

Characterization of native interaction of hsp110 with hsp25 and hsc70

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Abstract The 110 kDa heat shock protein (HSP) (hsp110) has been shown to be a diverged subgroup of the hsp70 family and is one of the major HSPs in mammalian cells [1,2]. In examining the native interactions of hsp110, we observed that it is found to reside in a large molecular complex. Immunoblot analysis and co-immunoprecipitation studies identified two other HSPs as components of this complex, hsc70 and hsp25. When examined *in vitro*, purified hsp25, hsp70 and hsp110 were observed to spontaneously form a large complex and to directly interact with one another. When luciferase was added to this *in vitro* system, it was observed to migrate into this chaperone complex following heat shock. Examination of two deletion mutants of hsp110 demonstrated that its peptide-binding domain is required for interaction with hsp25, but not with hsc70. The potential function of the hsp110-hsc70-hsp25 complex is discussed.

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Key words: Heat shock protein; Chaperone complex; Luciferase; Peptide-binding domain

1. Introduction

Heat shock proteins (HSPs) are a number of conserved proteins that can be induced in all organisms upon the exposure to stress conditions like high temperature. Many of them also function as molecular chaperones that prevent irreversible aggregation and assist protein folding or assembly. HSPs are divided into several major families based on their size and structure [3–6], the most well known being hsp110, hsp90, hsp70, hsp60/GroEL, hsp40/DnaJ and the small HSPs (sHSP, e.g. hsp25). All of these HSPs, except hsp110, have been extensively studied and their functions in cellular processes are broadly recognized today. Although the cloning of the hsp110 from hamster, mouse, yeast, *Arabidopsis*, fungi and a variety of other species has been recently described [7–14], the cellular functions of hsp110 are still not understood. Studies in our laboratory have been focusing on this large stress protein. We have previously shown that hsp110 is a significantly enlarged relative of hsp70 family, which also contains unique sequence elements not present in the hsp70s. We have also shown that hsp110 inhibits the aggregation of heat-denatured proteins in a highly efficient manner, sustains denatured proteins in a folding competent state, and confers thermotolerance when overexpressed *in vivo* [2]. In order to better understand this major stress protein in mammalian

cells, its native interactions were investigated. We describe here studies indicating that hsp110 interacts *in vivo* and *in vitro* with two other major stress proteins, hsc70 and hsp25, and define the domains of hsp110 involved.

2. Materials and methods

2.1. Reagents

The rabbit anti-hsp110 antibody has been characterized previously [1]. Affinity-purified mouse anti-hsc70 monoclonal antibody, rabbit anti-murine hsp25 antibody, rat anti-hsp90 antibody and rat anti-TCP-1a monoclonal antibody, as well as recombinant hsc70 and murine hsp25, were all obtained from Stressgen Biotechnological Corp (Victoria, Canada). Anti-His Antibody was purchased from Amersham. Colon 26 tumor cells were cultured in DMEM supplemented with 10% calf serum in a 5% CO₂ incubator.

2.2. Plasmid construction and expression

Purification of recombinant His-tagged hsp110 and two deletion mutants used here has been described elsewhere [2,15]. Briefly, for the construction of hsp110 mutants, primers 5'-GCTAGAG-GATCCTGTGCATTGCAGTGTGCAATT-/-CAGCGCAAGCT-TACTAGTCCAGGTCCATATTGA-3' (mutant #1, amino acids 375–858) and 5'-GACGACGGATCCTCTGTCTCGAGGCGAGCAT-GGA-/-CAGCGCAAGCTTACTAGTCCAGGTCCATATTGA-3' (mutant #2, amino acids 508–858) were used in the polymerase chain reaction (PCR). The PCR products were cloned into pRSETA vector (Invitrogen), and a His₆-(enterokinase recognition sequence) and additional Asp-Arg-Trp-Gly-Ser (for mutant #1) or Asp-Arg-Trp (for mutant #2) were added to the N-terminal of hsp110 mutants. Plasmids were transformed into *Escherichia coli* strain JM109 (DE3) and expression products were purified by Ni₂-nitrilotriacetic acid-agarose column (QIAGEN). The protein concentration was measured using the Bio-Rad protein assay kit.

