Different combinations of the heat-shock cognate protein 70 (hsc70) C-terminal functional groups are utilized to interact with distinct tetratricopeptide repeat-containing proteins

Shin-Jen WU*, Fu-Hwa LIU*†, Su-Ming HU*† and Chung WANG*[1]

*Institute of Molecular Biology, Academia Sinica, 128 Section 2, Academy Road, Taipei, Taiwan, and †Graduate Institute of Life Sciences, National Defense Medical Center, 161 Section 6, Ming-Chuang East Road, Taipei, Taiwan

A group of tetratricopeptide repeat (TPR)-containing proteins has been shown to interact with the C-terminal domain of the 70 kDa heat-shock cognate protein (hsc70). In the present study, the effect of the TPR-containing proteins, including the C-terminus of hsc70-interacting protein (CHIP), TPR1 and human glutamine-rich TPR-containing protein (hSGT), on refolding of luciferase by DnaJ and hsc70 was investigated. These proteins inhibited the restoration of luciferase activity by the chaperones. The inhibitory effect exerted by TPR1 and hSGT depended upon their binding to hsc70. However, the interaction with hsc70 did not appear to be required for the inhibition of luciferase refolding by CHIP. We also demonstrate that the peptide, GPTIEEVD, corresponding to the C-terminal end of hsc70, abolished the association of [^{3}H]hsc70 with CHIP, TPR1 and hSGT. This implied that the GPTIEEVD motif of hsc70 was responsible for interacting with these TPR-containing proteins. However, the GGXP-repeats (where X is any aliphatic residue), another C-terminal conserved motif of vertebrate hsc70s, were not essential for interacting with the TPR-containing proteins. On the basis of mutagenesis studies, it was clear that a unique combination of the functional groups in the GPTIEEVD motif were utilized to interact with each TPR-containing protein, suggesting that inhibitors can be designed and used to elucidate the functional role of these interactions.

Key words: heat-shock proteins, luciferase refolding, molecular chaperones, protein–protein interaction, tetratricopeptide repeats.

INTRODUCTION

It has been documented that the 70 kDa heat-shock cognate protein (hsc70) is an ATP-dependent molecular chaperone [1,2] and has high affinities for both ATP and ADP [3,4]. In the ADP-bound state, hsc70 binds tightly to peptides or unfolded protein substrates, whereas it releases the substrate more readily in the ATP-bound state [5–8]. It is also clear that hsc70 has to interact with its partners to exert its chaperoning functions. These hsc70-interacting proteins have been shown to co-operate with hsc70 in carrying out a variety of cellular functions, including protein transport into organelles and refolding of denatured proteins [1,9,10]. Thus it is conceivable that the interacting partner may provide functional specificity for hsc70. The best characterized hsc70-interacting proteins are members of the ubiquitous DnaJ protein family [11,12]. DnaJ of bacterial origin and many of its eukaryotic homologues is composed of several domains. The N-terminal J-domain is responsible for stimulating the ATP hydrolytic activity of hsc70 [13–15], and interacting with the 44 kDa N-terminal ATPase domain of DnaK (hsc70 in *Escherichia coli*) [16]. The cysteine-rich domain and the C-terminal region of DnaJ are capable of binding peptide substrates [17,18]. Moreover, DnaJ homologues in vertebrates have been shown to interact with the 30 kDa C-terminal domain of hsc70 [19,20]. Thus different regions of DnaJ-like proteins may interact with distinctive domains of hsc70 to exert its co-chaperone function.

Several other proteins have been shown to interact with the N-terminal 44 kDa domain of hsc70, including the heat-shock protein (hsp) 70-interacting protein (Hip, also known as p48) [21], hsp70/hsc70-associated protein [Hap, also known as the 46 kDa glucocorticoid receptor-associated protein (Rap-46) or Bcl-2 binding athanogene (BAG-1)] [19,22,23] and the hsp binding protein (hspBP1) [24]. Hip is capable of stabilizing the ADP-bound conformation of hsc70 and increases the affinity of substrates for hsc70 [21]. Hap also affects the binding of substrates to hsc70 [23,25]. However, Hap has an additional DNA binding activity and is capable of stimulating transcription [26]. In a system containing hsc70 and hsp40 [also known as human DnaJ-like protein 1 (HDJ1)], hspBP1 decreases ATP hydrolysis and luciferase refolding [24]. In addition, the hsc70/hsp90-organizing protein (Hop, also known as p60) and the C-terminus of Hsc70-interacting protein (CHIP, also known as NY-CO-7) have been shown to interact with the C-terminal domain of hsc70 [19,27]. Hop is an essential component in steroid receptor assembly [28–30]. Hop as well as CHIP may also inhibit luciferase refolding [19,27,31,32]. Using yeast two-hybrid screening, we [33] recently identified several proteins, including human glutamine-rich tetratricopeptide repeat (TPR)-containing protein (hSGT), TPR1 and TPR2 that interact with the C-terminal domain of hsc70. TPR1 and TPR2, first identified by Murthy et al. [34], are also known as TTC1 and TTC2 [35] respectively. The common feature among these proteins is the presence of the TPR. Indeed, their TPR domains appear necessary and sufficient for interacting

Abbreviations used: hsc, heat-shock cognate protein; CHIP, C-terminus of hsc70-interacting protein; GST, glutathione S-transferase; hsp, heat-shock protein; Hap, hsp70/hsc70-associated protein; HDJ1, human DnaJ-like protein 1; Hip, hsp70-interacting protein; Hop, hsc70/hsp90-organizing protein; TPR, tetratricopeptide repeat; hSGT, human glutamine-rich TPR-containing protein; hspBP1, heat-shock protein binding protein 1.

[1] To whom correspondence should be addressed (e-mail mbcw@ccvax.sinica.edu.tw).

with hsc70 [33]. However, a different set of TPR-containing proteins was shown to interact with hsp90 [36]. Interestingly, the last four residues (EEVD) of hsc70 and hsp90 are identical and play an important role in interacting with the TPR-containing proteins [33,35].

It is generally believed that TPR motifs found in many different proteins are responsible for protein–protein interaction [37]. Each motif is composed of 34 amino acids and folds into a well-defined helix-turn-helix structure [35,37,38], although there are few identical residues in each of these 34 positions among different TPR motifs. In a given subset of TPR motifs however, a number of residues are highly conserved. These residues may provide the binding sites for the interacting partners. For example, it has been shown previously that the N- and C-terminal TPR domains of Hop interact with hsc70 and hsp90 [39] respectively, and at certain positions in these two domains are either lysine or arginine residues. Recently, the structures of these two TPR domains complexed with the C-terminal peptides of hsc70 and hsp90 have been determined by X-ray crystallography [35]. Indeed, some of the conserved basic amino acid residues in these two TPR domains interact with the acidic groups in the EEVD motifs. Moreover, from these crystal structures it was also evident that the residues preceding the EEVD motif, which differ between hsc70 and hsp90, also play roles in interacting with their respective TPR domains. Therefore, in the present study, we have investigated whether the same C-terminal sequence of hsc70 was used to interact with other TPR-containing proteins. Furthermore, we have investigated whether identical functional groups in this sequence were used to interact with the TPR-containing proteins. Here, we demonstrate that PTIEEVD at the C-terminus of hsc70 is indeed responsible for interacting with several TPR-containing proteins. However, different TPR domains interact with a distinctive combination of functional groups in the PTIEEVD motif.

