Proteins interacting with the molecular chaperone hsp70/hsc70: physical associations and effects on refolding activity

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Abstract We investigated several hsp70/hsc70 interacting proteins and established by two independent techniques that hsp40 and Hop/p60 specifically interact with the 257 residue carboxy-terminal domain of hsp70 while Hap-46 and Hip/p48 bind the 383 residue amino-terminal ATP binding domain. Hap-46 and Hip/p48 competed for binding to hsc70, while Hap-46 had no effect on the binding of either Hop/p60 or hsp40 to hsc70. Hap-46 inhibited the refolding of thermally denatured firefly luciferase in an hsc70 and hsp40 dependent assay, and this effect was largely compensated by Hop/p60. These interacting proteins thus appear to cooperate in affecting the chaperoning activity of hsp70/hsc70.

Key words: Domain structure; hsp70/hsc70 molecular chaperone; Protein-protein interaction; Protein refolding

1. Introduction

The processes by which polypeptides and complex protein structures attain their native and biologically active conformations are of major importance in the field of protein biochemistry. Although such folding may occur spontaneously, it is in many cases assisted by proteins with chaperoning activity. Heat shock proteins of the 70 kDa protein family are certainly major chaperones involved in such ATP dependent folding reactions in eukaryotic cells (reviews: [1–3]), most notably the constitutively expressed heat shock cognate form hsc70. They have a distinct domain structure with an amino-terminally located ATP binding domain and a carboxy-terminal domain that has affinity for misfolded or aberrant proteins and polypeptides (Fig. 1).

Although hsp70/hsc70 by itself may exert some protective effects on denatured or misfolded proteins in that it is able to bind to them, it normally acts in concert with other cellular components which themselves associate with hsc70. In this respect, hsp40 and other members of the DnaJ-like protein family are of prime importance as cochaperones [4]. Recently, other hsp70/hsc70 interacting proteins have been identified: Hip/p48 was obtained as 'hsc70 interacting protein' and has an apparent molecular weight of 48 kDa [5,6]. Hop/p60 has been defined as 'hsp70/hsp90 organizing protein' [7] and was first described as a transformation sensitive human protein [8] and as the stress related protein p60 [9] with a molecular weight of roughly 60 kDa. Hap-46 is the acronym for 'hsp70/hsc70 associating protein'; it was originally identified as the nuclear receptor associating protein RAP46 [10] of apparent molecular weight 46 kDa. This protein has now been found to directly associate with hsp70 and several other members of the 70 kDa heat shock protein family [11]. Interaction occurs with the amino-terminal ATP binding domain of hsc70 [11], similar to that of Hip/p48 [5].

In the present investigation we further studied the binding of these proteins to hsp70/hsc70 and specific fragments thereof by use of in vitro assays and in vivo in yeast. We also examined the combined effects of these proteins in a protein refolding system using thermally denatured firefly luciferase as model protein.

2. Materials and methods

2.1. Expression of tagged proteins

The fusion protein of glutathione *S*-transferase (GST) with Hap-46 and His tagged human hsp40 were as before [10–12]. His tagged fragments of human hsp70 [13] were generated by PCR. For fragments N_1-hsp70 (codons 1–187), N_2-hsp70 (188–383), and C-hsp70 (384–640) primer pairs 5′-aattggatccgcatggccaaagccg-3′/5′-aattaagctttctgtccaggccgta-3′, 5′-aattggatccggcctggacagaacg-3′/5′-aattaagcttgctccccatcaggat-3′, and 5′-aattggatccgggacaagtccgaga-3′/5′-aattaagcttggcccctaatctacc-3′ were used. All sequences were verified and cloned into *Bam*HI/*Hin*dIII sites of pQE-30 and pQE-32 (Qiagen), resulting in plasmids pQE-32-N_1-hsp70, pQE-30-N_2-hsp70 and pQE-30-C-hsp70. Expression in *E. coli* JM109 and purification was as before [11]. Amino-terminally His tagged Hip/p48 and carboxy-terminally His tagged human Hop/p60 were expressed from plasmids pET-28a-Hip and pET-28a-Hop, respectively, in *Escherichia coli* BL21(DE3) [5] and purified on Talon resin (Clontech); after extensive washing with 1% Triton X-100, elution was with 100 mM imidazole.

