Specific Interaction of the 70-kDa Heat Shock Cognate Protein with the Tetratricopeptide Repeats*

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Using a yeast two-hybrid system with the 70-kDa heat shock cognate protein (hsc70) or its C-terminal 30-kDa domain as baits, we isolated several proteins interacting with hsc70, including Hip/p48 and p60/Hop. Both are known to interact with hsc70. Except for Hip/p48, all of the proteins that we isolated interact with the 30-kDa domain. Moreover, the EEVD motif at the C terminus of the 30-kDa domain appears essential for this interaction. Sequence analysis of these hsc70-interacting proteins reveals that they all contain tetratricopeptide repeats. Using deletion mutants of these proteins, we demonstrated either by two-hybrid or in vitro binding assays that the tetratricopeptide repeat domains in these proteins are necessary and sufficient for mediating the interaction with hsc70.

Members of the 70-kDa heat shock protein family (hsp70s) and their cognates (hsc70s) have been implicated as ATP-dependent molecular chaperones (for reviews, see Refs. 1 and 2). Structurally, hsc70 is composed of two major domains. While the N-terminal 44-kDa domain is an ATPase (3–5), the C-terminal 30-kDa domain is capable of forming complexes with unfolded polypeptides (6, 7). Now, it is clear that hsp70/hsc70s are acting in concert with other cellular proteins to exert the chaperoning functions such as folding of proteins, assembly, or disassembly of protein complexes or transport of proteins into organelles (for a review, see Ref. 1). For instance, hsc70 works together with auxilin to remove clathrin from coated vesicles (8). In Escherichia coli, DnaJ and GrpE are known to interact with DnaK (an hsc70 homolog) to assist protein folding (9). In yeast, interaction of SSA proteins (hsp70 homologs) with Ydj1p (a DnaJ homolog) was shown to play an important role in the translocation of some proteins across the endoplasmic reticulum membrane (10). Moreover, association of hsc70 with p60/Hop and p60 appears to play an essential role in the assembly of the progesterone receptor (11, 12). Using the yeast two-hybrid approach with the conserved 44-kDa ATPase domain of hsc70 as a bait, Hohfeld et al. (13) have isolated an hsc70-interacting protein, Hip. Later, Hip was found to be identical to p48, a protein that interacts transiently with the steroid hormone receptor (14). More recently, BAG-1/Rap-46/Hap-46 was also shown to interact with the N-terminal domain of hsc70 (15–17) and inhibit the release of bound unfolded protein substrates (18). A number of other cellular proteins, including p16 (19) and HspBP1 (20), have been identified as hsp70/hsc70-interacting proteins. Nevertheless, the biological significance of these interactions is not well understood.

During the last few years, the yeast two-hybrid system has been demonstrated as a powerful tool to identify interacting protein partners. However, it has not been investigated if intact hsc70 or its C-terminal 30-kDa domain can be effectively used as a bait in a two-hybrid screening, perhaps because the 30-kDa domain is known to bind short peptides or unfolded proteins (6, 7). In this study, we have explored such a possibility. Our result showed that the number of false positives was limited, and a small number of putative hsc70-interacting proteins occurred repeatedly. After sequencing these positive clones, it became evident that they all contain the tetratricopeptide repeats (TPR), a motif that was previously proposed to mediate protein-protein interaction (for a review, see Ref. 21).

EXPERIMENTAL PROCEDURES

Screening of hsc70-interacting Proteins with the Yeast Two-hybrid System—All of the two-hybrid experiments described in this study were performed using the yeast strain Y190 provided by CLONTECH. Yeast was grown either in YPD or in SD minimal medium. The plasmids used to construct the baits for the screening were pHsc70/15b (see below), containing the cDNA of hsc70, and pCt-30/R (6), containing the cDNA of the C-terminal 30-kDa domain. To insert these cDNA into pAS2–1 (CLONTECH), an EcoRI linker was first introduced into the aforementioned two plasmids at the unique NdeI site covering the initiating methionine. After digesting with EcoRI and BamHI, the cDNAs were isolated and cloned into pAS2-1 with these two sites resulting in plasmids pAS-hsc70 and pAS-30K.

