H., Kuno, T., Tanaka, H., Hirata, D., Miyakawa, T., and Tanaka, C. (1995) Gene 132, 57–66
24. Oh, H. J., Chen, X., and Subjeck, J. R. (1997) J. Biol. Chem. 272, 31636–31640
25. Milarski, K. L., and Morimoto, R. I. (1989) J. Cell Biol. 109, 1947–1962
26. Zhu, X., Zhao, X., Burkholder, W. F., Gragerov, A., Ogata, C. M., Gottesman, M. E., and Hendrickson, W. A. (1996) Science 272, 1606–1614
27. Welch, W. J., Garrels, J. I., Thomas, G. P., Lin, J. J., and Feramisco, J. R. (1983) J. Biol. Chem. 258, 7102–7111
28. Tomasovic, S. P., Steck, P. A., and Heitzman, D. (1983) Radiat. Res. 95, 399–413
29. Li, G. C., and Laszlo, A. (1985) J. Cell. Physiol. 128, 91–97
30. Chung, K. S., Hoe, K. L., Kim, K. W., and Yoo, H. S. (1998) Gene 210, 143–150
31. Freeman, B. C., and Morimoto, R. I. (1996) EMBO J. 15, 2969–2979
32. Ehrnsperger, M., Graber, S., Gaestel, M., and Buchner, J. (1997) EMBO J. 16, 221–229
33. Lee, G. J., Roseman, A. M., Saibil, H. R., and Vierling, E. (1997) EMBO J. 16, 659–671
34. Freeman, B. C., Myers, M. P., Schumacher, R., and Morimoto, R. I. (1995) EMBO J. 14, 2281–2292
35. Li, G. C., Li, L., Liu, R. Y., Rehman, M., and Lee, W. M. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 2036–2040
36. Boise, J. A., and Hightower, L. E. (1997) J. Biol. Chem. 272, 24825–24831
37. Wang, T. F., Chang, J. H., and Wang, C. (1993) J. Biol. Chem. 268, 26049–26051
38. Tsai, M. Y., and Wang, C. (1994) J. Biol. Chem. 269, 5958–5962
39. Lin, H. Y., Massen-Welch, P., Di, Y. P., Cai, J. W., Shen, J. W., and Subjeck, J. R. (1993) Mol. Biol. Cell 4, 1109–1119
40. Dierks, T., Volker, J., Schlenstedt, G., Jung, C., Sandholzer, U., Zachmann, K., Schlotterhose, P., Neifer, K., Schmidt, B., and Zimmermann, R. (1996) EMBO J. 15, 6931–6942
41. Chen, X., Easton, D., Oh, H. J., Lee-Yoon, D. S., Liu, X., and Subjeck, J. (1996) FEBS Lett. 390, 68–72
42. Craven, R. A., Tyson, J. R., and Stirling, C. J. (1997) Trends Cell Biol. 7, 277–282
43. Rost, B., Sander, C., and Schneider, R. (1994) Comput. Appl. Biosci. 10, 53–60
44. Rost, B., and Sander, C. (1994) Proteins 19, 55–72
45. Depiereux, E., Baudoux, G., Briffleul, P., Reginsier, I., De Bolle, X., Vinals, C., and Feytmans, E. (1997) Comput. Appl. Biosci. 13, 249–256