2.2. In vitro protein interaction experiments

Proteins were analyzed by standard 10% or 12% SDS-PAGE and transferred to Immobilon-P membranes (Millipore) as before [10]. Hsc70 was detected by antibody N27F3-4 (Stressgen) and His tagged hsp70 fragments by antibody ^{MRGS}His (Qiagen). Incubation with peroxidase conjugated second antibodies and detection with ECL (Amersham) were by standard procedures.

Far-Western blotting was as described [14] with the GST and hsp40 specific antibodies Z-5 and C-20 (Santa Cruz) and Hop/p60 specific antibody SRA-1500 (Stressgen).

Co-immunoprecipitations of bovine hsc70 (Stressgen) were carried out as before [11] with the above antibodies and Hip/p48 specific antiserum R-19 (Santa Cruz).

2.3. Yeast two hybrid experiments

Plasmids pGBT-Hsc70 and pGBT-N-Hsc70 containing codons 1–646 and 1–383 of rat hsc70 were as described [11]. The above plasmids pQE-30-N_2-hsp70 and pQE-30-C-hsp70 were cut with *Eco*RI and *Hin*dIII, and PQE-32-N_1-hsp70 with *Bam*HI and *Hin*dIII. The respective hsp70 cDNA fragments were converted at their 3′-ends into blunt ends by standard techniques [15] and cloned into *Eco*RI/*Sma*I or *Bam*HI/*Pst*I sites of pGBT9 in frame with GAL4BD [16], with the *Pst*I site converted into blunt ends. This resulted in plasmids pGBT-N_1-hsp70, pGBT-N_2-hsp70, and pGBT-C-hsp70, respectively.

Human Hip/p48 (codons 1–369) and Hop/p60 (only codons 1–446) cDNAs were ligated into the *Sma*I site of pGAD424 [16] in frame with GAL4AD as described before for Hap-46 [11]. Human hsp40

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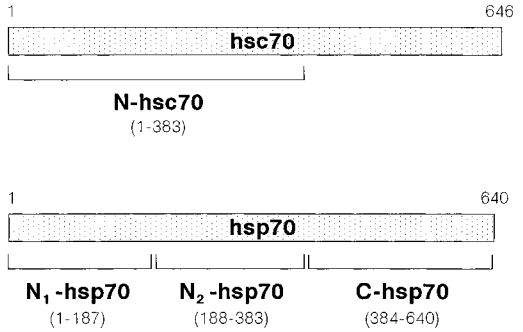

Fig. 1. Domain structure of hsp70 and hsc70. Human hsp70 used here contains a total of 640 amino acids, while bovine hsc70 is of 646 residues. Both have the same domain structure.

cDNA was cloned into *Eco*RI/*Pst*I sites of pGAD424. This resulted in plasmids pGAD-Hip, pGAD-Hop and pGAD-hsp40.

Cells of strain HF7c (Clontech) were cotransformed as before [11] with plasmids pGAD-RAP, pGAD-Hip, pGAD-Hop and pGAD-hsp40 and either pGBT-Hsc70, pGBT-N-Hsc70, pGBT-N_1-hsp70, pGBT-N_2-hsp70, pGBT-C-hsp70 or pGBT9. Growth on plates in medium lacking His, Leu and Trp was scored after 5 days at 30°C.