To screen for interacting proteins, plasmids of a human liver cDNA library in pACT2 (CLONTECH) were transformed into yeast harboring either pAS-hsc70 or pAS-30K. Double transformants were screened for histidine prototrophy on SD minimal plates containing 20 mg/mL 3-amino-1,2,4-triazole but lacking histidine, leucine, and tryptophan for 7 days at 30°C. The β-galactosidase activity of the transformants growing under these conditions was determined by filter assay (22). Blue colonies obtained were restreaked three times on the same selection plates to enrich the cDNA plasmids carrying the interacting proteins. The plasmids were then isolated and reintroduced into yeast Y190 together with the original bait for β-galactosidase activity assay or with an irrelevant bait, plasmid pLam5-1 (provided by CLONTECH), as a control for nonspecific interaction.

Construction of the Plasmids pHsc70/15b—Using polymerase chain reaction (PCR), we amplified the C-terminal fragment of pHsc70 (23) with two synthetic primers. Primer 1 (5′-TCGTCGACTTTAGATATGCT-3′) is identical to the coding sequence (nucleotides 1672–1688) of rat hsc70 (24), except a G/C substitution was made to introduce an XhoI site. Primer 2 (5′-AGAGTCTTAACTTACGTCCTCTC-3′) is complementary to the coding sequence around the termination codon, except that a BamHI site was added at the 3′-end of the coding strand. Subsequently, the PCR products were cloned into a pBluescriptSK+ vector (Stratagene) and were sequenced. Then the cDNA insert was excised, ligated with the

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‡ The abbreviations used are: hsc70, 70-kDa heat shock cognate protein; TPR, tetratricopeptide repeat(s); PCR, polymerase chain reaction; GST, glutathione S-transferase; SGT, small glutamine-rich tetratricopeptide repeat-containing protein; hSGT, human SGT.
NdeI–XhoI fragment of the insert in pHsc70-Ct (25) and pET-15b (Novagen) treated with both NdeI and BamHI. The resulting plasmid, pHsc7015b, contains the full-length hsc70 cDNA with a tag of six consecutive histidines (His tag) at the N terminus.

Construction of the C-terminal Deletion Mutants of hsc70 and the 30-kDa Domain—To construct the plasmid pHsc70-R for the expression of the hsc70 catalytic domain, we performed PCR with primers Fo and Re. Primer Re (5′-GAATTCATATGCTAGGCGCCTGAAGA) contains an EcoRI site and a stop codon at the 5′-end and is complementary to the coding sequence, with the last four residues (EEVD) of hsc70 being deleted. Primer Fo (5′-GAATAGACGAGAGACAGAGATAAG) is identical to the coding region for residues 530–535 of hsc70. The PCR products were cloned into pGEM-T vector and then were digested with EcoRI and XhoI. The resulting plasmid, pHsc701DEEVD, is used to express hsc70 without the EEVD motif (hsc701DEEVD)). This XhoI–EcoRI fragment was also used to replace the equivalent of pCt-30/R, resulting in plasmid pHct-30DEEVD/R. The plasmid was used to express the 30-kDa(ΔEEVD), the 30-kDa domain, or the 30-kDa(ΔEEVD)/R, corresponding to the 30-kDa(ΔEEVD)/R, encoding amino acids 20 to 303, 208 to 308, and 152 to 308, respectively; and, in both cases, the stop codon and an initiation methionine of TPR1, except that an alanine substitute the insert of pACT2-TPR2. Besides all of the tetratricopeptide repeats, upon expression, the resulting protein also contains an N-terminal His tag with a 9-base overhang.

