Chaperone ligand-discrimination by the TPR-domain protein Tah1

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Tah1 [TPR (tetratricopeptide repeat)-containing protein associated with Hsp (heat-shock protein) 90] has been identified as a TPR-domain protein. TPR-domain proteins are involved in protein–protein interactions and a number have been characterized that interact either with Hsp70 or Hsp90, but a few can bind both chaperones. Independent studies suggest that Tah1 interacts with Hsp90, but whether it can also interact with Hsp70/Ssa1 has not been investigated. Amino-acid-sequence alignments suggest that Tah1 is most similar to the TPR2b domain of Hop (Hsp-organizing protein) which when mutated reduces binding to both Hsp90 and Hsp70. Our alignments suggest that there are three TPR-domain motifs in Tah1, which is consistent with the architecture of the TPR2b domain. In the present study we find that Tah1 is specific for Hsp90, and is able to bind tightly the yeast Hsp90, and the human Hsp90α and Hsp90β proteins, but not the yeast Hsp70 Ssa1 isoform. Tah1 acheives ligand discrimination by favourably binding the methionine residue in the conserved MEEVD motif (Hsp90) and positively discriminating against the first valine residue in the VEEVD motif (Ssa1). In the present study we also show that Tah1 can affect the ATPase activity of Hsp90, in common with some other TPR-domain proteins.

Key words: ATPase activity, heat-shock protein 90 (Hsp90), heat-shock protein 70 (Hsp70), tetratricopeptide-repeat-containing protein associated with heat-shock protein 90 (Tah1), tetratricopeptide repeat (TPR) domain, stress-inducible protein 1/heat-shock protein organizing protein/p60 (Sti1/Hop/p60).

INTRODUCTION

The molecular chaperone Hsp (heat-shock protein) 90 plays a central role in the maturation and activation of key signalling proteins (reviewed in [1,2]) that include steroid hormone receptors [3,4] and protein kinases [5–9] among others [1,2,10,11]. An up-to-date comprehensive list can be found at http://www.picard.ch/downloads/downloads.htm. The activation of such proteins (client proteins) is regulated by the association of specific co-chaperones that complex with Hsp90 at various stages of the chaperone cycle [12,13]. A number of these co-chaperones play an apparent role in recruiting client proteins to the Hsp90 chaperone complex. For steroid hormone receptors it appears that the receptor is complexed with the TPR (teratricopeptide repeat)-domain containing co-chaperone Hop (Hsp-organizing protein)/Sti1 (stress-inducible protein 1; the yeast homologue of Hop) and Hsp70. Binding of Hop/Sti1 to the conserved MEEVD motif at the extreme C-terminal end of Hsp90 delivers the steroid hormone receptor to Hsp90 (reviewed in [4,14]). Protein kinases on the other hand are complexed with Cdc37 (cell-division cycle 37 homologue)/p50 (reviewed in [15]) that binds the N-terminal domain of Hsp90 [16]. In *Sacharomyces cerevisiae*, Tah1 (TPR-containing protein associated with Hsp90; YCR060W), consisting of 111 amino acid residues, was identified as an Hsp90-interacting co-chaperone in three independent studies [17–19]. Tah1 is thought to associate with Pih1 (protein interacting with Hsp90) and together complex with the essential DNA helicases, Rvb (RuVB-like)1 and Rvb2, that are key components of the chromatin remodelling complexes Ino80 and SWR-C [19]. Tah1 contains a single TPR domain with at least two TPR motifs [19].

Numerous crystallographic structures of TPR-domain-containing proteins have been solved [20–25]. These reveal a common motif that is composed of a degenerate 34-amino-acid sequence that forms an antiparallel α-helical hairpin, which in turn cluster to form a domain with a grooved surface that acts as a peptide-binding site. TPR domains mediate protein–protein interactions in numerous protein complexes [26–28]. The question of ligand discrimination by TPR domains has been addressed [29–31]. TPR-domain proteins such as Hip (Hsp-interacting protein) have been implicated in the regulation of the ATPase activity of Hsp70, whereas Hop/Sti1 has been shown to inhibit the ATPase activity of Hsp90, to activate Ssa1, but to have no affect on the vertebrate Hsp70 [32–36]. The TPR domains of Hsp70 and Hsp90 co-chaperones interact with the extreme C-terminal amino acid sequences of their respective chaperone [34,37–41], which share a common motif (EEVD). Residues immediately upstream of the conserved EEVD motif of Hsp70 and Hsp90 achieve selective binding to different TPR-domains [20–23,25]. In contrast, the co-chaperone CHIP (C-terminal of Hsp70-interacting protein), involved in the ubiquitylation of chaperone-bound client proteins [42,43], binds to both Hsp70 and Hsp90 by associating with the MEEVD (Hsp90) or the IEEVD (human Hsp70) motif and avoiding further interaction with upstream amino acid residues. This is achieved by a hydrophobic pocket that accommodates the methionine residue (M in MEEVD) from Hsp90 and the isoleucine residue (I in IEEVD) of human Hsp70 and in doing