3. Results

3.1. In vitro protein interactions

To investigate the interaction of hsp40, Hap-46, Hip/p48, and Hop/p60 with either complete hsc70 or domains of hsp70 we used far-Western blotting. Specific fragments of human hsp70 were bacterially expressed as His tagged polypeptides, purified, and run together with full length bovine hsc70 on SDS polyacrylamide gels. After blotting, proteins were renatured on the membrane using a standard protocol [14] and membranes were then incubated with the above proteins. Interactions were detected by use of specific antisera directed against these interacting proteins. As Hap-46 and Hip/p48 are known to interact with the amino-terminal 44 kDa ATP binding domain of hsc70 [5,11], we wondered whether hsp40 and Hop/p60 also bind to this domain. However, Fig. 2 shows that both proteins rather interact with the 257 amino acid carboxy-terminal portion of hsp70. The same result was obtained when we used co-immunoprecipitation with antisera specific for hsp40 and Hop/p60: the carboxy-terminal (residues 384–640), but not the amino-terminal (residues 1–383) domain of hsc70 was specifically retained (data not shown).

According to X-ray studies, the ATP binding domain of hsp70s and several other ATP binding proteins consists mainly of two subdomains N_1 and N_2 (cf. Fig. 1) separated by a deep cleft [17–19]. We therefore also checked constructs equivalent to these subdomains for interactions, i.e. polypeptides containing amino acids 1–187 and 188–383 of human hsp70. The experiments of Fig. 2 show that Hap-46 indeed binds to both fragments, albeit less avidly than to the entire ATP binding domain or to full length hsp70. This may suggest that Hap-46 upon binding possibly bridges the cleft between these hsp70 subdomains. As expected from the above results, both Hsp40 and Hop/p60 did not bind to subdomains N_1 and N_2 (Fig. 2).

Next we were interested in finding out whether some of the hsc70 interacting proteins hsp40, Hap-46, Hip/p48, and Hop/p60 might interfere with each other's binding to hsc70. We used immunoprecipitation with antibodies specific for the respective proteins and determined by Western blotting the relative amounts of hsc70 coprecipitated in the presence and absence of Hap-46. We found that Hap-46 competes for binding of Hip/p48 to hsc70, as one might expect for proteins which both interact with the ATP binding domain (Fig. 3A). The amount of hsc70 retained on the Hip/p48 specific

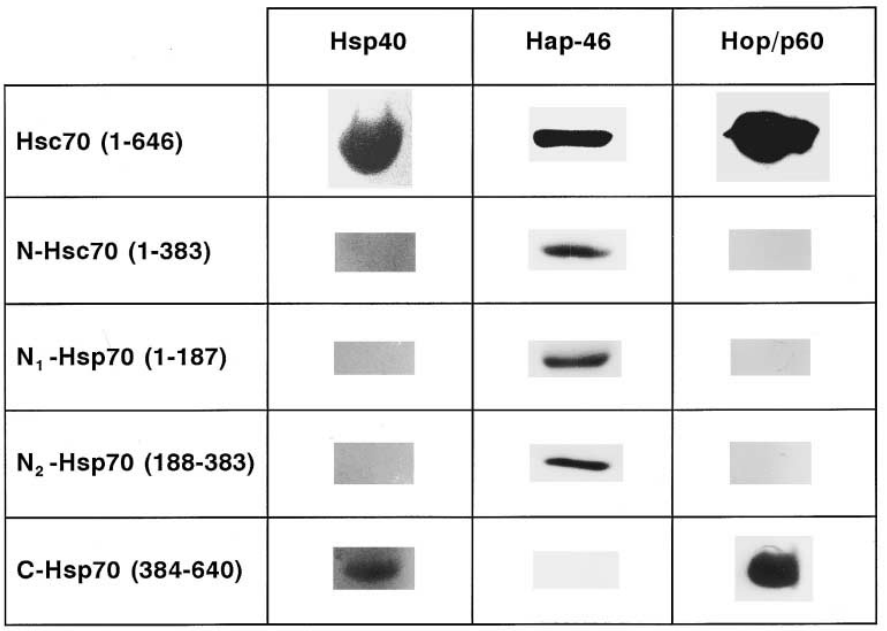

Fig. 2. Far-Western blots of hsp70/hsc70 with interacting proteins. Bovine hsc70 and the 44 kDa chymotryptic fragment thereof (N-Hsc70) were used. His tagged fragments N_1-Hsp70, N_2-Hsp70, and C-Hsp70 are described in Section 2 and have apparent molecular weights of 25, 26, and 33 kDa, respectively, in SDS-PAGE. After electrophoresis (roughly 2 μg each), these proteins were blotted and renatured [14]. Blots were incubated with His tagged hsp40 and Hop/p60 or GST-Hap-46 (50 μg/ml each), as indicated, followed by specific antibodies, as described in Section 2. Only relevant portions of blots are depicted.