Interaction of hsc70 with TPR Motifs—To construct the plasmids for the GST fusion proteins of the positive clones, the corresponding cDNA inserts of these clones were excised from the plasmids by digesting with EcoRI and XhoI. Using these two restriction sites, they were then ligated into pGEX-2T (Amersham) by using two primers (ADR, R208, and R152) were synthesized. Primer TP (5′-CTCGAGCTACAT-9GAATTCATATGGTG) and primer b (5′-CTCGAGTGTTTGTGTTC) with an NdeI site and stop codon at the 5′-end were released either by incubating with glutathione in phosphate-buffered saline or by boiling in SDS sample buffer. They were then resolved by gel electrophoresis. To construct the plasmids, one common forward primer (TP) and three reversed primers (ADR, R208, and R152) were synthesized. Primer TP (5′-CTCGAGCTACAT-9GAATTCATATGGTG) and primer II (5′-GGATCCCTAGGCGGCGCTTG) or primer II and primer III (5′-CATATGAGCACTAGATAAGGAG) contains an EcoRI site and a stop codon at the 5′-end and is identical to the coding sequence for amino acids 177–181. The reverse primer G (5′-CGGCGGCTAGGCGGCGCTTTGTTGTTC) with an XhoI site is complementary to the coding sequence covering amino acids 153–158 with an XhoI site and a stop codon. The PCR products were cloned into pGEM-T for sequence verification. The EcoRI–XhoI fragment was then excised and ligated into pAC2. However, the fragment between the EcoRI site and the initiation methionine of TPR2 in the original pACT2-TPR2, which was obtained from the two-hybrid screening, was not included in this plasmid. In this case, the EcoRI–XhoI fragment of the new plasmid was replaced with the equivalent of that from pACT2-TPR2. (The pACT2 contains a NdeI site at the multiple cloning sites, and TPR2 has a NdeI site around amino acid 104.) Identical approaches were used to clone the first four tetratricopeptide repeats into pACT2, except that PCR was performed with primers A and B. Primer C (5′-CTCGAGTATATTCTGCGAGCGAGGAG) is complementary to the coding sequence corresponding to amino acids 237–242. We also took advantage of a truncated TPR2 species (amino acid residues 1–270) obtained from the two-hybrid screening to generate additional mutants. To construct the mutants with the first five repeats, we carried out PCR with primers D (5′-CTCGAGAAATGGTGGGGCTGGTG) and E (5′-CTCGAGTGTTTGTGTTC) with an NdeI site and a stop codon at the 5′-end. The PCR products were cloned into pGEM-T vector and were sequenced. Then, the PsrI site was excised (there is a second NdeI site on the vector in the correct orientation) and replaced the corresponding fragment from the truncated plasmid.

A plasmid with all the tetratricopeptide repeats but without the J-domain was engineered by amplifying the TPR2 fragment containing amino acid residues 177–356. The forward primer F (5′-CTCGAGTACACAAATGGTGGGGCTGGTG) contains a XhoI site and is identical to the coding sequence for amino acid residues 177–181. The reverse primer G (5′-CTCGAGTGTTTGTGTTC) with an XhoI site is complementary to the coding sequence for amino acids 237–242, whereas primer E is complementary to the coding sequence for amino acids 274–279 with an XhoI site and stop codon at the 5′-end. The PCR products were cloned into pGEM-T vector and were sequenced. Then, the PsrI site was excised (there is a NdeI site on the vector in the correct orientation) and replaced the corresponding fragment from the truncated plasmid. The resulting plasmid has the same overhanging sequence as that of BamHI.

Construction of the Plasmids for hSGT Mutants—We isolated the SucI–PstI fragment (~1 kilobase pair) from the insert of the plasmid pACT2-hSGT and cloned it into a pBluescript vector (Stratagene) with the same sites. The SucI site is at residues 119 and 120 of hSGT, and the PstI site is at 490 base pairs downstream of the initiation methionine. When this fragment was digested with XhoI and ligated with the 5′-end of pACT2-TPR2 (with an NdeI site). The XhoI site is about 300 base pairs downstream of the stop codon. Once ligated, an additional XhoI site is created near the XhoI site in the resulting plasmid, phSGT-C/R. Simultaneously, the N-terminal region of hSGT was amplified by PCR with primer a (5′-CATATGGCATATCATATG) and primer b (5′-CTCGAGTATATCGTTTTTTC), complementary to the site.
sequence for amino acids 157–162. The products were cloned into pGEM-T, resulting in plasmid pHSGT-N7. Then both the NdeI–PstI fragment of pHSGT-N7 and the PstI–XhoI fragment of pHSGT-CKS were isolated and ligated together with pET-15b using the NdeI and XhoI sites. The resulting plasmid, pHSGT15b, can be used to express hSGT at the N terminus.

To construct the plasmid for hSGT without the glutamine-rich region, hSGT was amplified by primers c (5′-TAGAGCTCAACCCAGCC) and d (5′-CTCGAGTTAGAGCTGCGCCAGGTC) with the coding sequence of residues 119–124, and primer d is complementary to the sequence for residues 268–273 with an XhoI site and stop codon at the 5′ end. The PCR products were ligated into pGEM-T for sequencing. Then the plasmid was digested with SacI and XhoI, and the fragment was used to replace the SacI–XhoI fragment of pHSGT15b, resulting in pHSGTΔQ/15b. Using an identical method with primers c and e (5′-CTCGAGTTAGCTCCCGGGTAGCTAG) the plasmid pHSGT(N+TPR)15b was obtained, which can be used to express hSGT truncated at amino acid residue 208.