MATERIALS AND METHODS

Materials

The peptides used in this study were purchased from SynPep Corporation (Dublin, CA, U.S.A.) and were further purified by HPLC. Bovine hsc70 and recombinant hsc70(ΔGGXP) were produced and purified as described by Wang and Lee [4]. CHIP, Hop, TPR1 and hSGT proteins fused with glutathione S-transferase (GST) were produced as described previously [33]. DnaJ was purchased from StressGen (Victoria, BC, Canada). [^{3}H]-$NaBH_4$ was obtained from NEN (Boston, MA, U.S.A.) and was used to label hsc70 by reductive methylation, as described by Wang and Lee [4].

Table 1 Nucleotide sequences of the primers used in this study

Primers	Nucleotide sequences
A	5′-CCCACCATTGAAGAGGTCGATTAA
B	5′-ACCAGCACTCTGGTACAGCTTGG
C	5′-GAGAAGCAGAGAGATAAG
D	5′-GAATTCAATCGACCTCTTCGCCTGAAGAAGCACC
E	5′-GAATTCTTAGTCGACCTCCACAAATGGTGGG
F	5′-GAATTCTTAGTCGACACATTCAATGGT
G	5′-GAATTCTTAGTCGACACAACAAATGGTGGG
H	5′-GAATTCAATCGACCTCTTCAATGGTGGCGCC
I	5′-GAATTCCCGAGAAGAGCCCGA
J	5′-CTCGCGTCAGAAGTTCAGCCGCTGCTCCT

Generation of the hsc70 deletion mutant lacking the GGXP-repeat

The plasmid containing the 10 kDa C-terminal subdomain of hsc70 in pBluescript vector [40] was linearized by digesting with *Sph*I and then used as a template for the PCR with primers A and B (Table 1). Primer A is identical to the coding sequence of the rat hsc70 cDNA from amino acid residues 640 to the stop codon, and primer B is complementary to the cDNA corresponding to residues 615–608. PCR was performed using KlenTaq polymerase (Clontech, Palo Alto, CA, U.S.A.). The products (approx. 3kb) were self-ligated and sequenced. Subsequently, the *Xho*I–*Eco*RI fragment of the insert was excised and ligated into the *Xho*I–*Eco*RI sites of the plasmid pHsc70-Ct [41]. The resulting plasmid, pHsc70(ΔGGXP), was used to express hsc70 without the C-terminal glycine-rich region, including the GGXP-repeats (where X is an aliphatic residue). The insert was also substituted for the equivalent fragment in the plasmid pCt-30/R [42] to generate the plasmid p30(ΔGGXP)/15b. The cDNA insert of p30(ΔGGXP)/15b was isolated with *Nde*I and *Bam*HI digestion and was used to replace the cDNA of hsc70 in pAS-70K [33]. The resultant plasmid, pAS-30(ΔGGXP), was used for the yeast two-hybrid assays.

Generation of the hsc70 mutant lacking the PTI tripeptide

The hsc70 mutant lacking the PTI tripeptide (amino acids 640–642 in hsc70) was generated using PCR. The reaction was carried out with primers C and D (Table 1) and pCt-30/R [42] as the template. Primer C is identical to the coding region of the cDNA from amino acids 530–535. Primer D, having an *Eco*RI site and stop codon at the 5′-end, is complementary to the coding sequence of the cDNA from the C-terminus to amino acid 635, except that the codons for PTI were deleted. The PCR products were cloned into pGEM-T (Promega, Madison, WI, U.S.A.) for sequencing. The insert was excised and ligated as described above into pHsc70-Ct for use in expressing hsc70 without PTI and into pCt-30/R to generate pAS-30(ΔPTI) for use in the yeast-two hybrid assay.

Generating the hsc70 mutants with amino acid substitutions

Four mutants of hsc70, ECVD (E644C), CEVD (E643C), CCVD (E644C, E643C) and ATI (P640A) were generated using PCR. In each case, pCt-30/R [42] was used as the template and primer C as one of the primers. The other primer for each reaction was E, F, G and H (Table 1) for CEVD, ECVD, CCVD and ATI respectively. These primers are complementary to the cDNA corresponding to the C-terminal region of hsc70 and contain the nucleotide substitution(s) for the amino acids specified. The PCR products were ligated into pGEM-T for sequence verification, and the inserts were separately cloned into pHsc70-Ct [41], pET-15b (Novagene, Madison, WI, U.S.A.) or pAS2-1 (Clontech) for expressing the proteins or for the yeast two-hybrid assays.

Inhibition of [^{3}H]hsc70 binding to TPR-containing proteins by peptides

The interaction of peptides GPTIEEVD, containing either a C-terminal carboxy (-COOH) or amide (-$CONH_2$) group, and GPTIEEVDG with the TPR-containing proteins was assessed by determining their ability to prevent [^{3}H]hsc70 from complexing with the proteins. Briefly, the TPR-containing proteins fused with GST (10 μg) were incubated with 20 μl glutathione–Sepharose (Amersham Phamarcia Biotech, Piscataway, NJ, U.S.A.) in 100 μl of PBS for 1 h at room temperature. The resin

was then washed once with 1 ml of PBS and resuspended in 100 μl of PBS containing [^{3}H]hsc70 (1.4 μM) in the absence or presence of a 10-fold excess of the peptides. The mixtures were incubated overnight at 4 °C with constant rocking. After incubation, the resin was washed three times with 1 ml of PBS and the amount of bound [^{3}H]hsc70 in each sample was quantified by liquid scintillation counting.

In vitro assay of luciferase refolding

In vitro luciferase refolding was assayed using the method of Terada et al. [43] with slight modification [44]. Briefly, for renaturation assays, luciferase was denatured for 1 h in 6 M guanidinium chloride and diluted in 50 mM potassium acetate, 25 mM Hepes, 5 mM dithiothreitol, 1 mg/ml BSA, pH 7.2, before being added to the reaction mixtures containing 0.5 μM DnaJ and 1.7 μM hsc70 in the absence or presence of the TPR-containing proteins. The reaction mixtures were incubated at room temperature and, at given time points, aliquots were removed and the luciferase activity measured using a luciferase assay system (Promega). Equal amounts of native luciferase, diluted in 50 mM potassium acetate, 25 mM Hepes, 5 mM dithiothreitol, 1 mg/ml BSA, pH 7.2, was also measured and taken as 100 %.

Production of the TPR domain of CHIP–GST fusion protein

The cDNA fragment corresponding to the TPR domain of CHIP (amino acids 20–152) was amplified by PCR using CHIP in pACT2 [33] as a template with primers I and J (Table 1). Primer I contains an *Eco*RI site and primer J, with an additional *Xho*I site and stop codon, is complementary to the coding sequence. The PCR products were cloned into the pGEM-T vector and were sequenced. The insert was excised and cloned into the pGST-KS vector. The recombinant fusion protein was then expressed and purified with glutathione–Sepharose and used to determine *in vitro* binding according to the methods of Liu et al. [33].

Co-precipitation of TPR-containing proteins fused with GST and hsc70 and hsc70 (ΔEEVD)

TPR1, hSGT, Hop, CHIP and the TPR domain of CHIP (20 μg) fused with GST were incubated with 10 μl glutathione–Sepharose (Amersham Phamarcia Biotech) in 100 μl of PBS for 1 h at room temperature. The resin was then washed once with 1 ml of PBS and incubated with 50 μg of hsc70 or hsc70(ΔEEVD) for 30 min at 4 °C. In a separate study, the TPR domain of CHIP fused with GST was incubated as above, except 40 μg of hsc70 or the 30 kDa domain of hsc70 was added for 30 min at 4 °C. The proteins bound to the resin were eluted with 25 mM glutathione and a portion was resolved by SDS/PAGE and the bands were visualized using Coomassie Brilliant Blue.