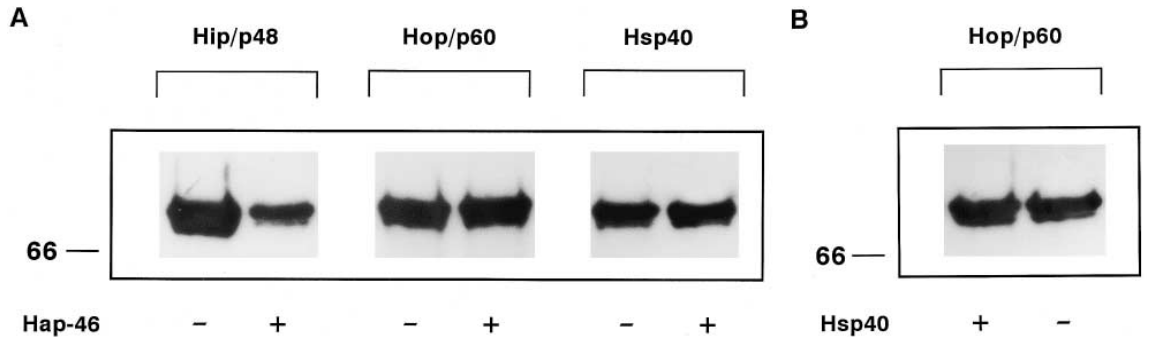

Fig. 3. Co-immunoprecipitation of hsc70 with interacting proteins. A: Bovine hsc70 (5 μg) was incubated for 16 h in the cold with hsp40, Hip/p48, or Hop/p60 (10 μg each) either in the presence or absence of Hap-46 (10 μg), as indicated, and the specific antibodies against hsp40, Hip/p48, or Hop/p60 (see Section 2), respectively, as well as protein G-Sepharose (Sigma); total volume 150 μl. ATP (5 mM) was included in each experiment. Analysis was by SDS-PAGE and immunoblotting with hsc70 specific antibody N27F3-4. B: Bovine hsc70 was similarly incubated with Hop/p60 and the Hop/p60 specific antibody in the presence or absence of hsp40 (10 μg each).

matrix was decreased 2–3-fold by Hap-46. On the other hand, Hap-46 showed no effect on the binding of hsp40 or Hop/p60 to hsc70 (Fig. 3A). Interestingly, hsp40 and Hop/p60 did not affect each other's binding to hsc70 with either Hop/p60 specifically bound to the matrix (Fig. 3B) or hsp40 bound correspondingly (data not shown).

3.2. *Protein interactions in yeast cells*

To ascertain whether the above described in vitro protein associations pertain to interactions within living cells, we turned to the yeast two hybrid system [16]. As outlined in Fig. 4, the cDNAs for hsp40, Hap-46, Hip/p48, and Hop/p60 were fused to the activating domain of the transcriptional activator protein GAL4 and tested against relevant constructs of mammalian hsp70/hsc70 fused to the DNA binding domain of GAL4. If protein associations occur, they will stimulate transcriptional activation of GAL4 responsive genes by putting both GAL4 domains in juxtaposition. In the cell strain HF7c used in our experiments, the yeast *HIS3* gene will then be turned on, resulting in growth in minimal medium.