In order to engineer the plasmid to express the TPR domain of hSGT, the cDNA was amplified with PCR using primers e and f (5′-CATAGTGTACGACGACGACGAG) . Primer f is identical to the sequence encoding amino acids 88–92 with a NdeI site at the 5′-end. The PCR products were cloned into pGEM-T, and the NdeI–XhoI fragment was then isolated and ligated into pET-15b with the same two restriction sites as used in the plasmid pHSGTTPR15b. Finally, to construct the plasmids for the two hybrid assays, the cDNA inserts of hSGT or its mutants in pET-15b were excised with restriction enzymes NcoI and XhoI. Then the fragments were cloned into pACT2 previously treated with these two enzymes.

Assaying for the Complexes Formed by the TPR Domain of hSGT and hsc70—Purified bovine hsc70 (100 μg) was incubated with an equal mass of the TPR motif of hSGT in 0.1 ml of buffer A (75 mM KCl, 40 mM HEPES, 5 mM dithiothreitol, 4.5 mM magnesium acetate, pH 7.0) at room temperature for 1 h. Then 80 μl of the mixtures were then applied to a Superdex 200 HR column (1 × 30 cm) in equilibrium with buffer A without dithiothreitol. The column was eluted with the same buffer at a flow rate of 0.5 ml/min, and 0.4-ml fractions were collected. Aliquots from fractions were analyzed by SDS gel electrophoresis to verify if the protein may form complexes.

Deletion Mutants with Disrupted TPR Motifs—To construct the deletion mutant for NY-CO-7, the cDNA was amplified with EcoRI and PstI. The products were treated with Klenow fragment and self-ligated, which resulted in an N-terminal 105 amino acid residues. Second, to construct the plasmid without the first three repeats, the PCR products were ligated into pGEM-T for sequencing. To remove the C-terminal region of TPR1 (residues 180–292), we digested the plasmid pACT2-TPR2 with NcoI and BamHI. Then the plasmid was digested with SacI and StuI. The SacI site is near the end of the first tetratricopeptide repeats, and StuI is at the beginning of the third repeats. The product was treated with Klenow and self-ligated, which resulted in an in-frame deletion of residues 119–159. The insert was then excised with NcoI and XhoI and then cloned into pACT2 with the same two restriction sites. (NcoI site is on the 5′-end.)

The C-terminal domain of hSGT contains a glutamate-rich region in front of its tetratricopeptide repeats (21). Moreover, TPR2 has an additional J-domain, and the glutamine-rich (Q-rich) region in the linear sequences of these proteins, wherever applicable, are also shown. The GenBank™ accession number for hSGT is AJ133129. Also, the amino acid residues of NY-CO-7 obtained in this study at positions 272 and 280 are arginine and leucine, respectively, instead of glycine and phenylalanine.

RESULTS

Using a human liver cDNA library and rat hsc70 as a bait, we screened for hsc70-interacting proteins. The sequences of rat and human hsc70s only differ in one amino acid. The 579th residue in rat hsc70 is serine (24), whereas it is asparagine in human hsc70 (32). After screening 2.5 × 10^6 transformants, we obtained three colonies that grew on the histidine dropout medium and showed a significant level of β-galactosidase activity in a bait-dependent manner. Nucleotide sequencing revealed that two of the positive clones belong to the same gene, NY-CO-7 (33), except for the difference in two amino acid residues, and the third one is Hipp(p48) (13).

We also used the C-terminal 30-kDa domain of hsc70 (6) as a bait to identify the interacting proteins in the same cDNA library. In this case, after screening 1.8 × 10^6 transformants, 23 colonies from 4 distinct genes were obtained. One of the cDNAs obtained is p60/Hop, a component of the steroid receptor coactivator complex (12, 34). The other two genes, the TP1 and TPR2 genes, were previously reported (33). The third one, hSGT, showed 88% identity in amino acid sequence with SG7 (36) shown to interact with NS1 of parvovirus. Interestingly, all of these putative hsc70-interacting proteins contain tetratricopeptide repeats (21). Moreover, TPR2 has an additional J-domain, while TPR1 contains a glutamate-rich region in front of its TPR motif, and hSGT has a glutamine-rich C terminus (Fig. 1).