Yeast two-hybrid assay

Yeast two-hybrid assays were performed by colony filter assay, essentially as recommended by Clontech. Briefly, the cDNAs corresponding to the protein pairs of interest were separately cloned into the pAS-1 and pACT2 vectors. The resulting plasmids were co-transformed into the yeast strain Y190 (Clontech) and the transformants grown on synthetic dextrose minimal medium lacking leucine and tryptophan for 3 days at 30 °C. The colonies were lifted on to paper filters (Whatman, Maidstone, Kent, U.K.) and the filters were quickly frozen in liquid N_2. The filters were thawed at room temperature and incubated with 5-bromo-4-chloroindol-3-yl β-D-galactopyranoside ('X-Gal', 0.33 mg/ml) in 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0, containing 40 mM 2-mercaptoethanol for colour development. Yeasts co-transformed with pLam5′-1 (Clontech) and the pACT2 plasmids were used as controls for non-specific interactions.

RESULTS

Inhibition of luciferase refolding by the TPR-containing proteins

We initially used a refolding system containing only hsc70 and DnaJ to examine if some of the TPR-containing proteins, including CHIP, TPR1 and hSGT, inhibited the refolding of luciferase. Using this refolding system, the restoration of luciferase activity was slightly lower, but the interplay of additional co-chaperones in the reticulocyte lysates can be completely ignored [43,44]. The results in Figure 1(A) show that over 50 % of the original activity of denatured luciferase was restored within 90 min in the presence of hsc70 and DnaJ. Addition of a comparable amount of CHIP into the reaction mixture resulted in an 80 % reduction of the refolding activity (Figure 1B). Hop, TPR1 and hSGT were also capable of inhibiting the luciferase

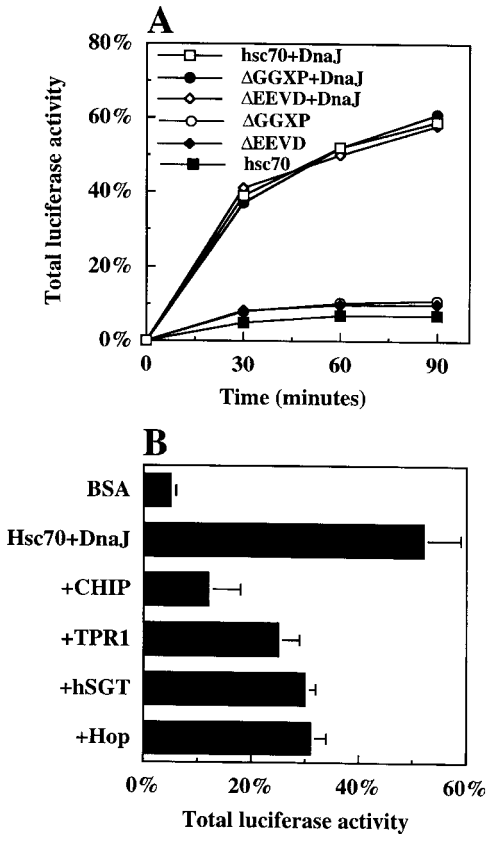

Figure 1 Time course of luciferase refolding and the effect of TPR-containing proteins

(**A**) The time course of luciferase refolding was determined using 1.7 μM of hsc70 proteins [hsc70, hsc70(ΔEEVD) and hsc70(ΔGGXP)] alone or in combination with 0.5 μM DnaJ. The refolding activities of DnaJ alone and BSA control were similar to that of hsc70 alone. The variations were less than 5 % of the total activity (results not shown). Results are means from three determinations. The activity of an equal amount of native luciferase was measured and taken as 100 %. (**B**) The effect of GST fusion proteins of CHIP, TPR1, hSGT and Hop (2 μM) on the restoration of luciferase activity by hsc70 and DnaJ was determined after incubation for 60 min. Results are means ± S.D. of three separate experiments using different preparation of proteins. The activity of an equal amount of native luciferase was measured and taken as 100 %.

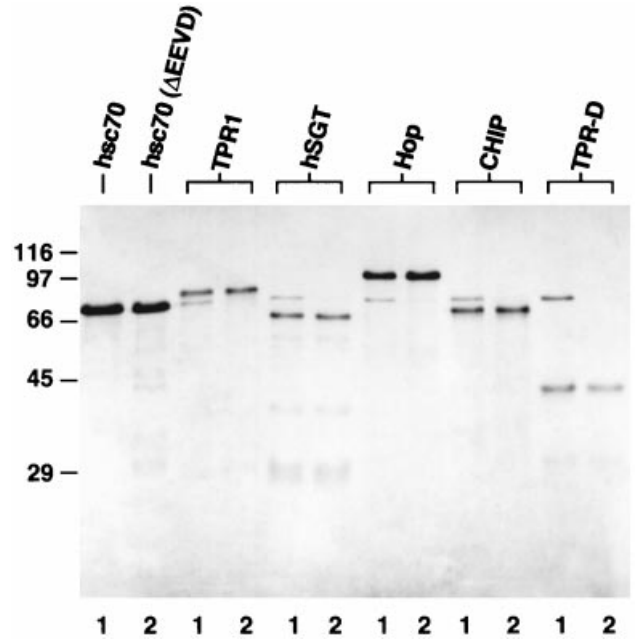

Figure 2 Hsc70(ΔEEVD) did not form complexes *in vitro* with the TPR-containing proteins

GST fusion proteins of TPR1, hSGT, Hop, CHIP and the TPR domain of CHIP (TPR-D) (20 μg) were added to glutathione–Sepharose and then incubated with 50 μg of hsc70 or hsc70(ΔEEVD). Subsequently, the proteins bound to the resins were eluted with 25 mM of glutathione and a portion of the eluates were resolved by SDS/PAGE and visualized with Coomassie Brilliant Blue. Lanes marked 1 are with hsc70 and lanes marked 2 are with hsc70 (ΔEEVD). The molecular-mass markers (in kDa) are indicated on the left.

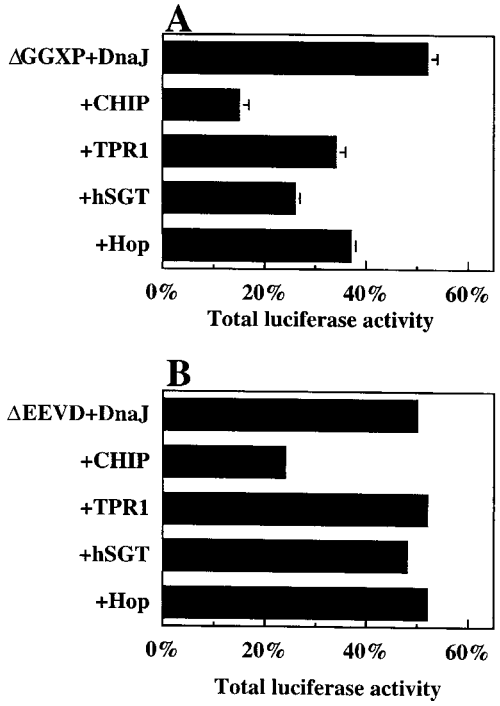

Figure 3 Effect of TPR proteins on the refolding by hsc70(ΔEEVD) and hsc70(ΔGGXP)

GST fusion proteins of TPR1, hSGT, Hop, CHIP and the TPR domain of CHIP (TPR-D) (2 μM) were added to the refolding assay mixtures containing DnaJ and either hsc70(ΔGGXP) (**A**) or hsc70(ΔEEVD) (**B**). After incubation for 60 min, the luciferase activity was measured as described in the Materials and methods section. Results are means ± S.D. of three experiments.

refolding, although the level of inhibition was only approx. 50% (Figure 1B). These results are consistent with the ability of Hop and CHIP to inhibit luciferase refolding by hsc70/hsp70 and hsp40 [19,27,31,32].