As shown in Fig. 4 (lines 2 and 4), Hap-46 associated with the ATP binding domain of hsc70 and was now found to mainly interact with subdomain N_2 of the hsp70 ATP binding domain (residues 188–383). While Hip/p48 readily interacted in this assay system with the complete ATP binding domain (Fig. 4, line 2), it rather appeared to require both subdomains N_1 (residues 1–187) and N_2 (residues 188–383) for interaction. On the other hand, both Hop/p60 and hsp40 (Fig. 4, lines 1 and 5) associated only with full length hsp70/hsc70 and with the carboxy-terminal domain (residues 384–640). Control experiments showed that the DNA binding domain (BD) of GAL4 by itself does not interact with the activating domain (AD) fused to the various proteins (Fig. 4, line 6). The reverse control, GAL4AD tested against the GAL4BD-hsc70 constructs, was also negative (data not shown).

3.3. *Effects on refolding of heat denatured luciferase*

An important question relates to the effects of these interacting proteins on the chaperoning activity of hsp70/hsc70. To this end we used thermally denatured firefly luciferase as a model protein and a published refolding system that depends on hsc70, hsp40, ATP, and a minimal amount of reticulocyte lysate [12]. We had previously found that Hap-46 efficiently inhibits reactivation of the denatured enzyme in a dose dependent manner [11]. We now observed that the addition of Hip/p48 to this system interfered only slightly with the inhibition caused by Hap-46 (Fig. 5, column 4) and did not affect the level of reactivation if added by itself (column 3). Interestingly, Hop/p60 on its own significantly inhibited refolding (Fig. 5, column 5) and this inhibition was again not abolished

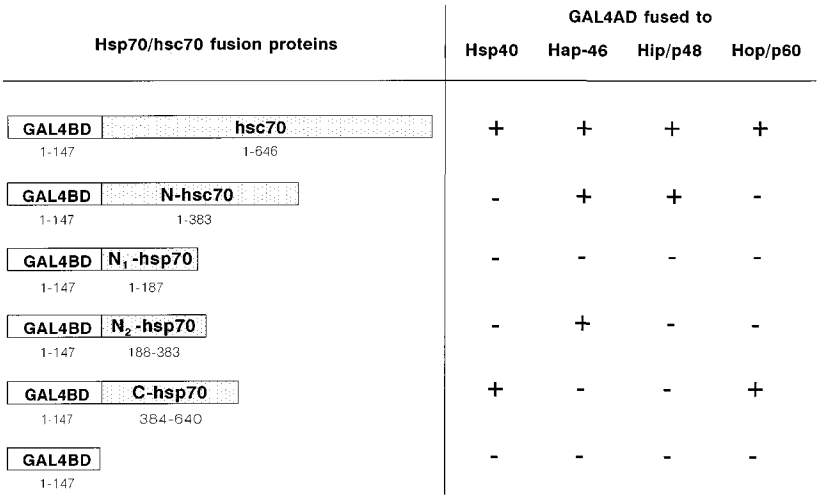

Fig. 4. In vivo interaction of hsp70/hsc70 with associating proteins. Constructs used in the yeast two hybrid system are described in Section 2. Growth in deficient medium was scored.

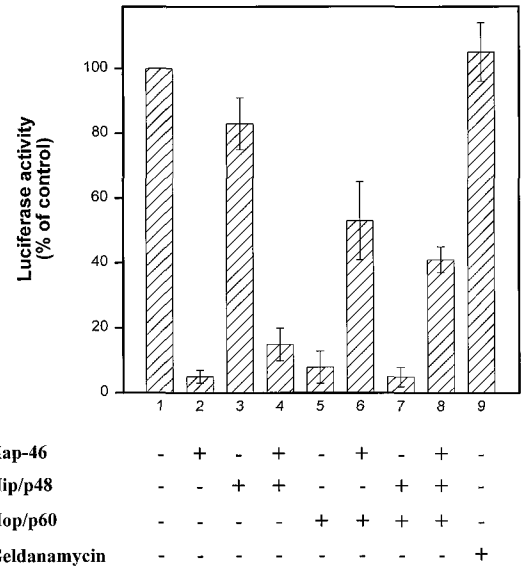

Fig. 5. Reactivation of denatured luciferase. Firefly luciferase (Sigma) was thermally denatured and reactivated as before [11,12] with purified hsc70 and hsp40 and 20-fold diluted, untreated rabbit reticulocyte lysate (Promega). In controls without further additions, set here as 100%, reactivation levels were roughly 40%. Other assays contained 10 μM concentrations of proteins, as indicated, or 40 μM geldanamycin. Routinely, experiments were in triplicate. The average of three independent experiments is shown with error bars indicating standard deviations.