Sequence analysis indicates that these previously undeni-
fied hsc70-interacting proteins are not prematurely terminated short peptides. It nevertheless was not entirely ruled out that some of them might interact as unfolded polypeptides with the 30-kDa domain. Therefore, we used the 30-kDa(Δshp1), a deletion mutant without both the GGXP repeats and the EEVD motif (29), as a bait to test for their interaction. Since the 30-kDa(Δshp1) is capable of complexing with the unfolded proteins (29), the clones should remain positive with the mutant if they are interacting as unfolded proteins with the 30-kDa domain. However, none of the clones appears to interact with the 30-kDa domain. Moreover, none of the clones obtained from screening with hsc70 were evaluated. For instance, TPR2 is composed of tetratricopeptide repeats to associate with hsc70. Deletion mutants without the J-domain and the three repeats in p60/Hop were shown to be responsible for its interaction with hsc70 (38), we decided to investigate if other hsc70-interacting proteins that we isolated also utilize their interaction in our two-hybrid assays between hsc70 and the three clones, p60/Hop, hSGT, and TPR1, might reflect that the essential role for the association of these clones with the 30-kDa domain.

We then investigated if NY-CO-7 may interact with other domains of hsc70 in the yeast two-hybrid system. The result of the two-hybrid experiments indicates that NY-CO-7 interacts with the C-terminal 30-kDa domain but not with the N-terminal 44-kDa domain of hsc70 (data not shown). Subsequently, we examined whether the four clones obtained from screening with the 30-kDa domain as a bait may interact with hsc70. Surprisingly, only TPR2 showed significant interaction with hsc70 (data not shown). Using color assay, the interaction between hsc70 and the other three clones, including p60/Hop, cannot be demonstrated (data not shown). However, using a similar two-hybrid system but using growth on dropout medium as an assay method, Gebauer et al. (17) and Demand et al. (37) showed that p60 interacts with full-length hsc70. Suffice it to say the plasmid constructs, yeast strain, and selection scheme differ slightly among these systems. To investigate the possible cause for this disagreement, we used our plasmid constructs and adopted their selection scheme for the two-hybrid experiments. Indeed, yeast (strain HF7c) harboring pACT-p60 and pAS-hsc70 can grow on complete medium lacking tryptophan, leucine, and histidine (data not shown). We also have fused the proteins with the GST to perform an in vitro binding assay. As expected, GST-TPR2 is capable of complexing with hsc70 and the 30-kDa domain but not with the deletion mutants without the EEVD motif (Fig. 3). We next assessed whether or not other fusion proteins may complex with hsc70 or the 30-kDa domain. Evidently, they form complexes with both hsc70 and the 30-kDa domain (Fig. 4). Moreover, the addition of ATP or ADP in the reaction mixtures had little effect on the results. These GST fusion proteins also failed to pull down the mutants of hsc70 or the 30-kDa domain without the EEVD motif (data not shown). Thus, the lack of interaction in our two-hybrid assays between hsc70 and the three clones, p60/Hop, hSGT, and TPR1, might reflect that the assay conditions we used are more stringent than selecting by growth in medium lacking histidine.

Except for Hip/p48, all the proteins shown in Fig. 1 interact with the C-terminal 30-kDa domain of hsc70, and they all contain at least three tetratricopeptide repeats. Since the first three repeats of p60/Hop were shown to be responsible for its interaction with hsc70 (38), we decided to investigate if other hsc70-interacting proteins that we isolated also utilize their tetratricopeptide repeats to associate with hsc70. Deletion mutants of these clones were engineered, and their interactions with hsc70 were evaluated. For instance, TPR2 is composed of seven tetratricopeptide repeats together with a J-domain (Fig. 1). We first deleted the J-domain and checked the interaction of this deletion mutant with the 30-kDa domain. The result clearly indicates that the TPR2 mutant without the J-domain remains capable of interacting with the 30-kDa domain (Fig. 5A, mutant 1–356). Furthermore, the last three repeats in TPR2 are dispensable, since the first four repeats (mutant
1–242) are sufficient to support relatively strong interaction with the 30-kDa domain. However, removal of the fourth tetratricopeptide (mutant 1–158) results in a significant loss in such interaction. These results imply that, for TPR2, the tetratricopeptide repeats are responsible for its interaction with hsc70 and that the J-domain is not needed for this interaction.