2.3. Purification of native hsp110

Cells were washed with phosphate-buffered saline (PBS) and homogenized with a Teflon homogenizer with five volumes of buffer (30 mM NaHCO₃, pH 7.5, 1 mM phenylmethylsulfonyl fluoride). The homogenates were centrifuged for 20 min at 12000×g, supernatants were further centrifuged for 2 h at 100000×g. Cell extracts were first applied to Con-A-Sepharose column, unbound proteins were collected and loaded on ion exchange column (Mono Q, Pharmacia) equilibrated with 20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.1 mM dithiothreitol (DTT). Bound proteins were eluted with a linear salt gradient (200 mM ~ 350 mM NaCl). hsp110 pooled fractions were concentrated using centricon 30 (Amicon) and applied to size exclusion column (Superose 6, Pharmacia) for high performance chromatography equilibrated with 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM DTT, then eluted with a flow rate of 0.2 ml/min. Thyroglobulin (669 kDa), ferritin (440 kDa), catalase (158 kDa), albumin (67 kDa) and ovalbumin (43 kDa) were used as protein markers.

2.4. Western blot analysis

Cells were washed with PBS and lysed in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100 and protease inhibitors. After incubation on ice for 30 min, cell extracts were boiled with an equal volume of sodium dodecyl sulfate (SDS) sample buffer

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(50 mM Tris-HCl, pH 6.8, 5% β -mercaptoethanol, 2% SDS, 10% glycerol) for 10 min and centrifuged at $10000\times g$ for 20 min. Equivalent protein samples were subjected to 7.5–10% SDS-polyacrylamide gel electrophoresis (PAGE) and electrotransferred onto Immobilon-P membrane (Millipore, UK). Membranes were blocked with 5% non-fat milk in TBST (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 0.05% Tween-20) for 1 h at room temperature, and then incubated for 2 h with primary antibodies diluted 1:1000 in TBST. After washing, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG diluted 1:2000 in TBST. Immunoreactivity was detected using the enhanced chemiluminescence detection system [16,17] (Amersham, Arlington Heights, IL, USA).

2.5. Immunoprecipitation

Immunoprecipitation was performed as previously described [18,19]. In brief, cells were washed three times with cold PBS and lysed in buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP40, 10 μ g/ml leupeptin, 25 μ g/ml aprotinin, 1 mM ABESF, 0.025% NaN_3). The lysates were centrifuged and supernatant was presorbed with 0.05 volume pre-immune serum together with 30 ml protein A beads for 1 h. The lysates were incubated overnight at 4°C with hsp110 antibody (1:100) or hsc70 antibody (1:200) or hsp25 antibody (1:100). For in vitro analysis of interaction within chaperones, recombinant wild-type hsp110 and hsp110 mutants were first incubated with hsc70 or hsp25 at 30°C. Then, hsc70 antibody or hsp25 antibody were added and further incubated overnight at 4°C. Immune complex was precipitated with protein A-agarose (30 μ l) for 2 h. Precipitates were washed three times with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40, 30–40 μ l SDS sample buffer was added and boiled for 5 min. Supernatants were loaded to 7.5–12% SDS-PAGE and analyzed by immunoblotting.

2.6. Interaction between luciferase and HSPs

Luciferase (Boehringer Mannheim) was incubated with hsp110, hsc70 and hsp25 (150 nM each) in 25 mM HEPES, pH 7.9, 5 mM magnesium acetate, 50 mM KCl, 5 mM β -mercaptoethanol, and 1 mM ATP at room temperature or 43°C for 30 min. The solution was centrifuged at $16000\times g$ for 20 min, the supernatant was loaded on the Sephacryl S-300 column (Pharmacia) equilibrated with 20 mM Tris-HCl, pH 7.8, 150 mM NaCl and 2 mM DTT. The protein was eluted at a flow rate of 0.24 ml/min at 4°C. Fractions were collected and analyzed by Western blotting.

3. Results

3.1. Existence of hsp110 as a large complex containing hsc70 and hsp25

In order to investigate the physiological role of hsp110, our efforts have focused on the characterization of native hsp110 in Colon 26 cells. After cell extracts were applied to successive chromatography on Con-A-Sepharose and Mono Q columns, partially purified hsp110 fraction was loaded onto the Superose 6 size exclusion column (maximum resolution of 5000 kDa). It was observed that the Con-A and ion exchange-purified hsp110 fraction eluted from the Superose column in those fractions of size range between 200 and 700 kDa (Fig. 1A). Work was repeated using Sephacryl 300 (allyl dextran/bisacrylamide matrix) column and analysis provided similar data (data not shown).

