Heat shock cognate protein 70 chaperone-binding site in the co-chaperone murine stress-inducible protein 1 maps to within three consecutive tetratricopeptide repeat motifs

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Murine stress-inducible protein 1 (mSTI1) is a co-chaperone homologous with the human heat shock cognate protein 70 (hsc70)/heat shock protein 90 (hsp90)-organizing protein (Hop). The concomitant interaction of mSTI1 with hsp70 and hsp90 at its N- and C-termini respectively is mediated by the tetratricopeptide repeat (TPR) motifs in these regions. With the use of co-precipitation assays, we show here that the N-terminal TPR domain of mSTI1 without extensive flanking regions is both necessary and sufficient to mediate a specific interaction with hsc70. In contrast, other TPR-containing co-chaperones require TPR flanking regions for target substrate recognition, suggesting different mechanisms of TPR-mediated chaperone–co-chaperone interactions. Furthermore, the interaction between mSTI1 and hsc70 was analysed to ascertain the effect of replacing or deleting conserved amino acid residues and sequences within the three

TPR motifs constituting the N-terminal TPR domain of full-length mSTI1. Replacement of a bulky hydrophobic residue in TPR1 disrupted the interaction of mSTI1 with hsc70. A highly conserved sequence in TPR2 was altered by deletion or single amino acid replacement. These derivatives retained a specific interaction with hsc70. These results are consistent with a model in which conserved residues within the N-terminal TPR region of mSTI1 contribute differentially to the interaction with hsc70, and in which TPR1 has a significant role in targeting mSTI1 to hsc70. The contribution of the TPR domain mutations and deletions are discussed with respect to their effect on target substrate interactions.

Key words: heat shock protein 70, heat shock protein 90, protein phosphatase 5, protein–protein interaction, stress protein.

INTRODUCTION

Murine stress-inducible protein 1 (mSTI1) is a co-chaperone that is homologous with the human heat shock cognate protein 70 (hsc70)/heat shock protein 90 (hsp90)-organizing protein (Hop) [1]. Hop is an indispensable component of a dynamic hetero-complex chaperoning machine that is involved in the functional maturation of a number of unrelated protein substrates [2–4]. The earliest detectable components of this heterocomplex include hsp70, hsp40 and the hsp70-interacting protein (Hip). This is followed by a transient interaction between hsc70 and hsp90 mediated by Hop. With the release of Hop, a number of cohort proteins associated with the mature heterocomplex interact with hsp90, including members of the class of immunophilins (FKBP52, FKBP51 and CyP-40) [5–7].

A conserved sequence motif evident in Hip, Hop and the immunophilins [8] is the tetratricopeptide repeat (TPR) motif. The TPR motif is a degenerate 34-residue repeat [9], the functional importance of which has been demonstrated in chaperone–co-chaperone interactions. In some instances, TPR flanking regions in addition to the TPR motifs themselves might have an essential or modulatory role in these TPR-mediated interactions.

The immunophilins CyP-40, FKBP51 and FKBP52 contain three TPR motifs that mediate their interactions with hsp90 [10–12]. Acidic and basic α -helical regions flanking the respective N- and C-terminal ends of the immunophilin TPR domains are

also necessary for hsp90 binding [12]. In addition, the association of FKBP51 with hsp90 requires a more extensive TPR C-terminal flanking region than FKBP52 [13]. Protein phosphatase 5 (PP5) is another TPR-containing protein associated with hsp90 in which extensive TPR flanking regions are necessary for chaperone recognition [14]. The N-terminal TPR domain of PP5 comprising three consecutive TPR motifs and 51 C-terminal flanking residues is targeted to the chaperone heterocomplex machinery via direct interaction with hsp90 [14]. The central and C-terminal TPR domain of mSTI1/Hop mediates its interaction with hsp90 [2,3,15]. Unlike the immunophilins and PP5, Hop is able to retain a moderate hsp90-binding ability even after removal of the C-terminus including the 23 residues of the most C-terminal TPR motif [3]. Removal of the N-terminal domain of Hop to within 11 residues of the central TPR domain did not affect hsp90 interaction [3]. Competitive binding studies between the immunophilins, PP5 and Hop suggest that they bind to an overlapping TPR acceptor site on hsp90 [16–18].