by Hip/p48 (Fig. 5, column 7). By contrast, Hop/p60 was able to compensate the inhibitory action of Hap-46 on luciferase reactivation by roughly 50% (Fig. 5, column 6). Further addition of Hip/p48, however, did not further improve the refolding efficiency (Fig. 5, column 8).

As hsp40 is an essential component of this refolding system [11,12] we wondered whether the inhibitory effect of Hap-46 could be compensated by extra amounts of hsp40. However, this was not so in assays in which we added up to twice the amount of hsp40 as in the experiments of Fig. 5 (data not shown).

We also asked whether hsp90 is involved in this refolding system as this heat shock protein is certainly contained in the reticulocyte lysate used in our experiments. We employed the hsp90 specific inhibitor geldanamycin [20,21] but did not observe any effect of this drug on the refolding of heat denatured luciferase (Fig. 5, column 9).

4. Discussion

The 274 amino acid protein Hap-46 of apparent molecular weight 46 kDa was originally detected as a steroid hormone receptor associating factor [10]. Recently it was observed to directly interact with hsc70 and selected members of the hsp70 family while the association with nuclear receptors and a variety of other cellular regulators rather depends on hsp70s as mediators of interaction [11]. In the presence of ATP, Hap-46 dramatically reduced the association of several unrelated, but misfolded proteins to hsc70 and significantly inhibited the refolding of thermally denatured luciferase [11]. A related protein of mouse origin, called BAG-1, has also been obtained [22]; it contains only 219 amino acids and is of roughly 27 kDa size [23]. Despite significant divergence in the amino-terminal region, this protein also interacts with hsp70 family members and similarly affects their chaperone function [24]. We now suggest the introduction of a uniform nomenclature and propose that BAG-1 should be called Hap-27. However, it appears possible that still other related polypeptides exist which nevertheless may serve somewhat divergent cellular functions.

We previously found that Hap-46 specifically interacts with the ATP binding domain of hsc70 [11]; so does Hip/p48 [5]. We now show that Hap-46 significantly competes with Hip/p48 for binding to hsc70 (cf. Fig. 3A). Nevertheless, Hap-46 and Hip/p48 are very different proteins, both in amino acid sequence and in structural organization. While Hap-46 does not self-associate [11], Hip/p48 is a homo-oligomer [25]. On the other hand, Hip/p48 and Hop/p60 do contain some sequence similarities, in particular tetratricopeptide repeats which are thought to be involved in protein-protein interactions [2].

In the present study we further used defined fragments of hsp70/hsc70 for interaction experiments. Employing the far-Western technique we observed that Hap-46 readily binds to both subdomains N_1 (residues 1–187) and N_2 (residues 188–383) of the ATP binding domain (Fig. 2). However, in intact yeast cells we saw interaction only with subdomain N_2 (cf. Fig. 4). Although the yeast two hybrid system is known for its tendency to produce false positives [26], we suspect that this difference is due to inadequate folding of the N_1 subdomain within yeast. Alternatively, the GAL4BD fusion partner might block the interaction of the N_1 subdomain with Hap-46. Interestingly, neither of these subdomains N_1 and N_2 interacted with Hip/p48 in our yeast experiments (Fig. 4). This is not surprising in view of the fact that hsc70 has been reported to require ADP for efficient binding of Hip/p48 [25] but cleavage of the ATP binding domain into two separate portions certainly results in loss of nucleotide binding ability.