Abbreviations used: CHIP, C-terminal of heat-shock protein 70-interacting protein; Cpr6, cyclosporin-sensitive proline rotamase 6; FKBP51, FK506-binding protein 51; GST, glutathione transferase; Hop, heat-shock-protein-organizing protein; Hsp, heat-shock protein; ITC, isothermal titration calorimetry; Sti1, stress-inducible protein 1 (the yeast homologue of Hop); cSti1, C-terminal of Sti1; Tah1, tetratricopeptide-repeat-containing protein associated with Hsp90; TPR, tetratricopeptide repeat.

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so twists the upstream peptide, which differs between the two chaperones, and hoists it clear of the TPR-domain groove [24]. In contrast, structural information showed that for Hop/Sti1 the N-terminal TPR domain (TPR1) interacts with Hsp70, whereas the central TPR domain (TPR2a) binds to Hsp90 [21]. However, the situation might be more complex in that mutations in the third TPR domain (TPR2b) disrupt association with both Hsp70 and Hsp90 [39]. A high-affinity ligand for the TPR2b domain has not been found [21,29].

In the present study we show that the TPR domain of Tah1 most closely resembles the TPR2b domain of Hop/Sti1 and address whether Tah1 is an Hsp90-specific co-chaperone or whether it can also associate specifically with Ssa1. We show that Tah1 is specific for Hsp90 and that ligand discrimination is achieved by favourable binding of the methionine residue of the conserved MEEVD motif of Hsp90 and by positively discriminating against the first valine residue of the VEEVD motif of Ssa1. We also characterize the oligomeric state of Tah1 and investigate its ability to regulate the ATPase activity of Hsp90.

MATERIALS AND METHODS

Sequence alignments

The amino acid sequences for Tah1, Sti1 and Hop were obtained from GenBank®. The accession numbers were: human Hop, NM_006819; *S. cerevisiae* Sti1, Z74935; and *S. cerevisiae* Tah1, NC_001135. The sequence alignment was generated using ClustalW [44] on-line at the EMBL-EBI (European Molecular Biology Laboratory-European Bioinformatic Institute).

Protein production and Hsp90 ATPase activity assays

Expression and purification of wild-type and mutant forms of His-tagged yeast Hsp90, His-tagged Hsp90β, mouse His-PreScission-tagged CHIP and His-tagged C-terminal domain of Sti1 (cSti1) have been described previously [24,35,45]. The yeast Tah1, the human Hsp90α and the C-terminal domain of yeast Hsp90 were cloned into pRSETA as NheI-EcoRI, NheI-PstI and NheI-HindIII DNA fragments respectively, and expressed as His-PreScission-tagged proteins. The yeast Ssa1 gene was cloned into pRSETA as an NheI-HindIII DNA fragment and expressed as a His-tagged fusion protein. The same protein purification procedure as was used for yeast Hsp90 was employed to purify the C-terminal domain of Hsp90, Hsp90α, Ssa1 and Tah1. PreScission cleavage of the His tags was carried out overnight at 4 °C, following the manufacturer's protocol (GE Healthcare). The cleaved sample was then passed through a 1 ml GST (glutathione transferase)-HiTrap column, equilibrated in 20 mM Tris/HCl (pH 7.5), 140 mM NaCl and 2.7 mM KCl, to remove GST-tagged PreScission. The flow-through was collected, desalted and then subjected to Talon-affinity chromatography to remove uncleaved His-PreScission protein. The Talon resin flow-through was then concentrated and subjected to Superdex 75- or 200-HR gel-filtration chromatography, as appropriate. All purified proteins were dialysed against 20 mM Tris/HCl (pH 7.5) containing 1 mM EDTA and 1 mM DTT (dithiothreitol) and then concentrated using Vivaspin concentrators (Sartorius) with an appropriate molecular mass cut-off. The ATPase assays were performed in triplicate as previously described [45,46], using 2 μM yeast Hsp90 and either 20 μM Tah1 or 30 μM geldanamycin. The protein concentration was determined by using the molar absorption coefficient (ε) of the protein (wild-type and mutant yeast Hsp90, 54 050 $M^{-1} \cdot cm^{-1}$; cleaved C-terminal domain of yeast Hsp90, 18 260 $M^{-1} \cdot cm^{-1}$; cleaved Hsp90α, 59 625 $M^{-1} \cdot cm^{-1}$; Hsp90β, 58 135 $M^{-1} \cdot cm^{-1}$; Ssa1, 20 045 $M^{-1} \cdot cm^{-1}$; cleaved CHIP, 29 380 $M^{-1} \cdot cm^{-1}$; cSti1, 34 480 $M^{-1} \cdot cm^{-1}$; and cleaved Tah1, 10 295 $M^{-1} \cdot cm^{-1}$).