TPR domains of both mSTI1/Hop and Hip mediate their interactions with hsp70 [2,3,15,19,20]. However, these co-chaperones do not share a common TPR acceptor site on hsp70 [21,22]. The proposed TPR acceptor sites for Hip and Hop are the N-terminal ATPase domain and C-terminal domain of hsp70 respectively, suggesting that their mechanism of protein recognition differs. In addition to the TPR domain itself, the interaction of Hip with hsp70 requires the participation of

Abbreviations used: GST, glutathione S-transferase; Hip, hsp70-interacting protein; Hop, hsc70/hsp90-organizing protein; hsc, heat shock cognate protein; hsp, heat shock protein; IPTG, isopropyl β -D-thiogalactoside; mSTI1, murine stress-inducible protein 1; PP5, protein phosphatase 5; TPR, tetratricopeptide repeat.

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an acidic, positively charged α -helix after the TPR domain [19,20].

TPR motifs therefore confer different binding specificities on TPR proteins towards distinct sets of partner proteins, despite their underlying structural similarity. At the primary structural level, eight consensus residues have been identified on the basis of their high level of conservation at positions 4, 7, 8, 11, 20, 24, 27 and 32. The crystal structure of the N-terminal TPR domain of PP5 has been solved to a resolution of 2.5 Å [23]. Each TPR motif consists of a pair of anti-parallel α -helices constituting domains A and B respectively, with domain A spanning consensus residues 4, 7, 8 and 11, and domain B spanning consensus residues 20, 24, 27 and 32 [9,24]. Consecutive TPR motifs generate a series of tandemly arranged anti-parallel α -helices, a sufficient number of which form a right-handed superhelix encompassing an amphipathic groove or channel. It has been proposed that the amphipathic TPR channel might accommodate the α -helix of an interacting polypeptide sequence. Evidence in support of a TPR-binding groove for PP5 [25] suggested that conserved, basic residues directed towards such a binding groove interact with acidic residues within the C-terminal domain of hsp90. Although this mechanism of TPR-mediated interaction might be conserved in hsp90-interacting TPR proteins, it is important to ascertain whether a similar mechanism underlies the interaction of TPR proteins with other chaperones. In expanding such a model, it is also important to elucidate the TPR features that direct differential protein-binding specificities.

In the present study, the ability of mSTI1 derivatives to recognize specifically, and interact stably with, hsc70 was examined, and the implications of these changes for TPR-mediated interactions are discussed. The C-terminal domain of mSTI1 was truncated to generate a fragment including the N-terminal TPR domain and 11 C-terminal flanking residues. We report here that this N-terminal TPR domain of mSTI1 is both necessary and sufficient for specific recognition of hsc70. The results support the classification of TPR co-chaperones into two distinct subdivisions. In addition, highly conserved TPR consensus residues in the N-terminal TPR domain of full-length mSTI1 were replaced by single amino acid substitutions, each with a distinct impact on hsc70 recognition. Finally, a highly conserved region was removed by in-frame deletion. Our results indicate that TPR1 within the N-terminal TPR domain of mSTI1 has a more significant role than TPR2 in the targeting of mSTI1 to hsc70.

EXPERIMENTAL

Materials

All reagents were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) or Roche Molecular Biochemicals (Indianapolis, IN, U.S.A.) unless stated otherwise. Restriction enzymes, specialist molecular biology enzymes and Hybond enhanced chemiluminescence (ECL[®]) nitrocellulose were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A.). All tissue culture media and supplements were obtained from Highveld Biological (Pty) Ltd. (Gauteng, S.A.). Mutagenesis kits and the pQE30 plasmid were purchased from Stratagene (La Jolla, CA, U.S.A.) and Qiagen (Valencia, CA, U.S.A.) respectively. Hsc70 purified from bovine brain was generously provided by Dr. M. Cheetham (Department of Ophthalmology, University College London, London, U.K.). The monoclonal antibody SPA-820 was purchased from StressGen Biotechnologies Corp. (Victoria, BC, Canada) and nickel-chelating Sepharose Fast Flow was obtained from Pharmacia Biotech (Uppsala, Sweden). The

hydroxyapatite Bio-Gel HTP and gel-filtration molecular mass standards were both obtained from Bio-Rad Laboratories (Hercules, CA, U.S.A.).