For NY-CO-7, TPR1, and hSGT, a series of deletions surrounding their respective TPR motifs were constructed (Figs. 5, B and C, and 6A) and then subjected to the two-hybrid assays. The results clearly show that most of the regions flanking the tetratricopeptide repeats of these three proteins can be removed without affecting their interaction with the 30-kDa domain. Moreover, we examined whether the deletion mutants with the smallest size of these four proteins interact with the 30-kDa (Δshp1). The results demonstrate that this is not the case (data not shown). They are not likely to interact as unfolded polypeptides with the 30-kDa domain. Since we also expressed hSGT and several deletion mutants thereof in bac-

![Diagram A](image1.png)

**Fig. 5. Interaction of the deletion mutants with the 30-kDa domain.** Two-hybrid assays were utilized here to assess the interaction of the mutants of TPR2 (A), NY-CO-7 (B), and TPR1 (C), respectively, with the 30-kDa domain in pAS2–1. In all of the cases, the upper panel is a schematic showing the primary structure of the proteins, including the deletion mutants, in pACT2. The locations of the tetratricopeptide repeats are indicated by a hatched bar. The lower panel shows the blue filters, and pLam5-1 was used as a negative control. In A, sequence 1–356 contains all seven repeats, whereas 1–279, 1–242, and 1–158 contain the first five, four, and three repeats, respectively. The first four tetratricopeptide repeats (1–242) are capable of supporting the interaction with the 30-kDa domain. In B, the filter for NY-CO-7 and pAS-hsc70 also is given. For both NY-CO-7 (B) and TPR1 (C), the three tetratricopeptide repeats together with about 20 amino acid residues after the C terminus of the third repeat are sufficient for the interaction with the 30-kDa domain.

![Diagram B](image2.png)

**Fig. 6. Interaction of hSGT mutants with the 30-kDa domain.** A, the two-hybrid assays. The upper panel shows the primary structures of the polypeptides, and the position of the tetratricopeptide repeats is shown as a hatched bar. The lower panel shows the results of the two-hybrid assays. Single colonies of yeast harboring pACT2-hSGT or the mutants and pAS-30K were selected by growth in medium lacking leucine and tryptophan. They were then patched and used for a β-galactosidase assay. The blue filters are shown here. pLam5-1 from CLONTECH was used as control. B, in vitro binding assay. The GST fused with the 30-kDa domain of hsc70 was mixed with hSGT or with the truncated hSGT, and the complexes were pulled down with glutathione-Sepharose for gel electrophoresis. A Coomassie Blue-stained gel is shown here. Lane 1, the GST fusion protein (Mr = 51,000); lane 2, hSGT (Mr = 36,000); lane 3, hSGT without the Q-rich region (Mr = 30,000); lane 4, hSGT without most of the residues after the tetratricopeptide repeats (Mr = 25,000); lane 5, the TPR domain of hSGT (Mr = 15,000). The major upper band in lane 3, varied from preparation to preparation, was subjected to amino acid sequencing. Only the sequences of the His tag were obtained. Therefore, they probably resulted from aggregation of the polypeptides. Lanes 6–9, the mixtures of fusion proteins with hSGT, with hSGT without Q-rich region, with C-terminal truncated hSGT, and with the TPR domain, respectively. GST itself did not pull down any of these hSGT-related proteins (not shown). Molecular weight markers are as follows: β-galactosidase (116,000), phosphorylase (97,000), bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (29,000), and lysozyme (14,000).

1–242) are sufficient to support relatively strong interaction with the 30-kDa domain. However, removal of the fourth tetratricopeptide (mutant 1–158) results in a significant loss in such interaction. These results imply that, for TPR2, the tetratricopeptide repeats are responsible for its interaction with hsc70 and that the J-domain is not needed for this interaction.