ITC (isothermal titration calorimetry): stoichiometry and K_d determination.

The heat of interaction was measured on a MSC system (Microcal) with a cell volume of 1.458 ml. For Tah1 and CHIP interactions, 9–11 aliquots of 27 μl of 300 μM Tah1 or CHIP were injected into 30 μM yeast Hsp90, yeast Hsp90 M705V, human Hsp90α, human Hsp90β or yeast Hsp90 C-terminal domain at 4 °C (Tah1 experiments) or 30 °C (CHIP experiments) in 20 mM Tris/HCl (pH 8.0) containing 1 mM EDTA and 5 mM NaCl. For peptide interactions, either 9–11 aliquots of 27 μl of 300 μM peptide or 14–15 aliquots of 400 μM peptide were injected into 30 μM Tah1 at 4 °C in 20 mM Tris/HCl (pH 8.0) containing 1 mM EDTA and 5 mM NaCl. For cSti1 interactions, 11 aliquots of 27 μl of 300 μM cSti1 were injected into 30 μM yeast Hsp90 or yeast Hsp90 M705V at 30 °C in 20 mM Tris/HCl (pH 8.0) containing 1 mM EDTA and 5 mM NaCl. The heat of dilution was determined in a separate experiment by diluting protein or peptide into buffer, and the corrected data were fitted using a non-linear least square curve-fitting algorithm (Microcal Origin) with three floating variables: stoichiometry, binding constant and change in enthalpy of interaction. For the Ssa1-Tah1 and Tah1-VEEVD peptide experiment the stoichiometry was fixed at $N = 1$. All peptides were accurately weighed on an analytical balance and dissolved in dialysis buffer.

Relative molecular mass determination by gel filtration

Aliquots of 0.5 ml of PreScission-cleaved Tah1 (1.4 $mg \cdot ml^{-1}$) were loaded on to a Superdex 75 HR 16/60 column equilibrated in 20 mM Tris/HCl (pH 7.4), 1 mM EDTA and 250 mM NaCl. The column was run at 0.5 $ml \cdot min^{-1}$ and was calibrated using gel-filtration standards from Bio-Rad (bovine thyroglobulin, M_r 670 000; bovine γ-globulin, M_r 158 000; chicken ovalbumin, M_r 44 000; horse myoglobin, M_r 17 000; and vitamin B12, M_r 1350) and GE Healthcare (blue dextran 2000, M_r 2 000 000; bovine albumin, M_r 67 500; chicken ovalbumin, M_r 43 000; bovine chymotrypsinogen A, M_r 25700 and bovine ribonuclease A, M_r 13 700). The relative molecular mass of Tah1 was determined by logarithmic interpolation.

Analytical ultracentrifugation

PreScission-cleaved Tah1 was dialysed against 20 mM Tris/HCl (pH 7.5) containing 1 mM EDTA and 5 mM NaCl. Sedimentation equilibrium analysis was performed at 20 °C in a Beckman XL-I analytical ultracentrifuge, following a standard operating procedure SE_IO_07.1 at sample concentrations of 7.0 and 0.5 $mg \cdot ml^{-1}$ (547 and 39 μM respectively). After initial scans at 726 ***g*** to check for the presence of any large aggregates, a final rotor speed of 39 030 ***g*** was selected. A 12 mm optical path length centrepiece was used in the cell. Scans were taken every 60 min and data were logged to disk using the Beckman software. After 24 h, no further change could be detected in the scans, and the run was terminated. The data were analysed by three methods using the INVEQ , the recently derived Mfit and a local (OS-X-profit) version of the widely used NONLIN algorithm. K_a values were converted into K_d values by simple reciprocation.