Preparation of mSTI1 cDNA derivatives

For the heterologous production of mSTI1 in *Escherichia coli*, the mSTI1 cDNA was inserted into the expression vector pGEX3X [26] to generate the recombinant plasmid pGEX3X2000 coding for the fusion protein of glutathione S-transferase (GST) with mSTI1, GST-543 [15]. Mutant constructs were generated from the pGEX3X2000 parental plasmid. The deletion mutant pGEX3X1032 encoding the C-terminal truncated protein GST-N109 was generated by digestion of pGEX3X2000 with *Afl*II/*Not*I, treatment of the restricted fragments with T4 DNA polymerase to generate fully base-paired ends, and ligation of the ends with T4 DNA ligase. The mutant plasmids coding for the mSTI1 recombinant derivatives GST-543(Y27A) (in which Tyr²¹ had been replaced with Ala) and GST-543(Y41S) were generated by site-directed mutagenesis with a double-stranded whole-plasmid linear amplification procedure (QuikChange Site-Directed Mutagenesis Kit). For the construction of the GST-543(Y27A)-encoding plasmid, the overlapping primer pair 5'-CCTTACAGTGCCTAGCGAGGCAATTAAAC-3' (forward primer) and 5'-GTTTAATTGCCTCGCTAGCGCACTGTAAGG-3' (reverse primer) were used to introduce a unique *Nhe*I restriction site by silent mutation as well as the desired site-directed mutation encoding a Y27A replacement through a single base-pair change. The mutant plasmid encoding GST-543(Y41S) was generated in a similar manner and contained a unique *Cla*I restriction site introduced by silent mutation as well as a single base-pair substitution that replaced Tyr⁴¹ with Ser in the expressed protein. The overlapping primer pair used was 5'-CATGTGCTCTCCAGCAATCATCTGCAGCCTAC-3' (forward primer) and 5'-GTAGGCTGCAGATCGATTGCTGGAGAGCAGATG-3' (reverse primer). A double-stranded whole-plasmid PCR-based exponential amplification procedure (ExSite Site-Directed Mutagenesis Kit) was used to generate a third mutated plasmid from pGEX3X2000 encoding GST-543(Δ 37-47). The primer pair 5'-TACGCCAAGAAAGGAGACTACC-3' (forward primer) and 5'-CTGGGGATCTAGTTTAATTGC-3' (reverse primer) flanked the internal coding region to be deleted; it resulted in the loss of a *Pst*I restriction site for selection of the mutated plasmid. The mSTI1 cDNA previously cloned into the expression vector pBluescript SK(-) (Stratagene) to generate pSK2000 [1] was PCR-amplified with the forward primer 5'-ATAGCATGCATGGAGCAGGTGAAT-3' and the universal reverse primer M13UP (5'-GTAAAACGACGGCCAGT-3'). PCR amplification with the forward primer generated a *Sph*I restriction site immediately 5' of the mSTI1 start ATG codon in the resultant PCR product. PCR amplification with the M13UP reverse primer encompassed a *Sal*I restriction site 3' of the mSTI1 stop codons. The PCR product was digested with *Sph*I/*Sal*I and cloned into *Sph*I/*Sal*I-restricted pQE30 to generate pQE30-2000 coding for histidine-tagged mSTI1, HIS-543. For all mutant constructs, the presence of the desired mutations and the absence of undesired mutations were confirmed by DNA sequencing [27].

Tissue culture and harvesting of NIH 3T3 crude extracts

NIH 3T3 fibroblasts were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) foetal calf serum and penicillin (100 i.u./ml)/streptomycin (100 µg/ml) solution. Once the cells had reached confluence, they were washed three times

Figure 1 Alignment of amino acid sequences of the N-terminal TPR domain of mSTI1 homologues

A multiple sequence alignment of mSTI1 homologues was generated. Abbreviations: mmSTI1, *Mus musculus*; hsSTI1, *Homo sapiens*; cgSTI1, *Cricetulus griseus*; dmSTI1, *Drosophila melanogaster*; gmSTI1, *Glycine max*; scSTI1, *Saccharomyces cerevisiae*; lmSTI1, *Leishmania major*; spSTI1, *Schizosaccharomyces pombe*; acSTI1, *Acanthamoeba castellanii*. The consensus code is shown above the multiple sequence alignment. Symbols: letter, conserved residue; +, conserved positive charge; -, conserved negative charge; #, conserved hydrophobic residue; ^, conserved small residue; ~, conserved polar residue; full stop, non-conserved position; blank, a gap (inserted or deleted residue) is present at this position. The three N-terminal TPR motifs in mSTI1 are shown below the multiple sequence alignment. In each TPR motif, domains A and B have been underlined in bold and the TPR consensus positions have been designated. The amino acid residues targeted for substitutions and deletions have been highlighted in black and unfilled boxes respectively.