Since hsp110 was eluted as one broad peak of high molecular mass, it is reasonable that this large in situ hsp110 complex might also contain additional components, potentially including other molecular chaperones and/or cellular substrates that may interact with hsp110. In order to investigate this possibility, we examined the purified hsp110 fraction derived from both ion exchange and size exclusion columns by immunoblotting for other HSPs using available antibodies. As shown in Fig. 1B, antibodies for hsp90, hsc70, T-complex polypeptide 1 (TCP-1) and hsp25 were used. All four proteins were readily detectable in the total cell lysate (lanes 1, 3, 5 and 7). When the hsp110 fraction was examined, TCP-1 and hsp90 were not observed (lanes 2 and 6). However, both hsc70 and hsp25 were found to co-purify with hsp110 with a significantly greater fraction of total cellular hsc70 present than of hsp25. Having found that hsc70 and hsp25 were also present, we determined their chromatographic profiles in the purified system (also shown in Fig. 1A).

To determine whether this co-purification also reflected an interaction between these three molecular chaperones, a recip-

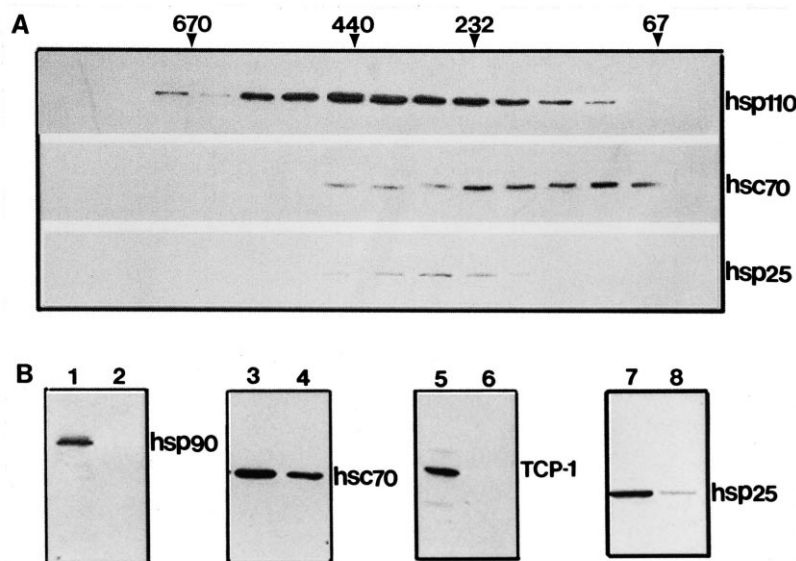


Fig. 1. Characterization of hsp110 complex. A: Chromatography profiles of native hsp110 separated by size exclusion column for FPLC. hsp110 was partially purified by successive chromatography on Con-A-Sepharose and Mono Q column. Pooled fraction was loaded on the Superose 6 column, proteins in each fraction were detected by immunoblotting with antibodies for hsp110, hsc70 and hsp25 (1:1000). B: Composition analysis of native hsp110 complex. Purified hsp110 fraction was detected by antibodies for hsp90 (lane 1, 2), hsc70 (lane 3, 4), TCP-1 (lane 5, 6) and hsp25 (lane 7, 8). Total cell extracts were also used as a positive control (lane 1, 3, 5 and 7).

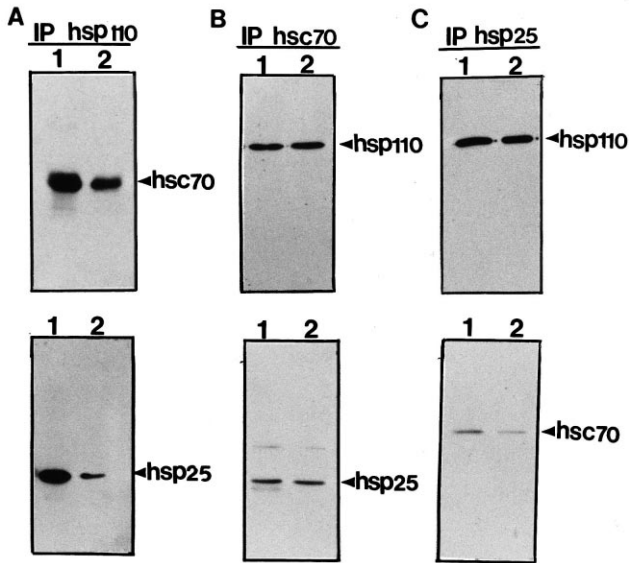


Fig. 2. Reciprocal immunoprecipitation between hsp110 and hsp25. Cell lysates (lane 2) were incubated with antibodies for hsp110 (1:100) (A), hsp70 (1:200) (B) and hsp25 (1:100) (C), protein A-Sepharose was added and further incubated at 4°C overnight, immunoprecipitates were examined by immunoblotting with hsp110, hsp70 and hsp25 antibodies. Total cell extracts were also used as a positive control (lane 1).