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Gel filtration was used to demonstrate the association of hsc70 with the TPR domain of hSGT. Briefly, bovine hsc70 (A), the TPR domain of hSGT (B), and the mixtures of hsc70 and the TPR domain (C) were applied to a Superdex 200 HR column, and fractions were collected. Either 25 µl (A and B) or 35 µl (C) of the samples from each experiment were withdrawn and resolved by SDS gels. The gels corresponding to column fractions 22–47 are given here. The proteins were exclusively in these fractions. At least two distinct peaks can be observed for bovine hsc70 (A), and the peak around fractions 33 and 34 (indicated by vertical lines) is the monomer. The TPR domain (B) elutes as a single peak around fractions 42–44 (horizontal lines). In the mixtures (C), however, the TPR domain of hSGT also appears in fractions 32 and 33 (indicated by the circle), one fraction ahead of the hsc70 monomer (see A).

teria (Fig. 6B, lanes 2–5), we therefore performed in vitro binding assay to assess their association with the 30-kDa domain. The purified polypeptides were incubated with the 30-kDa domain fused to GST (Fig. 6B, lane 1), the complexes formed were isolated by glutathione-Sepharose and displayed by gel electrophoresis. As shown in Fig. 6B (lanes 6–9), all of the mutants that we prepared are capable of complexing in vitro with the 30-kDa domain. Considering together the results for all of the clones shown in Fig. 1, a fragment containing the tetratricopeptide repeats with a few flanking residues is sufficient for their specific interaction with the 30-kDa domain of hsc70.

In order to determine if the TPR domain of hSGT may interact with the native hsc70, we first incubated the purified protein (Fig. 6B, lane 5) with bovine hsc70. The mixtures were then subjected to gel filtration analysis. Under the experimental conditions, hsc70 separated into two major peaks, and the peak with elution volumes of about 13–14 ml represents hsc70 in monomeric form (Fig. 7A, fractions 33 and 34). On the other hand, the TPR domain of hSGT elutes as a single peak with an elution volume of about 16.5–18 ml (Fig. 7B, fractions 41–45). However, in the mixtures of hsc70 and the TPR domain of hSGT, the TPR domain appears in two distinct fractions (Fig. 7C). It either elutes at the same location as the polypeptide by itself or coelutes with hsc70. The result suggests that the TPR domain of hSGT indeed forms complexes with hsc70. Moreover, from Fig. 7C, this TPR domain appears only associated with monomeric hsc70.

The next question considered in this study was whether or not the tetratricopeptide repeats in these proteins are necessary for their interaction with hsc70. Therefore, we disrupted the tetratricopeptide repeats and then determined if the interactions might be affected. On the basis of the two-hybrid assays, removal of a portion of the repeats in NY-CO-7, TPR1, and hSGT resulted in a complete loss of interaction with the 30-kDa domain (Fig. 8). However, TPR2 without the first two and one-half repeats (Δ1–102) remained capable of interacting with the 30-kDa domain, although the interaction became much weaker (Fig. 8). This relatively weak signal is not a manifestation that TPR2(Δ1–102) interacts as an unfolded protein with the 30-kDa domain, since TPR2(Δ1–102) failed to show any significant interaction with the 30-kDa (Δph1) (Fig. 9A). Moreover, this weaker interaction is not due to a reduction in the expression of fusion proteins (Fig. 9, B and C). Removal of the first three repeats (Δ1–199), nevertheless, rendered TPR2 incapable of interaction with hsc70 (Fig. 8), although both the AD-TPR2(Δ1–199) and the BD-30-kDa domain were expressed in yeast cells (Fig. 9, B and C). Therefore, despite the fact that the situation for TPR2 is slightly more complicated, these results support the view that the tetratricopeptide repeats in NY-CO-7, TPR1, TPR2, and hSGT are necessary for interacting with hsc70.

From the results shown in Figs. 5–8, one may conclude that three tetratricopeptide repeats arranged in tandem appear as the major determinant for the association with the C-terminal 30-kDa domain of hsc70. To investigate whether or not there are unique structural features in these tetratricopeptide repeats, we aligned the amino acid sequences of each repeat to identify the conserved residues. The first three tetratricopeptide repeats of p60/Hop were also included, since they are responsible for the association with hsc70 (38). As shown in Fig. 10A, it is evident that the first two repeats are quite conserved and the last one is less conserved. The consensus sequences were shown in Fig. 10A. Since the structure of the TPR domain of protein phosphatase 5 was determined (27), we used it to build a model to localize the conserved residues in three dimensions (Fig. 10B). The model reveals that a number of the conserved residues are clustered at the interface of the first and the second repeats. While some of these conserved residues are there solely for structural reasons, others may be responsible for the specific interaction. However, from the model, it is not obvious where the surface that is responsible for interacting with the 30-kDa domain resides.