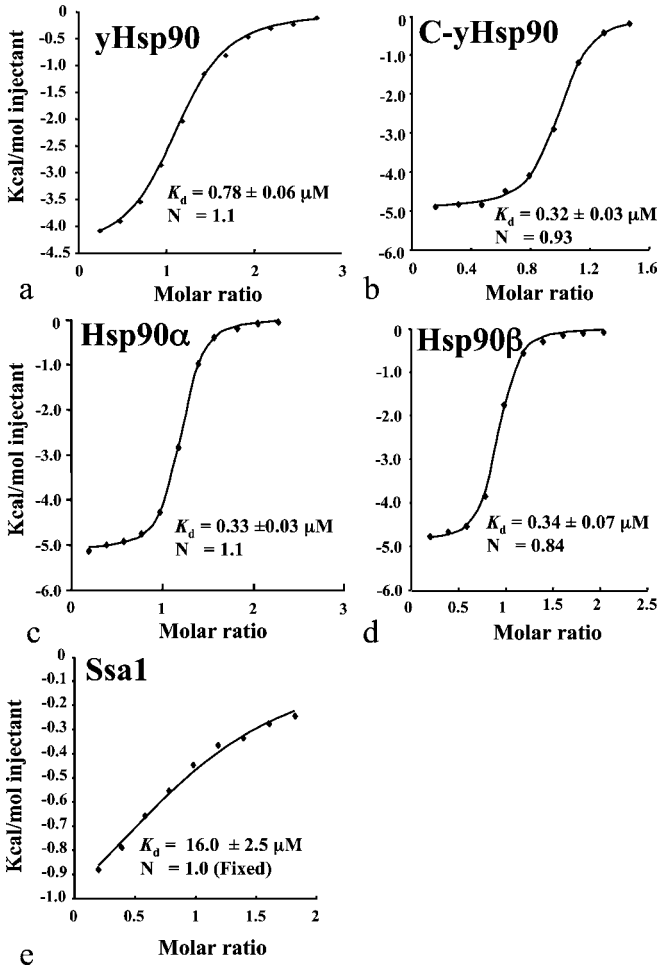

Figure 1 ITC of Tah1 with Hsp90 and Ssa1

ITC by injecting Tah1 into (**a**) yeast Hsp90 (yHsp90), (**b**) the C-terminal domain of yeast Hsp90 (C-yHsp90), (**c**) Hsp90α, (**d**) Hsp90β and (**e**) Ssa1. The results show that the Tah1 interaction with full-length yeast Hsp90, Hsp90α, Hsp90β and C-yHsp90 is significantly tighter than against the yeast Hsp70, Ssa1.

RESULTS

Tah1 selectively binds Hsp90

Tah1 has been identified as a TPR-domain protein that interacts with Hsp90. Our sequence alignments suggest that Tah1 is most similar to the TPR2b-domain of Hop/Sti1 (Supplementary Figure S1 at http://www.BiochemJ.org/bj/413/bj4130261add.htm), which influences the binding of Hop to both Hsp70 and Hsp90 [39]. In order to investigate the ability of Tah1 to bind both Ssa1 and Hsp90 we used ITC to determine the extent of any interaction. Figure 1(a) shows that Tah1 binds to the full-length yeast Hsp90 ($K_d = 0.78 \pm 0.06$ μM) with a stoichiometry of 1:1, whereas Figure 1(b) shows that the binding site is solely located in the C-terminal domain of Hsp90 ($K_d = 0.32 \pm 0.03$ μM). Table 1 summarizes all ITC data in the present study. Tah1 was also shown to be able to interact with the human Hsp90α ($K_d = 0.33 \pm 0.03$ μM) and Hsp90β ($K_d = 0.34 \pm 0.07$ μM) paralogues with a similar affinity (Figures 1c and 1d). However, its interaction with the yeast Ssa1 was significantly weaker ($K_d = 16 \pm 2.5$ μM; Figure 1e), where the strength of the interaction is reflected in the shape of the binding curve for which a steeper gradient in the observed curve indicates tighter binding (compare Figures 1a and 1e), and suggests that Tah1 is an Hsp90-specific co-chaperone.