rocal co-immunoprecipitation analysis was conducted with Colon 26 cell extracts and hsp110 fractions. Hsc70 and hsp25 were shown to precipitate with hsp110 using an anti-hsp110 antibody (Fig. 2A). Conversely, hsp110 was co-precipitated by an anti-hsc70 antibody or anti-hsp25 antibody

(Fig. 2B,C, top). Pre-immune serum was also used to perform immunoprecipitation as a negative control with a correspondingly negative outcome (data not shown). Finally, interaction between hsc70 and hsp25 was analyzed by using antibodies for hsc70 and hsp25. Again, these two proteins were observed to co-immunoprecipitate with one (Fig. 2B,C, bottom). From the above study, we can conclude that hsp110, hsc70 and hsp25 interact in situ, either directly or indirectly.

3.2. Analysis of interaction of hsp110 with hsc70 and hsp25 in vitro

Next, we wished to determine whether hsp110, hsc70 and hsp25 interacted in vitro and whether they also were capable of forming a large molecular weight complex by using purified protein components. We then added luciferase as a potential substrate to this mixture since we have previously shown that hsp110 can solubilize this reporter protein following heat denaturation. Luciferase, with hsp110, hsc70 and hsp25 mix (at 1:1 molar ratio) were incubated at room temperature or at 43°C for 30 min. The soluble fractions were loaded onto a Sephacryl S-300 column, eluted fractions were run on SDS-PAGE and analyzed by immunoblotting with antibodies for hsp110, hsc70, hsp25 and luciferase. The results of this study are presented in Fig. 3. It was found that hsp110, hsc70 and hsp25 are again present in high molecule weight fractions, however, these fractions were eluted at a significantly larger molecular size than that seen in vivo (Fig. 3A). Moreover, it was seen that heat treatment does not change the elution pattern for hsp110, hsc70 or hsp25. However, luciferase, which does not co-elute with the hsp110 complex prior to heating (being present as a monomer), was observed to move into a high molecule weight structure after the heat

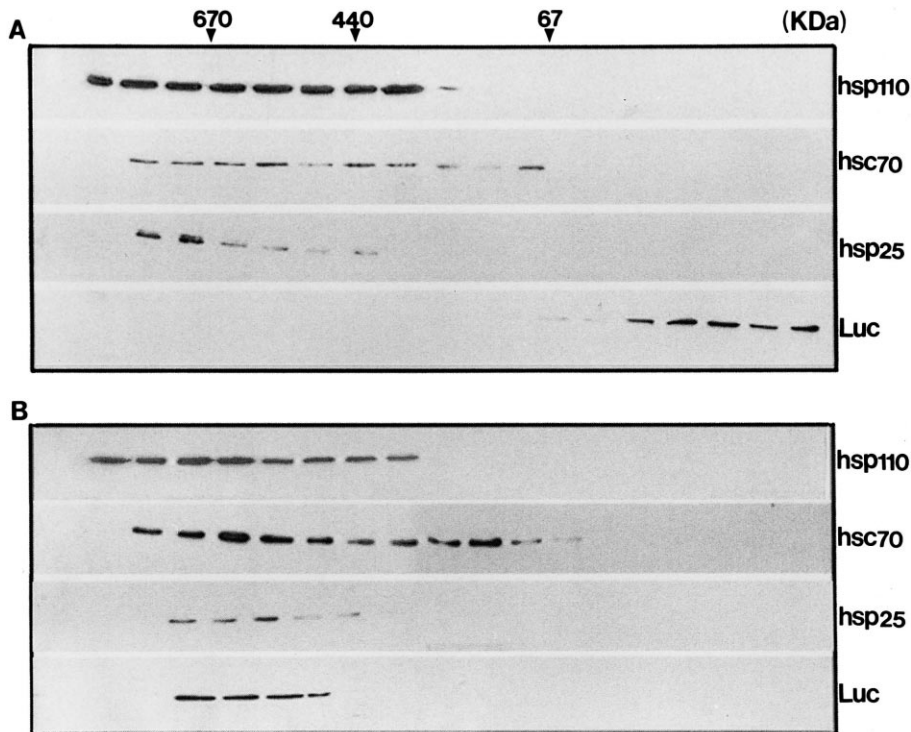


Fig. 3. Interaction between luciferase and HSPs complex. Luciferase and HSPs were incubated at room temperature (A) or 43°C (B) for 30 min and soluble fraction after centrifugation at 16 000×g was loaded on Sephacryl S-300 column. The eluted fractions were analyzed by immunoblotting with antibodies for HSPs and luciferase.