DISCUSSION

Evidently, it is feasible to use the yeast two-hybrid system with either hsc70 or its C-terminal 30-kDa substrate binding domain as baits to screen for proteins interacting with hsc70. However, under our assay system, some positive clones obtained by the 30-kDa domain, including p60/Hop, fail to show significant interaction with the bait composed of full-length hsc70. Nevertheless, these clones should be bona fide hsc70-interacting proteins, since they are capable of forming complexes with native hsc70 (Fig. 4). Recently, contrary to our results, Gebauer et al. (17) and Demand et al. (37) using a distinct assay method showed that p60/Hop interacts with the...
full-length hsc70. We have adopted their selection schemes for the two-hybrid experiments. Indeed, interaction between full-length hsc70 and p60/Hop could be demonstrated (data not shown). The system used by us thus appears to be more stringent than simply selecting by growth in histidine dropout medium. Therefore, while we might have reduced the number of false positives, we could also have missed pairs with relatively weak interaction.

All of the hsc70-interacting proteins isolated in this investigation (Fig. 1) contain tetratricopeptide repeats. For NY-CO-7, TPR1, and hSGT, three repeats arranged in tandem with a few flanking residues appear necessary and sufficient for their association with the C-terminal domain of hsc70. The sequences of the flanking residues do not share any significant homology. Conceivably, they might be important for maintaining the structural integrity of the respective TPR domains. For instance, they could play a role in stabilizing the termini of the TPR domains. For NY-CO-7, hSGT, and TPR1, the secondary structures of the repeats for the four repeats are needed for interacting with hsc70 (Fig. 5A).

Based on sequence comparison, one would predict that the first three repeats are more likely to mediate the interaction with hsc70. Indeed, if growth on medium lacking histidine was used as the selection criterion in the two-hybrid assay, the interaction between TPR2-(1–158) with the 30-kDa domain can be expected, since Hip/p48 does not interact with the 30-kDa domain of hsc70. Moreover, these consensus sequences are different from those of the tetratricopeptide repeats in proteins involved in the cell cycle regulation. Thus, while tetratricopeptide repeats appear as a common structural motif in proteins, they can be divided into distinctive subgroups according to their interacting partners.

However, TPR2 appears to be more intricate. The results of the two-hybrid analysis with single colonies show that the first four repeats are needed for interacting with hsc70 (Fig. 5A).

Based on sequence comparison, one would predict that the first three repeats are more likely to mediate the interaction with hsc70. Indeed, if growth on medium lacking histidine was used as the selection criterion in the two-hybrid assay, the interaction between TPR2-(1–158) with the 30-kDa domain can be demonstrated. Yet, it fails to interact with the 30-kDa (data not shown). However, removal of the first two and one-half repeats in TPR2 did not completely abolish the interaction with the 30-kDa domain. On the other hand, the deletion mutant containing the last four repeats and the J-domain did not show significant interaction with the 30-kDa domain (Fig. 8; Δ1–199). The lack of interaction did not result from a reduction in the expression of the fusion proteins (Fig. 9, B and C, lane 3). Thus, the C-terminal region of the third repeat might be important for mediating the interaction with the 30-kDa domain. If so, one needs to elucidate its role in interacting with hsc70.
More recently, Young et al. (41) showed that the TPR domains in some other proteins, including FKBP52, FKBP54, and hTOM34, are responsible for their interaction with hsp90. Thus, we decided to align the sequences of the tetratricopeptides in these proteins. After comparison, it becomes clear that the consensus sequences of the tetratricopeptide repeats of hsp90-interacting proteins are quite similar to those of hsc70-interacting proteins. They share identical residues in a number of locations (Fig. 10B). Interestingly, just like that of hsc70, the C terminus of hsp90 also ends with the sequence of EEVD. Although it remains to be proven, it is likely that some of these conserved residues in both types of tetratricopeptide repeats are responsible for interacting with the EEVD motif. However, it remains to be determined what are the structural elements in these two classes of TPR domains governing the specificity toward hsp90 or hsc70.

In conclusion, we have used the yeast two-hybrid system and isolated several putative hsc70-interacting proteins. These proteins all contain the tetratricopeptide repeats. These repeats are necessary and perhaps also sufficient for their interactions with the C-terminal region of hsc70. However, the functional significance of these interactions still needs to be elucidated.

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Addendum—During revision of this manuscript, Ballinger et al. (42) identified a new hsc70-interaction protein, CHIP. CHIP is identical to NY-CO-7, except for a difference in one amino acid residue. In NY-CO-7, amino acid 52 is alanine instead of valine.

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