Table 1 Summary of the binding affinities of Tah1, CHIP and cSti1

(a)

Tah1 interactions with . . .	K_d (μM)	Stoichiometry (N)
Yeast Hsp90	0.78 ± 0.06	1.1
C-terminal domain of Hsp90	0.32 ± 0.03	0.93
Hsp90α	0.33 ± 0.03	1.1
Hsp90β	0.34 ± 0.07	0.84
Ssa1	16 ± 2.5	1.0 (fixed)
Yeast Hsp90 peptides		
MEEVD	35.4 ± 4.4	1.1
TEMEEVD	0.95 ± 0.04	1.2
PADTEMEEVD	0.73 ± 0.04	1.0
PADAAMEEVD	0.75 ± 0.04	0.81
PADTEAEEVD	3.3 ± 0.2	0.89
PADTEVEEVD	9.8 ± 0.7	1.1
Human Hsp90 peptides		
DDTSRMEEVD	0.9 ± 0.06	1.1
EDASRMEEVD	1.0 ± 0.19	0.81
Ssa1 peptides		
VEEVD	48.8 ± 5.5	1.0 (fixed)
PTVEEVD	10.6 ± 1.4	1.0
AEGPTVEEVD	28.9 ± 3.1	1.2
AEGPTAEEVD	14.8 ± 0.7	1.1
AEGPTMEEVD	4.2 ± 0.5	0.98

(b)

Hsp90 and M705V interactions	K_d (μM)	Stoichiometry (N)
CHIP–Hsp90	4.2 ± 0.63	1.2
CHIP–Hsp90 M705V	6.7 ± 0.62	1.1
cSti1–Hsp90	0.04 ± 0.01	0.88
cSti1–Hsp90 M705V	0.2 ± 0.03	0.98
Tah1–Hsp90 M705V	13 ± 1.7	0.83

Comparison of MEEVD peptide binding to Tah1 and TPR2a

In the TPR1–VEEVD and TPR2a–MEEVD complexes most of the direct hydrogen-bonded interactions with bound peptide involve the main chain and are therefore sequence independent. Both complexes have a highly conserved two-carboxylate clamp. In the TPR2a complex the terminal main chain carboxylate of MEEVD is hydrogen bonded to Lys^{229}, Asn^{233} and Asn^{264}. These residues are also conserved in Tah1 (Lys^{8}, Asn^{12} and Asn^{43} respectively; see Supplementary Figure S1 and Figure 2). However, in contrast, residues that interact with the side chain of the terminal aspartate residue, which is bound by Lys^{301} and Gln^{298} in TPR2a, are not conserved in Tah1 (Thr^{70} and His^{73} respectively). Consequently, if a two-carboxylate clamp exists in the peptide-bound Tah1 complex it is clearly different to that seen in TPR2a. Other electrostatic interactions include a tetrahedrally co-ordinated water molecule that interacts with the carboxylate side chain of the terminal aspartate residue, and with the main chain carbonyl of Thr^{263} (Ser^{42} in Tah1), the guanidinium group of Arg^{305} (Arg^{77} in Tah1) and the side chain carbonyl of Asn^{264} (Asn^{43} in Tah1). The side chain amide of Asn^{264} is also involved in main chain interactions with the backbone amide of the terminal aspartate residue, whereas the guanidinium group of Arg^{305} also contacts the main chain carbonyl and amide (via a water molecule) of Glu^{-2} (S^{-6}RMEEVD^{0}). Furthermore, the hydroxy group of Tyr^{236} (Phe^{15} in Tah1; Supplementary Figure S1 and Figure 2) contacts the main chain carbonyl of Glu^{-3}, an interaction not possible with Phe^{15} of Tah1, whereas the side chain carbonyl group of Glu^{271} (Lys^{50} in Tah1) contacts the main chain amide