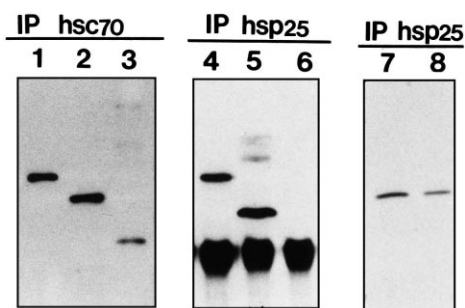


Fig. 4. Interaction analysis of hsp110 mutants and hsp70, hsp25 in vitro. *E. coli* expressed full-length hsp110 (lane 1, 4) and mutant #1 (lane 2, 5), mutant #2 (lane 3, 6) were incubated with hsc70 or hsp25 at 30°C for 1 h, then anti-hsc70 or anti-hsp25 antibodies were added. Immunoprecipitates were detected by anti-His antibody. In vitro interaction between hsc70 and hsp25 was also analyzed by the same method described above, hsc70 antibodies were used to test immunoprecipitate (lane 8). Total cell lysate was used as a positive control (lane 7). Equal amounts of protein (2 µg) for wild-type hsp110, hsp110 mutants, hsc70 and hsp25 were included in each assay.

exposure (Fig. 3B). Almost all of the luciferase was sustained in a soluble form in these experiments. When heated alone, luciferase became rapidly insoluble ([2] and data not shown). Heat shock did not affect the solubility of the three hsp110, hsc70 or hsp25.

The above data indicate that hsp110, hsc70 and hsp25 co-purify in a large molecular weight structure in vitro, as does luciferase (if present) after heating. This does not indicate how these proteins interact with themselves or that any two of them interact at all, although, that heated luciferase remains soluble is evidence for its interaction with at least one of the chaperones. Specifically, do both hsp110 and hsp25 bind to hsc70 but not to one another or can hsp110 and hsp25 interact on their own, etc.? To determine how these proteins interact, we again performed co-immunoprecipitation experiments using the pairs of purified proteins. Hsc70 and hsp110 were found to interact in the absence of hsp25 (Fig. 4, lane 1) and correspondingly hsp110 was observed to precipitate with hsp25 alone, in the absence of hsc70 (lane 4). Lastly, hsc70 and hsp25 also co-precipitate in the absence of hsp110 (lane 8).

Finally, we extended this in vitro study defining the interactions between hsp110, hsc70 and hsp25 by examining two deletion mutants of hsp110 which have previously been shown to represent the most simplistic (i.e. functional and non-functional) forms of this chaperone [15]. The first mutant examined (#1) lacks the N-terminal ATP-binding domain of hsp110, but contains the remaining sequence: i.e. the adjacent β sheet peptide-binding domain and other C-terminal sequences (size: 75 kDa and containing amino acids 375–858). This mutant has been shown to be fully functional in its ability to stabilize heat-denatured luciferase in a folding competent state. The second mutant used here (#2) again lacked the ATP-binding domain as well as the adjacent β sheet (peptide-binding) domain, but contained the remaining C-terminal sequence (size: 62 kDa and containing amino acids 508–858). This mutant has recently been shown to be incapable of performing the chaperoning function of sustaining heat-denatured luciferase in a soluble state. Mutant #1 (no ATP-binding domain) was observed to co-precipitate with both hsp70

(lane 2) and hsp25 (lane 5), indicating that these interactions do not involve its ATP-binding domain. However, mutant #2 (lacking both the ATP region and the peptide-binding region of hsp110) was observed to only associate with hsp70 (lane 3). This indicates that hsp25 and hsp70 can interact with hsp110 at different sites and that the association of hsp110 with hsp25 requires the peptide-binding domain of hsp110.