with ice-cold PBS and treated with trypsin/versene. The cells were collected by centrifugation, washed three times with ice-cold PBS and resuspended in 50 mM Tris/HCl (pH 8.0)/150 mM NaCl/0.02 % sodium azide/100 µg/ml PMSF/1 µg/ml aprotinin/1 % (v/v) Triton X-100. Lysis occurred at 4 °C with gentle agitation for 10 min before the cellular debris was removed by centrifugation for 30 min at 4 °C and 12000 *g*. The supernatant was removed and used immediately or stored for up to 1 week at -70 °C.

Production and purification of GST-tagged mSTI1

The production of GST fusion proteins was induced in a 500 ml culture volume of exponential-growth-phase *E. coli* XLI Blue cells by the addition of isopropyl β-D-thiogalactoside to a final concentration of 1 mM. Induction was continued for 5–7 h at 37 °C before the *E. coli* cells were harvested and lysed by mild sonication in a minimal volume of PBS. The cellular debris was removed by centrifugation for 20 min at 4 °C and 12000 *g*; the crude cell extract was added to 500 µl of a 50 % (w/v) slurry of glutathione-agarose beads in PBS. Binding was allowed to occur for 1 h at 4 °C with gentle inversion. The beads were washed three times in ice-cold PBS before the bound GST fusion proteins were eluted by the addition of 1 ml of elution buffer [50 mM Tris/HCl (pH 8.0)/5 mM GSH]. Purified proteins were removed and used immediately or stored at -70 °C until usage the following day.

HSC70-mSTI1 glutathione-agarose co-precipitation assays

Each GST fusion protein and GST on its own were added to PBS and 50 µl of a 50 % (w/v) slurry of glutathione-agarose beads

such that the final sample volume was 1.0 ml and the final protein concentration was 0.3 µM. Recoupling of GST and GST fusion proteins was done for 1 h at 4 °C with gentle inversion. The beads were collected by centrifugation and washed three times with ice-cold PBS as before. After the final wash, hsc70 purified from bovine brain was added to a final concentration of 0.03 µM in the presence or absence of additional salt (up to 600 mM NaCl, final concentration), and the volume was made up to 1 ml with PBS. Alternatively, 250 µg of NIH 3T3 crude extract rather than purified hsc70 was used in the binding reaction. Binding was done for 1 h at 4 °C with gentle inversion. The beads were washed three times with 1 ml volumes of ice-cold PBS, then resuspended in 70 µl of SDS/PAGE reducing sample-loading buffer [28]. The beads were collected by centrifugation and 30 µl of supernatant from each sample was separated by SDS/PAGE and transferred to Hybond ECL Nitrocellulose membrane. The co-elution of hsc70 with the GST-mSTI1 fusion proteins was revealed by immunodetection and chemiluminescent autoradiography [BM Chemiluminescence Western Blotting Kit (Mouse/Rabbit)]. The monoclonal primary antibody (product no. SPA-820) specific for hsc70 and hsp70 was diluted 1:500 in Tris-buffered saline blocking solution [50 mM Tris/HCl (pH 7.5)/150 mM NaCl/1 % (w/v) non-fat milk powder].

Production and purification of histidine-tagged mSTI1

Production of HIS-543 in *E. coli* XLI Blue (pQE30-2000) cells was induced by the addition of isopropyl β-D-thiogalactoside to a final concentration of 1 mM. Growth was continued for at least 5 h at 37 °C before the cells were harvested by centrifugation. The cells were lysed by mild sonication in buffer A [100 mM sodium phosphate (pH 7.4)/300 mM NaCl/80 mM imidazole/1 % (v/v) Triton X-100/100 µg/ml PMSF/1 mM aprotinin] and

the cellular debris was removed by centrifugation. The supernatant from the cells of a 1 litre culture was added to 10 ml of nickel-chelating Sepharose Fast Flow and binding occurred for 30 min at 4 °C with gentle inversion. The Sepharose was collected by centrifugation and washed with 10 column vol. of buffer A. The bound HIS-543 fusion protein was eluted by the addition of buffer A containing imidazole at a final concentration of 0.3 M. The eluent was buffer-exchanged into 10 mM potassium phosphate, pH 7.5, with a Sephadex G25 column, and then loaded on a hydroxyapatite Bio-Gel HTP column. A linear gradient from 10 to 200 mM potassium phosphate, pH 7.5, at a flow rate of 0.275 ml/min was used to elute the histidine-tagged mSTI1. Fractions containing HIS-543 were pooled and stored at 4 °C for up to 1 week.