Next we determined whether this inhibitory effect correlated with the binding of the TPR-containing proteins to the C-terminus of hsc70. Two hsc70 mutants, hsc70(ΔEEVD) and hsc70(ΔGGXP), were used for this investigation. Although hsc70(ΔEEVD) failed to interact with the TPR-containing proteins (Figure 2, also see reference [33]), hsc70(ΔGGXP) was capable of interacting with these proteins (see below). As shown in Figure 1A, both hsc70(ΔEEVD) and hsc70(ΔGGXP) also supported luciferase refolding in a DnaJ-dependent manner. Moreover, with hsc70(ΔGGXP) and DnaJ, all the TPR-containing proteins examined were capable of inhibiting the luciferase refolding (Figure 3A). Here the reduction in refolding appeared to correlate with the binding of the TPR-containing proteins to hsc70 [33]. In contrast, addition of TPR1, hSGT and Hop to the reaction mixtures containing hsc70(ΔEEVD) and DnaJ did not affect the refolding of luciferase (Figure 3B). For these three proteins, the lack of inhibitory effect on the refolding was correlated with their inability to interact with hsc70(ΔEEVD) [33]. CHIP, however, resulted in an inhibition of refolding (Figure 3B), although it does not interact with hsc70(ΔEEVD) [33].

It is conceivable that regions other than TPR domain of CHIP may affect the luciferase refolding by interfering with the interaction of hsc70 with DnaJ [27]. We therefore investigated whether the TPR domain of CHIP alone affected the luciferase refolding in an hsc70-binding-dependent manner. As shown in Figure 4, the TPR domain of CHIP fused with GST interacted with both hsc70 and the 30 kDa domain of hsc70 (Figure 4A) *in vitro*, but did not interact with hsc70(ΔEEVD) (Figure 2). Moreover, the TPR domain of CHIP inhibited the luciferase refolding supported by hsc70 and hsc70(ΔGGXP) (Figure 4B). Interestingly, it also inhibited the restoration of luciferase activity in a system containing hsc70(ΔEEVD) and DnaJ (Figure 4B). Thus the TPR domain of CHIP alone is capable of inhibiting luciferase refolding even though it does not interact with hsc70(ΔEEVD).

Interaction of TPR-containing proteins with the PTIEEVD sequence

It has been shown previously that only the last seven residues of hsc70 (PTIEEVD) are responsible for interacting with the N-terminal TPR domain of Hop [35]. Therefore we examined whether the peptide GPTIEEVD, containing a C-terminal carboxy group (-COOH), inhibited the association of hsc70 with the Hop. [^{3}H]Hsc70 and the peptide were incubated with the Hop–GST fusion protein, and the amount of [^{3}H]hsc70 bound to the fusion protein quantified. The results shown in Figure 5 clearly demonstrate that a 10-fold molar excess of GPTIEEVD-COOH was indeed capable of inhibiting the association of hsc70 with Hop. Subsequently, we investigated whether the octapeptide may inhibit the association of hsc70 with hSGT, TPR1 and CHIP. Under the same experimental conditions, the amount of [^{3}H]hsc70 bound to TPR1 and CHIP was reduced to a similar level to that of Hop (Figure 5). The interaction of the peptide with TPR1 and CHIP may therefore be similar to that of Hop. However, GPTIEEVD was not effective in blocking the binding of [^{3}H]hsc70 to hSGT (Figure 5). In this case, the binding was

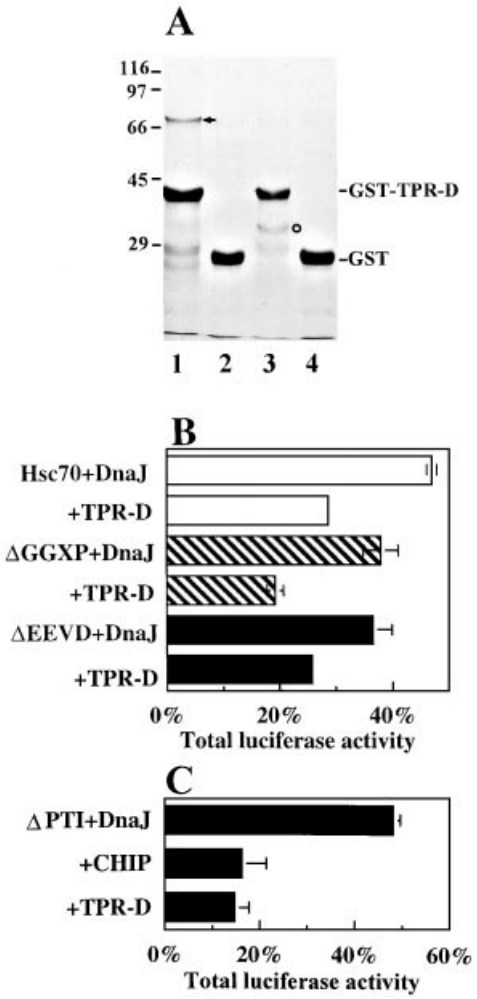

Figure 4 Effect of TPR domain of CHIP on luciferase refolding

(**A**) The TPR domain of CHIP fused with GST (GST-TPR-D; 20 μg) was immobilized with glutathione–Sepharose (10 μl) and then mixed with 40 μg of hsc70 (lane 1) or the 30 kDa domain (lane 3). Proteins bound to the resins were eluted with 25 mM of glutathione and then resolved by SDS/PAGE and visualized with Coomassie Brilliant Blue. GST incubated with the same amount of hsc70 (lane 2) and the 30 kDa domain (lane 4) were used as controls. Hsc70 and the 30 kDa domain are indicated by the arrow and circle respectively. Molecular-mass markers (in kDa) are indicated at the left. (**B**) The refolding of luciferase by 1.7 μM hsc70 proteins [hsc70, hsc70(ΔEEVD) and hsc70(ΔGGXP)] and 0.5 μM DnaJ was assayed in the absence or presence of 4 μM of the TPR domain of CHIP (TPR-D). (**C**) The refolding of luciferase by 1.7 μM hsc70(ΔPTI) and 0.5 μM DnaJ was assayed in the absence or presence either of the TPR domain of CHIP (TPR-D; 2 μM) or CHIP (2 μM). Results are means ± S.D. of three experiments.