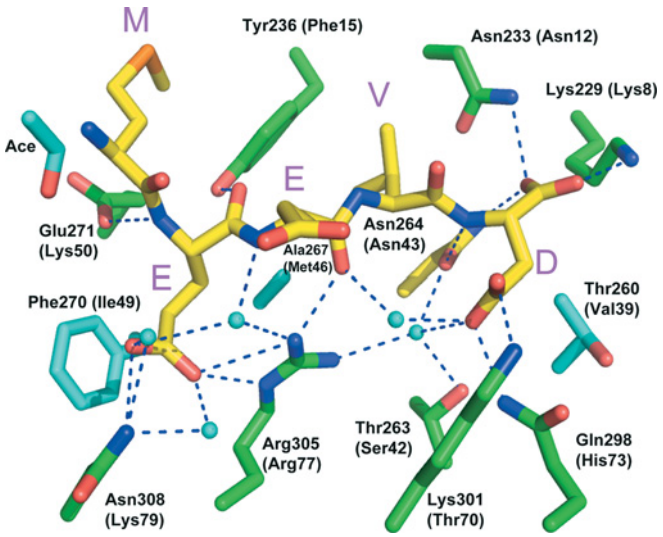

Figure 2 Pymol diagram showing the interaction of the MEEVD peptide with the TPR2a domain of Hop

Residues are labelled and those residues in brackets are the equivalent residues of Tah1. The single letter amino acid code is used for the MEEVD peptide sequence. Water molecules are shown as cyan coloured balls and polar interactions are shown as broken lines.

of Glu^{-3}. The only interactions to side chains (excluding the two carboxylate clamp) are between the guanidinium group of Arg^{305} and the side chain amide of Asn^{308} (Lys^{79} in Tah1) to the carboxylate group of Glu^{-3}. Additionally, Val^{-1} is involved in a hydrophobic interaction with a pocket formed by Asn^{233}, Tyr^{236} (Phe^{15} in Tah1), Asn^{264} and Ala^{267} (Met^{46} in Tah1), whereas the side chain of Met^{-4} binds between Tyr^{236} (Phe^{15} in Tah1) and Glu^{271} (Lys^{50} in Tah1). Two further van der Waals contacts involve the side chain of Glu^{-3} and Asp^{-0} with Phe^{270} (Ile^{49} in Tah1) and Thr^{260} (Val^{39} in Tah1) respectively. It should be noted that the Glu^{-2} side chain is not involved in interactions. In summary, our alignments suggest that there are significant residue differences between the TPR2a and the Tah1 TPR domain that point to differences in the precise way that they would bind the MEEVD peptide (Supplementary Figure S1 and Figure 2).

Ligand discrimination by Tah1 is similar to that of the TPR2a domain of Hop

For Hop, the residue at position −4 of Hsp70 ($I^{-4}EEVD^{0}$ or VEEVD for SsaI) and Hsp90 (MEEVD) primarily determines the specificity for the TPR1 and TPR2a domains respectively [29]. To determine whether Tah1 ligand discrimination was similar to that of TPR2a we investigated the ability of Tah1 to bind peptides representing the C-terminal ends of Hsp90 and Ssa1. The results in Figure 3 show that the binding affinity for the C-terminal peptides of yeast Hsp90 increases with increasing peptide length (MEEVD, $K_d = 35.4 \pm 4.4\ \mu M$; TEMEEVD, $K_d = 0.95 \pm 0.04\ \mu M$; PADTEMEEVD, $K_d = 0.73 \pm 0.04\ \mu M$; Figures 3a–3c) and that the decapeptide most probably represents the intact binding site for Tah1 (PADTEMEEVD, $K_d = 0.73 \pm 0.04\ \mu M$ and full-length yeast Hsp90, $K_d = 0.78 \pm 0.06\ \mu M$; Figures 1a and 3c respectively). Indeed, the binding of Tah1 to the human Hsp90α and Hsp90β decapeptide was also similar to that of the corresponding full-length proteins (Hsp90α decapeptide, DDTSRMEEVD, $K_d = 0.9 \pm 0.06\ \mu M$; Hsp90$\beta$ decapeptide, EDASRMEEVD, $K_d = 1.0 \pm 0.19\ \mu M$; full-length Hsp90$\alpha$, $K_d = 0.33 \pm 0.03\ \mu M$ and full-length Hsp90β, $K_d = 0.34 \pm 0.07\ \mu M$; Figures 1c, 1d, 3d and 3e). In contrast with the Hsp90 decapeptides, peptides representing the C-terminal end of Ssa1 showed a significantly weaker affinity for Tah1 (VEEVD, $K_d = 48.8 \pm 5.5\ \mu M$; PTVEEVD, $K_d = 10.6 \pm 1.4\ \mu M$; and AEGPTVEEVD, $K_d = 28.9 \pm 3.1\ \mu M$; Figures 4a–4c).