Molecular mass determination by gel filtration

Aliquots (50 μ l) of HIS-543 over a range of concentrations were loaded on a Phenomenex Biosep SEC-S2000 gel-filtration column equilibrated in 20 mM sodium phosphate (pH 7.5)/100 mM NaCl. The column was run at 1 ml/min and was calibrated with Bio-Rad gel-filtration molecular mass standards. The molecular mass of HIS-543 was determined by logarithmic interpolation.

RESULTS

Generation of mSTI1 derivatives

An amino acid alignment of mSTI1 and its homologues was established to target potentially important conserved TPR residues (Figure 1). A BLAST search [29] was performed to identify mSTI1 homologues; closely related high-scoring sequences were aligned. Some of the sequences, notably those from *Caenorhabditis elegans*, *Trypanosoma cruzi*, *Plasmodium falciparum* and *Methanobacterium thermoautotrophicum*, were not included in the representation of the multiple sequence alignment because their N-terminal domains did not share significant identity with mSTI1, despite a significant overall identity. The alignment of mSTI1 homologues revealed highly conserved residues in their respective N-terminal domains. Certain conserved residues were targeted for amino acid substitutions and deletion; these are shown in Figure 1 in black and unfilled boxes respectively. In GST-543(Y27A), the replacement of a conserved tyrosine residue by alanine was encoded by a single base-pair replacement in TPR1 of mSTI1. This single amino acid substitution mapped to the TPR consensus position 24 of domain B (Figure 1). A Tyr $\rightarrow$ Ser substitution in TPR2 of mSTI1 [GST-543(Y41S)] mapped to the domain A consensus position 4 (Figure 1). Finally, a highly conserved region in mSTI1 that coincided with the first ten residues of TPR2 domain A (Figure 1) was removed by in-frame deletion mutagenesis. Additional GST-mSTI1 derivatives used in this study included GST-N109 and GST-C334 [15], the former being a C-terminal truncation of mSTI1 to within 11 residues of the N-terminal TPR domain, and the latter being an N-terminal truncation of mSTI1 to within 16 residues of the central TPR domain. All the GST-mSTI1 fusion proteins remained soluble on heterologous expression and purification; there was no evidence of aggregation as monitored by spectrophotometric analysis at 340 and 600 nm. Furthermore, for those GST-mSTI1 fusion proteins that were able to associate with hsc70, these interactions were sensitive to a progressive increase in ionic strength and were finally abolished by the addition of 0.4–0.5 M NaCl (see Figures 2E, 3C and 4C). Hence hsc70 exhibited recognition of these GST-mSTI1 fusion proteins as specific partner proteins rather than as misfolded substrates. The sequestration of a misfolded substrate away from the cellular

Figure 2 Interaction of GST-N109 with hsc70

As indicated above the lanes, 0.3 μ M GST-543, GST-C334, GST-N109 or GST were coupled to glutathione-agarose beads. NIH 3T3 mouse cell extract (250 μ g) or purified hsc70 at a final concentration of 0.03 μ M was added to the reaction mixtures in the presence or absence of NaCl; the reaction was left to reach equilibrium for 1 h at 4 °C. The glutathione-agarose-bound GST-mSTI1 fusion proteins and their co-precipitating partner proteins were extracted into SDS/PAGE sample buffer, separated by SDS/PAGE [15% (w/v) gel] and transferred to nitrocellulose. The co-elution of hsc70 with the GST-mSTI1 fusion proteins was detected by immunodetection and chemiluminescent autoradiography. (A) The presence of hsc70 in NIH 3T3 crude extracts was used as a positive control for Western blot analysis. (B) The Western blot was stained with Ponceau S to detect the relative transfer efficiency of the GST fusion proteins. (C) The interaction of the GST-mSTI1 fusion proteins with purified hsc70 resulted in heavy background staining. (D) Native hsc70 in the presence of complex competitive inhibitors (NIH 3T3 extract proteins) decreased this background. (E) To provide evidence that the GST-mSTI1 derivatives were correctly folded and specifically recognized by hsc70, the sensitivity of the interactions of GST-543 and GST-N109 with hsc70 to increased ionic strength were examined. A final concentration of 0.2–0.5 mM GST-543 and GST-N109 was used in determining the effect of increased ionic strength.

environment by the molecular chaperone function of hsc70 typically cannot be disrupted by an increased ionic strength. Taken together, these observations suggested that the GST-mSTI1 derivatives were not misfolded.