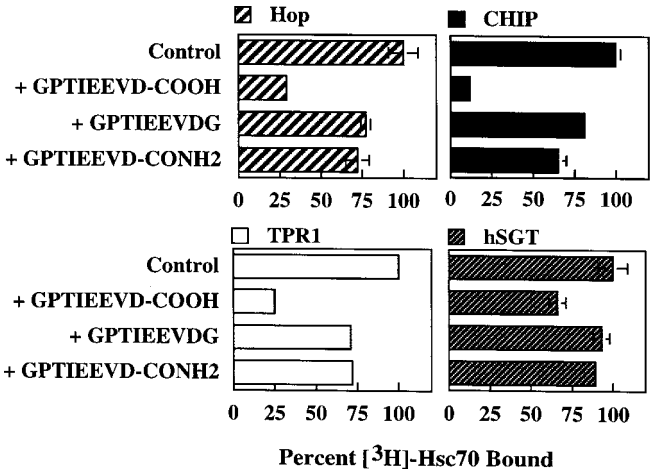

Figure 5 Effect of peptides on the binding of [^{3}H]hsc70 to the TPR proteins

TPR-containing proteins fused with GST were incubated with [^{3}H]hsc70 and a 10-fold molar excess of GPTIEEVD-COOH, GPTIEEVD-CONH$_2$ or GPTIEEVDG. [^{3}H]Hsc70 associated with the TPR-containing proteins was quantified and the non-specific radioactivity associated with GST alone, as a control, was subtracted. The amount of [^{3}H]hsc70 in the complexes in the absence of GPTIEEVD was taken as 100%. Results are means ± S.D. of two determinations.

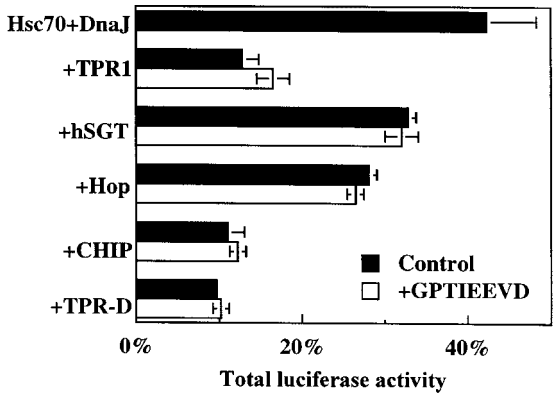

Figure 6 Effect of GPTIEEVD on preventing the inhibition of luciferase refolding by the TPR-containing proteins

GPTIEEVD (20 μM) was added to the refolding reaction mixtures comprising hsc70 (1.7 μM), DnaJ (0.5 μM) and 2 μM TPR1, hSGT, Hop, CHIP or the TPR domain of CHIP (TPR-D). After incubation for 60 min, the luciferase activity was measured. Results are means ± S.D. of three determinations.

only reduced by 30%. Nevertheless, with a 50-fold molar excess of GPTIEEVD, the amount of [^{3}H]hsc70 bound was reduced to 25% of the control level (results not shown). Thus the situation with hSGT appeared to be slightly different from that of Hop. Since only the last seven residues of hsc70, PTIEEVD, were shown to bind to the N-terminal TPR domain of Hop [35], the glycine residue in GPTIEEVD probably contributed little to its binding to CHIP and TPR1.

As the peptide GPTIEEVD prevented hsc70 from associating with the TPR-containing proteins, we therefore examined whether it prevented the inhibition of luciferase refolding by the TPR-containing proteins. A 10-fold molar excess of the peptide was added to the refolding reaction mixtures and the restoration of luciferase activity was then measured. As shown in Figure 6, GPTIEEVD did not reduce the level of inhibition by the TPR-containing proteins. This was an unexpected result. However, we have shown in Figures 3 and 4(B) that binding of CHIP to hsc70 might not be a prerequisite for its inhibition of luciferase refolding. It is possible that CHIP may exert its inhibitory effect through some, as yet, unidentified interaction and thus, GPTIEEVD might not have an effect. Since GPTIEEVD was not very effective in blocking the interaction of hsc70 with hSGT (Figure 5), it was not surprising that GPTIEEVD did not affect the inhibiton by hSGT (Figure 6). However, it was not anticipated that the peptide would fail to prevent TPR1 and Hop from inhibiting luciferase refolding (Figure 6). One possibility for this is that transient interaction of the TPR-containing proteins with hsc70 during the hsc70/DnaJ refolding cycle may be sufficient to inhibit this process. Further investigations are needed to elucidate

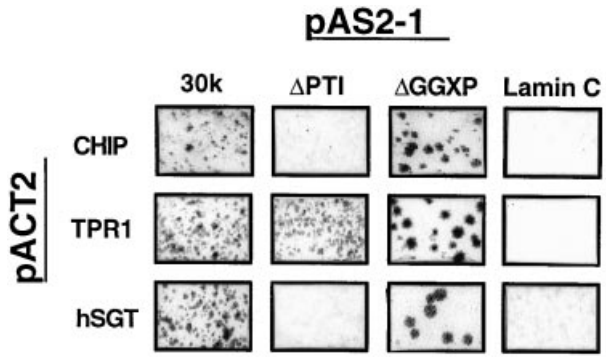

Figure 7 Interaction of TPR-containing proteins with the hsc70 deletion mutants

The interaction between two deletion mutants of the 30 kDa domain, 30 kDa(ΔPTI) and 30 kDa(ΔGGXP), and CHIP, TPR1 and hSGT were determined using the yeast two-hybrid assay. The 30 kDa domain proteins were fused to the GAL4 DNA binding domain (pAS2-1), whereas the TPR-containing proteins were fused with the GAL4 transcription activation domain (pACT2). The assays were then carried out and the filters after colour development are shown. The 30 kDa domain and lamin C were used as positive and negative controls respectively.

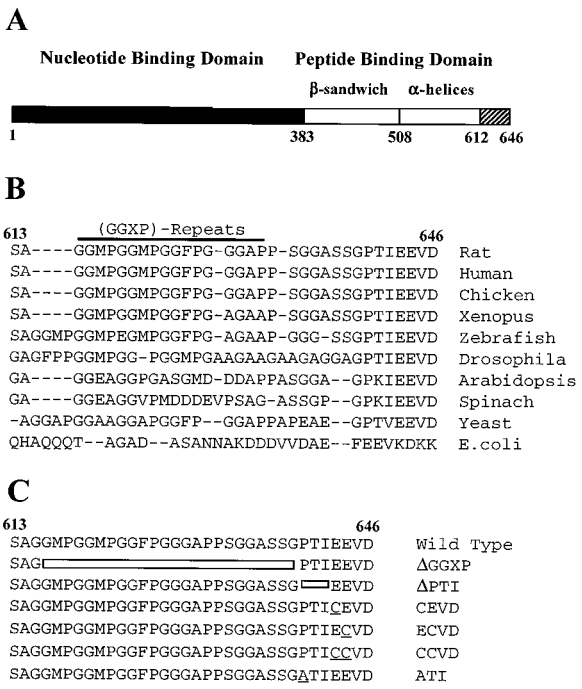

Figure 8 Hsc70 domain structure and comparison of the C-terminal sequence

(**A**) The linear structure of rat hsc70. The N-terminal 383 residues is the nucleotide binding domain which possesses the ATP-hydrolytic activity. The C-terminal peptide binding domain (amino acids 384–612) is composed of a β-sandwich and an α-helical subdomains. The structure and the functional significance of the C-terminal region (amino acids 613–646) are largely unknown. (**B**) The C-terminal sequences of various hsc70s (amino acids 613–646 of rat hsc70) were aligned for comparison. The GGXP-repeats are indicated located near the C-terminus of the α-helical subdomain of hsc70. (**C**) The hsc70 mutants used in this study. Mutants were constructed as described in the Materials and methods section. The deleted regions are shown as open bars, whereas the substituted amino acids are underlined.

the mechanism for hsc70/DnaJ dependent refolding and the inhibitory effect exerted by the TPR-containing proteins.