With hsp40 we found in far-Western blots and in the two hybrid system that it clearly interacts with the carboxy-terminal portion of hsp70 (cf. Figs. 2 and 4). This is consistent with the finding that the very carboxy-terminus of hsp70 is involved in the interaction with HDJ-1 [27], like hsp40 a member of the DnaJ-like protein family [4]. Our observation is also reminiscent of the interaction between DnaK and DnaJ proteins, the prokaryotic homologs of hsp70 and hsp40, respectively. Deletion of the carboxy-terminal 94 amino acids of DnaK abolished the recruitment of DnaJ [28].

A protein equivalent to human Hop/p60 [7] and termed RF-hsp70 (recycling factor for hsp70) has been purified from rabbit reticulocyte lysate [29,30]. It is thought to bind near the ATP binding site of hsp70 [29], i.e. within the amino-terminal portion. By contrast, our experiments unequivocally establish that Hop/p60 directly interacts with the carboxy-terminal domain of hsp70 rather than the ATP binding domain (cf. Figs. 2 and 4). Nevertheless Hop/p60 affects the chaperoning activity and this finding suggests interdomain communication within hsp70 molecules, as has been observed before [27,31,32] and is further discussed below.

The potential roles of the hsp70/hsc70 partner proteins Hip/p48 and Hop/p60 in the hsc70/ATPase reaction cycle have recently been discussed [2]. In essence, this cycle involves repeated binding and release of polypeptides to be folded with concomitant ATP hydrolysis and nucleotide exchange on the chaperone. With the data presented above, Hap-46 now also

needs to be taken into account as an inhibitor of hsp70/hsc70 in protein folding reactions, although the mechanistic details of this inhibition still need to be elucidated. On the other hand, the physiological functions of Hip/p48 and Hop/p60 are also far from being clear. Although RF-hsp70, the homolog of Hop/p60, has been reported to promote nucleotide exchange on hsp70 [29], we observed no significant enhancement in experiments with hsc70 and Hop/p60 (unpublished data). Hip/p48 has been found to stimulate the refolding of luciferase denatured by guanidinium-HCl [5] but had no effect on the reactivation of the same model protein after heat treatment (cf. Fig. 5, column 3). Moreover Hip/p48 may be substituted by hsp40 in protein folding reactions [12]. Refolding of thermally damaged rather than chemically denatured proteins is certainly of more relevance to what happens in intact cells. We suspect that Hap-46 may play a role in the decision on whether, upon stress conditions, misfolded proteins are refolded within cells or rather degraded [11,33]. Further experiments are required along these lines.

The X-ray structure of the carboxy-terminal domain of bacterial DnaK again shows two subdomains [34]. One of these, consisting of a sandwich of β strands, binds polypeptides in their extended configuration, while the other is built up of five α helices and may contact the ATP binding domain at the other end of the primary structure [34]. Because of homologies in sequences, it is inferred that the basic structure of eukaryotic hsp70s is quite similar. This then provides an explanation for our observation that the inhibitory effect of Hap-46 on protein folding can be compensated by Hop/p60 (cf. Fig. 5) even though these hsc70 binding proteins interact with distinct domains (cf. Figs. 2 and 4) and do not compete for binding to hsc70 (cf. Fig. 3A). We suppose that by way of this contact between the α helical portion at the carboxy-terminus of hsc70 and the ATP binding domain molecular communication may occur between Hop/p60 and Hap-46. Similar considerations apply to Hap-46 and hsp40, which also interacts with the carboxy-terminal domain of hsc70 (cf. Figs. 2 and 4).

The data presented here extend the view that various cellular proteins cooperate with hsp70/hsc70. Also, cooperation between the chaperoning systems of hsp70/hsc70 and hsp90 has recently been shown and discussed [3,35–37]. Interestingly, we did not obtain any evidence for the participation of hsp90 in our protein refolding experiments when we used the hsp90 specific inhibitor geldanamycin [20,21]. However, the refolding system employed here uses a minimal amount of reticulocyte lysate and hence contains a rather low concentration of hsp90, i.e. in the order of 75 nM. It thus appears that a variety of protein folding systems may exist simultaneously and function concomitantly.

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