N-terminal TPR domain of mSTI1 is necessary and sufficient for hsc70 interaction

Purified bovine hsc70 or NIH 3T3 mouse cell extracts were added to equivalent concentrations of GST-mSTI1 fusion proteins bound to glutathione-agarose beads. The individual components comprising protein complexes and co-precipitated on glutathione-agarose beads were separated by SDS/PAGE, and transferred to nitrocellulose membrane; the presence or absence of hsc70 was determined by Western blot analysis (Figure 2A).

Figure 3 Interaction of GST-543(Y27A) and GST-543(Y41S) with hsc70

GST-543(Y27A) and GST-543(Y41S) were included in the glutathione–agarose co-precipitation assay. (A) Ponceau S staining of the samples transferred to nitrocellulose compared the relative transfer efficiencies of the GST–mSTI1 fusion proteins. GST-543(Y27A) transferred more efficiently than the other samples. (B) The interaction of the GST–mSTI1 fusion proteins with hsc70 in crude extracts of NIH 3T3 cells was detected by Western blot analysis and autoradiography. Co-precipitation assays with purified hsc70 gave the same result but with a higher background of hsc70 interaction for the GST-C334 and GST negative controls (results not shown). (C) Evidence that GST-543(Y41S) was correctly folded and specifically recognized by hsc70 was provided by the inclusion of NaCl in the co-precipitation assay.

The transfer of the GST fusion proteins to nitrocellulose occurred with equivalent efficiency such that hsc70 levels detected were quantitatively comparable (Figure 2B). The use of purified hsc70 in the glutathione–agarose pull-down assay (Figure 2C) resulted in a high background of binding, as was evident for GST and for GST-C334, which was previously shown to be unable to mediate hsc70 interaction [15]. The binding of hsc70 to GST-543 and GST-N109 clearly exceeded the level of background binding. When NIH 3T3 mouse cell extracts containing native hsc70 were used in the co-precipitation assay, the background association was undetectable (Figure 2D). In both assays it was clear that GST-N109 was able to associate with hsc70 at levels comparable to those of GST-543. Furthermore, this association of GST-N109 with hsc70 was similar to that of unmodified, full-length mSTI1 (GST-543) in that both were sensitive to a progressive increase in ionic strength and were finally abolished by the addition of 0.4–0.5 M NaCl (Figure 2E). Hence these GST–mSTI1 fusion proteins were not misfolded and were recognized as specific partner proteins to hsc70. Taken together, these

Figure 4 Interaction of GST-543(Δ37–47) with hsc70

GST-543(Δ37–47) was included in the glutathione–agarose co-precipitation assay. (A) Ponceau S staining of the samples transferred to nitrocellulose, showing that GST-543(Δ37–47) transfer occurred less efficiently. (B) Western analysis and chemiluminescent autoradiography were used to detect the interaction of the GST–mSTI1 fusion proteins with hsc70 in NIH 3T3 mouse cell extracts. (C) The addition of NaCl to the glutathione–agarose co-precipitation assay was used to provide evidence that GST-543(Δ37–47) was correctly folded and was therefore specifically recognized by hsc70.

results show that the mSTI1 N-terminal TPR domain with an 11-residue C-terminal flanking region (GST-N109) was necessary and sufficient for the interaction with hsc70.

Substitution at TPR consensus position 24 in TPR1 of mSTI1 disrupts hsc70 interaction

Because the N-terminal TPR domain of mSTI1 was sufficient to mediate a specific interaction with hsc70, TPR consensus residues were replaced to assess the impact on hsc70 interaction. Conserved residues targeted for replacement mapped to the TPR consensus positions 24 [GST-543(Y27A)] and 4 [GST-543(Y41S)] (Figure 1). Glutathione–agarose co-precipitation assays with NIH 3T3 mouse cell extracts (Figure 3B) and purified hsc70 (results not shown) rendered reproducible results. The interaction of each of GST, GST-C334 and GST-543(Y27A) with hsc70 was undetectable. The association of hsc70 with GST-543(Y27A) was

undetectable despite a greater efficiency of transfer of this GST fusion protein to nitrocellulose (Figure 3A). Conversely, the association of hsc70 with GST-543(Y41S) was clearly detectable at a slightly lower level than that of GST-543 (Figure 3B). This interaction was sensitive to an increase in ionic strength and was undetectable in the presence of high [NaCl] in the co-precipitation assay (Figure 3C). Therefore hsc70 associated with GST-543(Y41S) as a specific partner protein and not as a misfolded substrate.