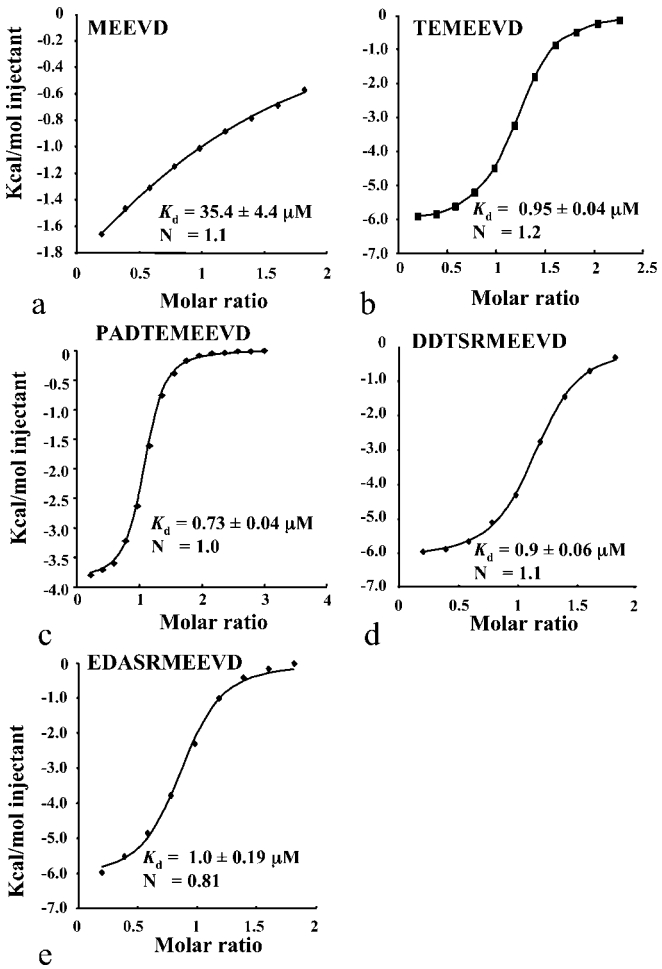

Figure 3 ITC of Tah1 with Hsp90 peptides carrying the MEEVD motif

ITC by injecting into Tah1 (**a**) the pentapeptide MEEVD, (**b**) the heptapeptide TEMEEVD and (**c**) the decapeptide PADTEMEEVD (all representing the C-terminal end of yeast Hsp90), and the decapeptides (**d**) DDTSRMEEVD and (**e**) EDASRMEEVD (representing the human Hsp90α and Hsp90β C-terminal ends). The results show that the Tah1 interaction increases in affinity with increasing length of the peptide and that decapeptides representing the extreme C-terminus of Hsp90α and Hsp90β bind with a similar affinity to the equivalent decapeptide of yeast Hsp90.

The methione residue of the conserved MEEVD motif confers tight Tah1 binding

The results so far show that Tah1 preferentially binds Hsp90 over Ssa1. Consequently, we next investigated the mechanism behind this selectivity. A comparison of the decapeptide sequences from a variety of cytoplasmic Hsp90s suggests that there is very little sequence conservation upstream of the conserved MEEVD motif, except at the −6 position (91 % conservation) and −5 position (68 % conservation) [29]. This suggests that position −6 (which is mainly a serine residue and rarely a threonine residue) may be critical for the specificity in the interaction with Tah1. Using ITC, the binding of the PAD*AA*MEEVD decapeptide (changes to wild-type sequence are shown in italic; $K_d = 0.75 \pm 0.04\ \mu M$; Figure 5a) was found to be similar to the decapeptide with