We next investigated whether the nonapeptide GPTIEEVDG inhibited the association of hsc70 with these proteins. This peptide was used to mimic PTIEEVD with minimal steric hindrance and to determine whether the PTIEEVD sequence had to be located at the C-terminus of the protein in order to interact with the TPR-containing proteins. The results shown in Figure 5 demonstrate that, with a 10-fold molar excess, the nonapeptide reduced the [^{3}H]hsc70 bound by only approx. 25 %. Thus it was much less effective in competing with [^{3}H]hsc70 for binding to the proteins. Therefore the PTIEEVD sequences in the middle of proteins were unlikely to interact with any of these TPR-containing proteins. Indeed, the octapeptide was a relatively weak inhibitor if its C-terminal carboxy group (-COOH) was substitued with an amide group (-$CONH_2$; Figure 5). Thus the carboxy group on the C-terminal aspartate residue plays an important role in interacting with these proteins.

As GPTIEEVD competed with hsc70 for the binding of the TPR-containing proteins (Figure 5) and the removal of the C-terminal EEVD motif of hsc70 abolished its interaction with these proteins [33], we examined whether the tripeptide PTI at the C-terminal region was required for the interaction with TPR1, CHIP and hSGT using the PTI deletion mutant, 30 kDa(ΔPTI), in the yeast two-hybrid assay. The 30 kDa(ΔPTI) failed to interact with Hop (results not shown) and was also unable to interact with CHIP and hSGT, but did interact with TPR1 (Figure 7). Thus the regions of hsc70 that interact with these TPR-containing proteins are overlapping, but not necessarily identical.

Since the PTI sequence preceding the EEVD motif in hsc70 also appeared important in interacting with CHIP, the question arose as to whether CHIP might inhibit the refolding supported by hsc70 and hsc70(ΔEEVD) through an interaction with PTI. Therefore we investigated whether CHIP inhibited refolding by hsc70(ΔPTI) and DnaJ. The results shown in Figure 4(C) demonstrate that hsc70(ΔPTI) was capable of assisting luciferase refolding in the presence of DnaJ. The restoration of luciferase activity by hsc70(ΔPTI) and DnaJ remained largely unchanged after the addition of Hop and hSGT (results not shown). However, in the presence of TPR1, this activity was reduced (results not shown). Moreover, both CHIP and the TPR domain of CHIP were capable of inhibiting the refolding of luciferase supported by hsc70(ΔPTI) and DnaJ (Figure 4C). Thus the inhibitor process exerted by CHIP on luciferase refolding appears complex and the mechanism of this inhibition remains to be determined.

In examining the amino acid sequences of the C-terminal region of the vertebrate hsc70s (Figures 8A and 8B), there are additional GGXP-repeats (where X is an aliphatic residue) located near the C-terminus of the α-helical subdomain of hsc70. Since the ΔPTI mutant interacted with TPR1 (Figure 7), and GPTIEEVD was not effective in competing with hsc70 for the binding to hSGT (Figure 5), we therefore investigated whether the GGXP repeats affected the interaction of hsc70 with TPR1 and hSGT. A 30 kDa(ΔGGXP) mutant (Δ616–639; Figure 8C) was generated and then subjected to a yeast two-hybrid assay with TPR1 and hSGT. The results shown in Figure 7 indicate that removal of the GGXP-repeats from the 30 kDa domain did not affect the interaction with TPR1 and hSGT. Furthermore, the 30 kDa(ΔGGXP) was also capable of interacting with CHIP (Figure 7) and Hop (results not shown). This finding was further supported by *in vitro* pull-down assays using GST fusion proteins (results not shown). Evidently, the GGXP-repeats of hsc70 contribute little to the interaction with the TPR-containing proteins examined here. It is therefore reasonable to treat the PTIEEVD motif at the C-terminus of hsc70 as a single unit, and

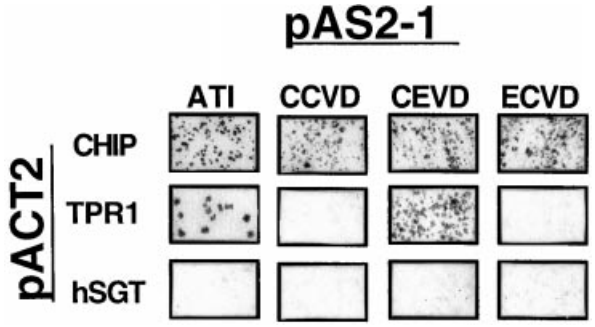

Figure 9 Interaction of the 30 kDa domain mutants with CHIP, TPR1 and hSGT

The interaction between the amino acid substitutions in the EEVD motif (AT1, CCVD, CEVD and ECVD) and CHIP, TPR1 and hSGT were determined using the yeast two-hybrid assay. The amino acid substitutions in the EEVD domain proteins were fused to the GAL4 DNA binding domain (pAS2-1), whereas the TPR-containing proteins were fused with the GAL4 transcription activation domain (pACT2). The assays were then carried out and the filters after colour development are shown.

the GGXP-repeats as a different structural motif. Thus the functional role of the C-terminal region of hsc70 appears complicated, and the significance of the GGXP-repeats needs to be determined.

Clearly, besides interacting with Hop, the PTIEEVD motif is also responsible for the interaction with TPR1, CHIP and hSGT. Therefore we investigated whether the same functional groups at the C-terminus of hsc70 were used to interact with these TPR-containing proteins. We carried out several amino acid substitutions in this motif, including replacing glutamic acid with cysteine and proline with alanine (Figure 8C). Subsequently we tested whether the interaction of the C-terminal 30 kDa domain of hsc70 with TPR1, CHIP and hSGT was affected by these substitutions. As shown in Figure 9, the interaction of these proteins with the mutants varied considerably. CHIP was capable of interacting with all of the mutants examined, but hSGT did not interact with any of the mutants. However, the yeast two-hybrid assay (even with liquid β-galactosidase assay) is not quantitative, as the copy number of the GAL4 DNA binding domain and transcription activation domain fusion proteins in yeast can vary considerably. Therefore even though some of the mutants were capable of interacting with these TPR-containing proteins, it is not clear whether the affinities of the mutants for the TPR domains remained unchanged. Nevertheless, it is likely that a unique combination of the functional groups in PTIEEVD are responsible for the interaction with a specific TPR-containing protein.

DISCUSSION

It has previously been shown that a group of TPR-containing proteins interact with the C-terminus of hsc70 [33]. In the present study, we have further characterized the interaction of hsc70 with three of TPR-containing proteins, CHIP, TPR1 and hSGT. Three conclusions can be drawn from the work described here.