Removal of a highly conserved block in TPR2 of mSTI1 does not disrupt hsc70 interaction

A highly conserved region has been identified in the N-terminal TPR domain of all mSTI1 homologues (Figure 1); a similar conserved sequence occurs in the C-terminal TPR domain [1,30]. These highly conserved regions might be important in heat shock protein interaction or they might have an as yet undetermined function. The N-terminal conserved region in mSTI1 was therefore targeted for deletion. In GST-543(Δ 37–47), the N-terminal conserved region mapped to the entire A domain of TPR2 (Figure 1). Despite the removal of the entire A domain of TPR2, this derivative was still able to bind hsc70 (Figure 4B). This interaction occurred at lower levels than that of GST-543, although the transfer efficiency of this modified mSTI1 protein to nitrocellulose was also lower than that of the remaining GST fusion proteins (Figure 4A). Furthermore, the interaction of this deletion derivative with hsc70 was rendered undetectable by the addition of high [NaCl] in the glutathione-agarose pull-down assay (Figure 4C), once again confirming specific recognition of partner protein.

Purification and gel filtration of histidine-tagged mSTI1

We purified histidine-tagged mSTI1 to 98% purity and determined the estimated molecular mass of the protein by gel-filtration chromatography (results not shown). Histidine-tagged mSTI1 was eluted as a single species with an apparent molecular mass of approx. 143 kDa, suggesting that it exists either in an extended conformation or as a dimer.

DISCUSSION

The TPR motif is a protein-binding structural module, the alteration of which has an impact on the biological function of TPR proteins and complexes within the cell. Despite the degenerate nature of the TPR motif, the basic structural unit comprising the TPR motif is conserved in all TPR domains, which in turn demonstrate different partner-protein-binding specificities. In elucidating the mechanism of TPR-mediated interactions, it is necessary to identify the parameters that impart functional specialization on different TPR proteins.

We have shown that the N-terminal TPR domain of mSTI1, including the three N-terminal TPR motifs and a limited C-terminal flanking region of 11 residues (Figure 2), is both necessary and sufficient to mediate the specific recognition of purified and endogenous hsc70. Unlike PP5, Hip and the immunophilins [12–14,19,20], extensive regions flanking the mSTI1 TPR domains are not imperative for mSTI1–hsp90 and mSTI1–hsc70 interactions. These results might support the classification of TPR proteins involved in chaperone-co-chaperone interactions into two different groups (Figure 5) encompassing those that do not require extensive TPR flanking regions (less than 20 residues) and those that require more extensive flanking regions (more than 20 residues). The necessity

Class I

Class II

Figure 5 Classification of TPR proteins interacting with hsp90 and hsp70

Class I represents the group of TPR co-chaperones in which interaction with heat shock protein does not require extensive TPR flanking regions. This class includes mSTI1/Hop [3,15]. Class II represents the group of TPR co-chaperones in which extensive TPR flanking regions (more than 20 residues) are necessary for interaction with heat shock protein, including the immunophilins (CyP40, FKBP51 and FKBP52) [10,12,13], Hip [19,20] and PP5 [14]. The TPR motifs in each of the TPR proteins are shaded in grey. In each of the immunophilins, the peptidylprolyl isomerase (PPase) domain is indicated, and the putative calmodulin binding domain is filled with black. For each co-chaperone the TPR boundaries are demarcated as shown above each diagram and the heat-shock-protein-binding region is delineated beneath each diagram. Arrowheads indicate the extent of flanking regions required for interaction with heat shock protein. In PP5, FKBP51 and FKBP52 the extent of N-terminal flanking region required for hsp90 binding has not been clarified, as indicated by the underlying dotted line. In FKBP51 and FKBP52, the extended dotted line delineating the hsp90-binding region is equivalent to the CyP40 region shown to be necessary for hsp90 interaction. It is thought that these regions are similarly necessary in FKBP51 and FKBP52. In Hip, removal of the N-terminus to within 14 residues of the TPR domain allowed hsp70 recognition, albeit at lower levels than full-length Hip [20]. Additional N-terminal flanking regions might be necessary for hsp70 recognition [19], as indicated by the underlying dotted line.

of TPR flanking regions, or a lack thereof, might complement the functional specialization of the TPR motifs defined by their degeneracy and might have mechanistic implications for TPR-mediated interactions. The TPR flanking regions might direct the TPR protein to the correct target sequence, recognition of which is then consolidated by the TPR motifs themselves.