4. Discussion

The present study describes investigations into the native interactions of hsp110 in Colon 26 cells. We have found that hsp110 co-purifies with both hsc70 and hsp25 and further, that the three proteins can be co-immunoprecipitated. To determine that the co-immunoprecipitation results can reflect direct interactions between these chaperones and to also define these interactions, in vitro studies using purified hsp110, hsc70 and hsp25 were undertaken. It was found that these three chaperones also spontaneously form a large molecular complex in vitro. Moreover, this complex forms in the absence of an added substrate, but substrate (luciferase) can be induced to migrate into the complex by a heat stress. It is also shown that each pair of these proteins can interact directly, i.e. hsc70 with hsp110, hsc70 with hsp25, and hsp110 with hsp25. This, together with the co-precipitation data obtained from cell lysates, strongly argues that these interactions naturally occur in situ. Moreover, use of two deletion mutants of hsp110 demonstrates that its peptide-binding domain is required for hsp25-binding, but not for hsc70-binding and that its ATP-binding domain is not required for the interaction with either hsc70 or hsp25. This suggests that hsp110 may bind to hsp25 through its peptide-binding domain. That hsc70-hsp110-binding occurs in the absence of the hsp110 peptide-binding domain suggests that hsc70 may be actively binding to hsp110 through its (i.e. hsc70's) peptide-binding domain, but does not exclude the possibility that the two proteins interact via the involvement of other C-terminal domains.

These interactions between hsp110 and hsc70 raise questions as to how these proteins may function cooperatively. Since the peptide-binding domain of hsc70 and hsp110 appears to represent the 'business end' of these chaperones in performing chaperoning functions, it would be anticipated that their peptide-binding domains would be actively associated with substrate and not one another. This raises the possibility that this complex represents a chaperone 'storage compartment' which awaits cellular requirements. However, the migration of heat-denatured luciferase into this fraction following heat shock argues for an active chaperoning activity of the complex itself. It is possible that hsc70 may piggy-back hsp110 in a manner which allows transfer of substrate from hsp110 to hsc70 with subsequent folding in conjunction with DnaJ homologs and other chaperones. hsp110 has not yet been shown to have a folding function in conjunction with DnaJ co-chaperones, as is the case with hsc70 [2,15]. However, hsp110 exhibits different ATP-binding properties than do the hsp70s [15] and possible co-chaperones of hsp110 may be awaiting discovery. Previous in vitro studies have demonstrated that while sHSPs (e.g. hsp25) bind non-native protein [20–23], refolding still requires the presence of hsp70 [24]. Perhaps, hsp110 and sHSPs may act in the differential binding of a broad variety of substrates for subsequent shut-

ting to hsp70-DnaJ containing chaperone machines. Studies indicate, however, that an *in vitro* hsp110-hsc70-hsp25 complex (at a 1:1:1 molar ratio) is slightly less effective than is hsp110 alone in inhibiting luciferase aggregation following heat shock and further studies of this nature are necessary to determine or exclude a direct chaperoning function (Wang et al., data not shown).

That these three chaperones interact may represent a general phenomenon. Plesofsky-Vig and Brambl have recently shown that the small HSP of *Neurospora crassa*, called hsp30, binds to two cellular proteins, hsp70 and hsp88. Cloning and analysis of hsp88 have shown that it represents the hsp110 of *N. crassa* [25], suggesting that the interactions described here are phylogenetically conserved. In addition, Hatayama has described an interaction between hsp110 (referred to as hsp105) and hsp70 in FM3A cells [26]. The size of the hsp110 complex and the interaction with hsc70 observed in the present study (which also employed the added step of ion exchange chromatography) are clearly similar to, and in excellent agreement with this recent report. That hsp110 and hsc70 interact is also suggested by earlier studies from our laboratory which have demonstrated that hsp110 and hsc70 can interact and are functional in the folding heat-denatured luciferase [2]. Finally, it is noteworthy that hsp90 and TCP-1 were not observed in the hsp110 complex in the present study, despite its previously identified association with hsc70 and other proteins in the steroid hormone receptor [27–31]. However, it has recently been shown that SSE1 encoding a yeast member of the hsp110 family is required for the function of glucocorticoid receptor and physically associates with the hsp90 [32].

Several important questions are raised by the observations described here. For example, does this complex offer an enhanced capacity to hold a greater variety of substrate proteins in a folding competent state and/or to do so more efficiently, and is there an enhanced ability gained to refold denatured proteins in the presence of additional chaperones? Further studies are needed to define the function(s) of this hsp110-hsc70-hsp25 complex and how these chaperones interact with one another in the processing of substrate.

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