The first conclusion is that the TPR-containing proteins are capable of inhibiting the restoration of luciferase activity by hsc70 and DnaJ. Several points can be made from these findings. First, with the exception of CHIP, the inhibition induced by these TPR-containing proteins is correlated with their binding to hsc70. However, the situation for CHIP appears complex, but our results are in agreement with the notion that CHIP binding to hsc70 is not required for inhibiting the luciferase refolding. Nevertheless, the mechanism by which CHIP exerts this inhibition remains to be elucidated. Secondly, we have shown that deletion of the EEVD motif in hsc70 has little effect on DnaJ-dependent refolding of luciferase (Figure 3). In contrast to our results, Freeman et al. [45] reported that hsp70(ΔEEVD)/hsc70(ΔEEVD) together with HDJ1 failed to support luciferase refolding. Nevertheless, Michels et al. [46] recently showed that, in intact cells, hsp70(ΔEEVD) attenuated heat inactivation of luciferase and assisted the refolding of luciferase. Expression of both hsp70(ΔEEVD) and HDJ1 in these cells, however, did not further enhance the refolding activity [46]. Taken together, the apparent discrepancy between our results and those of Freeman et al. [45] may be due to the difference in the DnaJ-like proteins used in the assays. For example, HDJ1 does not possess the cysteine-rich domain which may be important in supporting the restoration of luciferase activity by hsc70(ΔEEVD). Thirdly, the functional significance of the inhibitory effect needs to be determined. It is possible that, in intact cells, these TPR-containing proteins act as negative regulators of protein folding. However, the TPR domains in CHIP, TPR1 and hSGT represent only about 30% of their masses, suggesting that the rest of the protein is likely to have some other function(s). Therefore it is also possible that hsc70 may regulate an, as yet, unidentified function(s) by associating with the TPR domains of these proteins. This hypothesis will be able to be tested once the functions of these TPR-containing proteins are known.

The second conclusion is that the C-terminal residues of hsc70 are predominant in interacting with TPR1, CHIP and hSGT. Since the peptide GPTIEEVD is capable of inhibiting the binding of [^{3}H]hsc70 to TPR1 and CHIP to a similar degree as Hop (Figure 5), the affinities of TPR1 and CHIP for the peptides should therefore be similar to that for Hop ($K_d = 18\ \mu$M) [35]. However, the 30 kDa(ΔPTI) remains capable of interacting with TPR1 (Figure 7) and thus it appears that only the tetrapeptide EEVD is essential for interacting with TPR1. Therefore it is possible that TPR1 interacts with both hsc70 and hsp90 with similar affinities. However, higher concentrations of GPTIEEVD were needed to inhibit the binding of [^{3}H] hsc70 to hSGT (Figure 5 and results not shown). There are at least two possibilities to explain these results. One simple interpretation is that the conformation of the peptide PTIEEVD in solution is significantly different from that bound to hSGT. Thus extra energy is needed for efficient binding. Alternatively, regions of hsc70, other than the PTIEEVD motif, are also involved in the association with hSGT. In addition, it is evident from the crystal structure that the carboxy group of the C-terminus of PTIEEVD plays an important role in interacting with Hop [35]. The results shown in Figure 5 indicate that this is also true for TPR1, CHIP and hSGT, because replacing the C-terminal carboxy group with an amide group significantly reduced the ability of the peptide to displace bound [^{3}H]hsc70. Moreover, the PTIEEVD sequence has to be located in the C-terminal end of the protein in order to interact with the TPR-containing proteins, as the ability of GPTIEEVDG to inhibit the binding of [^{3}H]hsc70 to these proteins is greatly reduced (Figure 5).

The last conclusion is that different functional groups in PTIEEVD are utilized for the interaction with TPR1, CHIP and hSGT. Based on the results from the structure determination, the proline in PTIEEVD is important for interacting with Hop. Indeed, replacing the proline with alanine abolished the association of 30 kDa domain with Hop (results not shown). However, this substitution had little effect on the interaction of the 30 kDa domain with CHIP and TPR1 (Figure 9). In

addition, the interaction of each TPR-containing protein with the EEVD mutants was also different from one another (Figure 9). Determination of the structures of the protein complexes at the atomic level should reveal details of the interactions between the C-terminal region of hsc70 and various TPR domains. Nevertheless, it is clear that distinctive functional groups in hsc70 are used to interact with each TPR domain. It should therefore be possible to design inhibitors which specifically block the interaction of hsc70 with certain TPR-containing proteins.

We thank Dr Hsou-Min Li and Dr Ming F. Tam for comments on the manuscript. This work was supported by grants from the National Science Council (NSC-89-2311-B-001-076; NSC-89-2311-B-001-210) and from Academia Sinica of Taiwan.