Although the N-terminal TPR domain is necessary and sufficient for hsc70 binding in this assay, the mSTI1–hsc70 interaction might be modulated or regulated by additional determinants within the tertiary or quaternary conformation of mSTI1. Both rat Hop [22] and the yeast co-chaperone STI1

(47% identity with mSTI1) [31] behave as a dimer in solution. We have similarly found that histidine-tagged mSTI1 is eluted as a single species with an apparent molecular mass closely approximating that of a dimer on gel-filtration chromatography (results not shown). The functional significance of the dimerization of mSTI1 homologues and its role in chaperone recognition remains to be determined.

Having established the importance of the N-terminal TPR domain of mSTI1 in hsc70 interaction, TPR consensus residues were targeted for replacement (Figure 1). The proposed role of the TPR consensus residues in maintaining the structural integrity of the TPR motif has been rationalized by the resolution of the crystal structure of the N-terminal TPR domain of PP5 [23]. Non-conservative substitution of these consensus residues would be predicted to destabilize the TPR helical packing with functional repercussions depending on the extent of destabilization. Tyr²⁷ and Tyr⁴¹ of mSTI1, both of which are TPR consensus residues, were replaced; the impacts on hsc70 interaction were found to be different. Tyr²⁷, which maps to the TPR consensus position 24 in the B domain of TPR1, was replaced with an alanine. This single amino acid substitution resulted in the complete abrogation of hsc70 recognition (Figure 3). Tyr⁴¹, which maps to the TPR consensus position 4 in the A domain of TPR2, was replaced with serine. In contrast with GST-543(Y27A), GST-543(Y41S) maintained a specific interaction with hsc70 at levels comparable to those of full-length mSTI1 (Figure 3).

In addition to the Y27A and Y41S replacements, a highly conserved region mapping to the A domain of TPR2 (Figure 1) was removed by in-frame deletion. Surprisingly, GST-543(Δ 37–47) was able to recognize hsc70 specifically, albeit at lower levels than full-length mSTI1 (Figure 4). The replacement of the consecutive residues YSN with CGR in the highly conserved TPR2 A domain of Hop similarly resulted in a decreased association with hsp70 [32]. In addition, the replacement of Arg⁷⁴ with Ala in the A domain α -helix of TPR2 in PP5 diminished, but did not abrogate, hsp90 binding [25]. The results obtained for the Y41S substitution and Δ 37–47 deletion in mSTI1, the YSN to CGR replacements in Hop [32] and the R74A substitution in PP5 [25] all suggest that deletions and substitutions in domain A of TPR2 do not sufficiently destabilize the region of the TPR domain that is important for the interactions of these proteins with their respective chaperones. The interaction of hsc70 with mSTI1 might be more sensitive to alterations in TPR1 than in TPR2. The highly conserved sequence within TPR2 of mSTI1, although not critical to hsc70 interaction, might have a significant biological role in an alternative modulatory function of mSTI1.

The N-terminal TPR domain of mSTI1 shares significant amino acid identity (31.2%) with that of PP5. Homology modelling [33] of the N-terminal TPR domain of mSTI1, with the PP5 structure [23] as a template, predicted that the sequentially adjacent TPR motifs of mSTI1 define a series of anti-parallel α -helices potentially forming a groove or channel, as has been proposed for PP5 [23,25] (results not shown). We propose that TPR1 of mSTI1 might make a more significant contribution than TPR2 to a putative TPR groove involved in hsc70 recognition. Resolution of the three-dimensional structure of mSTI1 and mSTI1–hsc70 complexes, as well as a mutational analysis of solvent-exposed residues directed into this putative groove, would be necessary to confirm this proposal.

Note added in proof (received 1 December 1999)

It has recently been shown that removal of the TPR-flanking regions of the proteins NY-CO-7, TPR1 and hSGT did not affect their respective interactions with hsc70 [34]. Hence these proteins may be included in our classification of TPR proteins as Class I proteins.

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