REFERENCES

1 Hartl, F. U. (1996) Molecular chaperones in cellular protein folding. Nature (London) **381**, 571–579
2 Bukau, B. and Horwich, A. L. (1998) The Hsp70 and Hsp60 chaperone machines. Cell (Cambridge, Mass) **92**, 351–366
3 Gao, B., Emoto, Y., Greene, L. and Eisenberg, E. (1993) Nucleotide binding properties of bovine brain uncoating ATPase. J. Biol. Chem. **268**, 8507–8513
4 Wang, C. and Lee, M. R. (1993) High-level expression of soluble rat hsc70 in *Escherichia coli*: purification and characterization of the cloned enzyme. Biochem. J. **294**, 69–77
5 Palleros, D. R., Welch, W. J. and Fink, A. L. (1991) Interaction of hsp70 with unfolded proteins: effects of temperature and nucleotides on the kinetics of binding. Proc. Natl. Acad. Sci. U.S.A. **88**, 5719–5723
6 Palleros, D. R., Reid, K. L., Shi, L., Welch, W. J. and Fink, A. L. (1993) ATP-induced protein-Hsp70 complex dissociation requires K^+ but not ATP hydrolysis. Nature (London) **365**, 664–666
7 Schmid, D., Baici, A., Gehring, H. and Christen, P. (1994) Kinetics of molecular chaperone action. Science (Washington, DC) **263**, 971–973
8 McCarty, J. S., Buchberger, A., Reinstein, J. and Bukau, B. (1995) The role of ATP in the functional cycle of the DnaK chaperone system. J. Mol. Biol. **249**, 126–137
9 Becker, J., Walter, W., Yan, W. and Craig, E. A. (1996) Functional interaction of cytosolic hsp70 and a DnaJ-related protein, Ydj1p, in protein translocation in vivo. Mol. Cell. Biol. **16**, 4378–4386
10 Hendrick, J. P., Langer, T., Davis, T. A., Hartl, F. U. and Wiedmann, M. (1993) Control of folding and membrane translocation by binding of the chaperone DnaJ to nascent polypeptides. Proc. Natl. Acad. Sci. U.S.A. **90**, 10216–10220
11 Cyr, D. M., Langer, T. and Douglas, M. G. (1994) DnaJ-like proteins: molecular chaperones and specific regulators of Hsp70. Trends Biochem. Sci. **19**, 176–181
12 Cheetham, M. E. and Caplan, A. J. (1998) Structure, function and evolution of DnaJ: conservation and adaptation of chaperone function. Cell Stress Chaperones **3**, 28–36
13 Braun, J. E. A., Wilbanks, S. M. and Scheller, R. H. (1996) The cysteine string secretory vesicle protein activates Hsc70 ATPase. J. Biol. Chem. **271**, 25989–25993
14 Srinivasan, A., McClellan, A. J., Vartikar, J., Marks, I., Cantalupo, P., Li, Y., Whyte, P., Rundell, K., Brodsky, J. L. and Pipas, J. M. (1997) The amino-terminal transforming region of simian virus 40 large T and small t antigens functions as a J domain. Mol. Cell. Biol. **17**, 4761–4773
15 Leng, C. H., Brodsky, J. L. and Wang, C. (1998) Isolation and characterization of a DnaJ-like protein in rats: the C-terminal 10-kDa domain of hsc70 is not essential for stimulating the ATP-hydrolytic activity of hsc70 by a DnaJ-like protein. Protein Sci. **7**, 1186–1194
16 Greene, M. K., Maskos, K. and Landry, S. J. (1998) Role of the J-domain in the cooperation of Hsp40 with Hsp70. Proc. Natl. Acad. Sci. U.S.A. **95**, 6108–6113
17 Szabo, A., Korszun, R., Hartl, F. U. and Flanagan, J. (1996) A zinc finger-like domain of the molecular chaperone DnaJ is involved in binding to denatured protein substrates. EMBO J. **15**, 408–417
18 Lu, Z. and Cyr, D. M. (1998) The conserved carboxyl terminus and zinc finger-like domain of the co-chaperone Ydj1 assist Hsp70 in protein folding. J. Biol. Chem. **273**, 5970–5978
19 Gebauer, M., Zeiner, M. and Gehring, U. (1997) Proteins interacting with the molecular chaperone hsp70/hsc70: physical associations and effects on refolding activity. FEBS Lett. **417**, 109–113
20 Demand, J., Luders, J. and Hohfeld, J. (1998) The carboxy-terminal domain of Hsc70 provides binding sites for a distinct set of chaperone cofactors. Mol. Cell. Biol. **18**, 2023–2028
21 Hohfeld, J., Minami, Y. and Hartl, F. U. (1995) Hip, a novel co-chaperone involved in the eukaryotic Hsc70/Hsp40 reaction cycle. Cell (Cambridge, Mass) **83**, 589–598
22 Takayama, S., Bimston, D. N., Matsuzawa, S., Freeman, B. C., Aime, S. C., Xie, Z., Morimoto, R. I. and Reed, J. C. (1997) BAG-1 modulates the chaperone activity of Hsp70/Hsc70. EMBO J. **16**, 4887–4896
23 Zeiner, M., Gebauer, M. and Gehring, U. (1997) Mammalian protein RAP46: an interaction partner and modulator of 70 kDa heat shock proteins. EMBO J. **16**, 5483–5490
24 Raynes, D. A. and Guerriero, Jr, V. (1998) Inhibition of Hsp70 ATPase activity and protein renaturation by a novel Hsp70-binding protein. J. Biol. Chem. **273**, 32883–32888
25 Bimston, D., Song, J., Winchester, D., Takayama, S., Reed, J. C. and Morimoto, R. I. (1998) BAG-1, a negative regulator of Hsp70 chaperone activity, uncouples nucleotide hydrolysis from substrate release. EMBO J. **17**, 6871–6878
26 Zeiner, M., Niyaz, Y. and Gehring, U. (1999) The hsp70-associating protein Hap46 binds to DNA and stimulates transcription. Proc. Natl. Acad. Sci. U.S.A. **96**, 10194–10199
27 Ballinger, C. A., Connell, P., Wu, Y., Hu, Z., Thompson, L. J., Yin, L. Y. and Patterson, C. (1999) Identification of CHIP, a novel tetratricopeptide repeat-containing protein that interacts with heat shock proteins and negatively regulates chaperone functions. Mol. Cell. Biol. **19**, 4535–4545
28 Chen, S., Prapapanich, V., Rimerman, R. A., Honore, B. and Smith, D. F. (1996) Interactions of p60, a mediator of progesterone receptor assembly, with heat shock proteins hsp90 and hsp70. Mol. Endocrinol. **10**, 682–693
29 Chen, S. and Smith, D. F. (1998) Hop as an adaptor in the heat shock protein 70 (Hsp70) and hsp90 chaperone machinery. J. Biol. Chem. **273**, 35194–35200
30 Kosano, H., Stensgard, B., Charlesworth, M. C., McMahon, N. and Toft, D. (1998) The assembly of progesterone receptor-hsp90 complexes using purified proteins. J. Biol. Chem. **273**, 32973–32979
31 Gebauer, M., Zeiner, M. and Gehring, U. (1998) Interference between proteins Hap46 and Hop/p60, which bind to different domains of the molecular chaperone hsp70/hsc70. Mol. Cell. Biol. **18**, 6238–6244
32 Johnson, B. D., Schumacher, R. J., Ross, E. D. and Toft, D. O. (1998) Hop modulates Hsp70/Hsp90 interactions in protein folding. J. Biol. Chem. **273**, 3679–3686
33 Liu, F.-H., Wu, S.-J., Hu, S.-M., Hsiao, C.-D. and Wang, C. (1999) Specific interaction of the 70-kDa heat shock cognate protein with the tetratricopeptide repeats. J. Biol. Chem. **274**, 34425–34432
34 Murthy, A. E., Bernards, A., Church, D., Wasmuth, J. and Gusella, J. F. (1996) Identification and characterization of two novel tetratricopeptide repeat-containing genes. DNA Cell Biol. **15**, 727–735
35 Scheufler, C., Brinker, A., Bourenkov, G., Pegoraro, S., Moroder, L., Bartunik, H., Hartl, F. U. and Moarefi, I. (2000) Structure of TPR domain-peptide complexes: critical elements in the assembly of the Hsp70-Hsp90 multichaperone machine. Cell (Cambridge, Mass) **101**, 199–210
36 Young, J. C., Obermann, W. M. J. and Hartl, F. U. (1998) Specific binding of tetratricopeptide repeat proteins to the C-terminal 12 kDa domain of hsp90. J. Biol. Chem. **273**, 18007–18010
37 Lamb, J. R., Tugendreich, S. and Hieter, P. (1995) Tetratricopeptide repeat interactions: to TPR or not to TPR? Trends Biochem. Sci. **20**, 257–259
38 Das, A. K., Cohen, P. W. and Barford, D. (1998) The structure of tetratricopeptide repeats of protein phosphatase 5: implications for TPR-mediated protein-protein interactions. EMBO J. **17**, 1192–1199
39 Lassle, M., Blatch, G. L., Kundra, V., Takatori, T. and Zetter, B. R. (1997) Stress-inducible murine protein mSTI1. characterization of binding domains for heat shock proteins and *in vitro* phosphorylation by different kinases. J. Biol. Chem. **272**, 1876–1884
40 Wu, S.-J. and Wang, C. (1999) Binding of heptapeptides or unfolded proteins to the chimeric C-terminal domains of 70-kDa heat shock cognate protein. Eur. J. Biochem **259**, 449–455
41 Tsai, M.-Y. and Wang, C. (1994) Uncoupling of peptide-stimulated ATPase and clathrin-uncoating activity in deletion mutant of hsc70. J. Biol. Chem. **269**, 5958–5962
42 Hu, S.-M. and Wang, C. (1996) Involvement of the 10-kDa C-terminal fragment of hsc70 in complexing with unfolded protein. Arch. Biochem. Biophys. **332**, 163–169
43 Terada, K., Kanazawa, M., Bukau, B. and Mori, M. (1997) The human DnaJ homologue dj2 facilitates mitochondrial protein import and luciferase refolding. J. Cell Biol. **139**, 1089–1095
44 Chang, T.-C., Hsiao, C.-D., Wu, S.-J. and Wang, C. (2001) The effect of mutating arginine-469 on the substrate binding and refolding activities of 70-kDa heat shock cognate protein. Arch. Biochem. Biophys. **386**, 30–36
45 Freeman, B. C., Myers, M. P., Schumacher, R. and Morimoto, R. I. (1995) Identification of a regulatory motif in Hsp70 that affects ATPase activity, substrate binding and interaction with HDJ-1. EMBO J. **14**, 2281–2292.
46 Michels, A. A., Kanon, B., Bensaude, O. and Kampinga, H. H. (1999) Heat shock protein (Hsp) 40 mutants inhibit Hsp70 in mammalian cells. J. Biol. Chem. **274**, 36757–36763

Received 14 March 2001/3 July 2001; accepted 15 August 2001