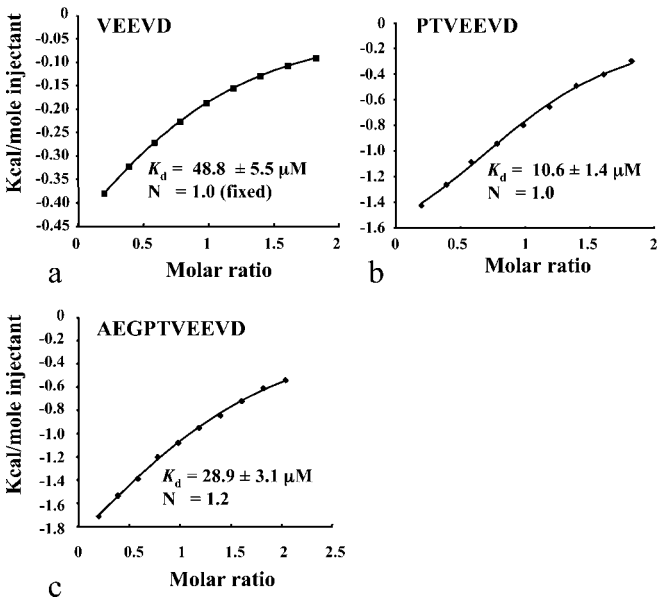

Figure 4 ITC of Tah1 and Ssa1 peptides containing the VEEVD motif

ITC by injecting into Tah1 (**a**) the pentapeptide VEEVD, (**b**) the heptapeptide PTVEEVD and (**c**) the decapeptide AEGEPTVEED, representing the extreme C-terminus of Ssa1. The results show that the Tah1 interaction with Ssa1 peptides remains weak and does not increase in affinity with increasing peptide length, in contrast with equivalent peptides based on Hsp90.

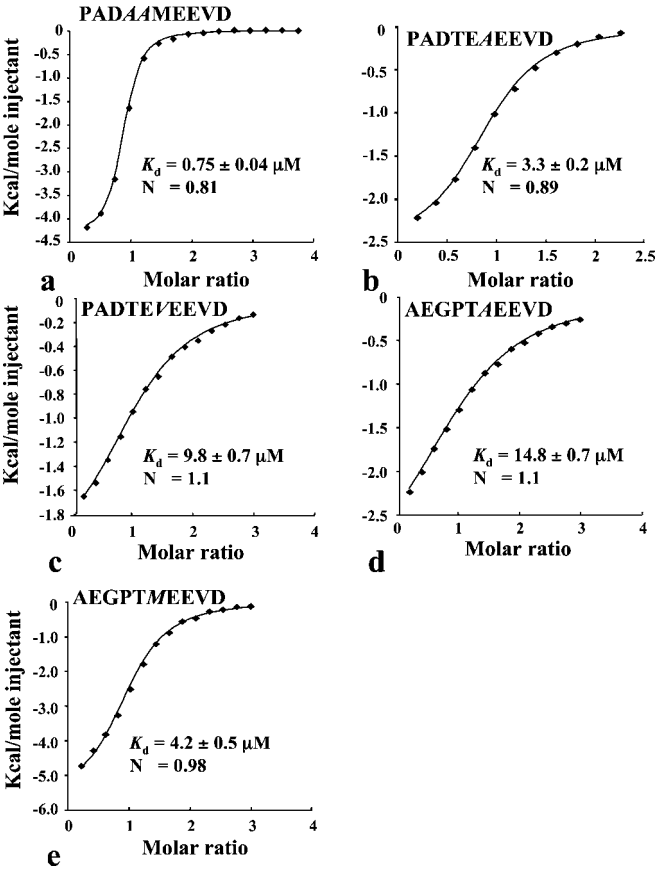

Figure 5 ITC with Tah1 and peptides with altered Hsp90 and Ssa1 sequences

ITC by injecting into Tah1 the modified decapeptides (**a**) PAD*AA*MEEVD, (**b**) PADTE*A*EEVD, (**c**) PADAA*V*EEVD, (**d**) AEGPT*A*EEVD and (**e**) AEGPT*M*EEVD. The results show that the upstream conserved TE motif (in PADTEMEEVD) of Hsp90 when replaced by Ala–Ala does not disrupt binding to Tah1, and that at position −4 a methionine residue is favoured over an alanine or valine residue. The results also show that a methionine residue, and to a lesser extent an alanine residue, at position −4 of the Ssa1 decapeptide increases the binding affinity of the peptide over that containing a valine resdiue at this position.

wild-type sequence (PADTEMEEVD, $K_d = 0.73 \pm 0.04\ \mu$M; Figure 3c). Consequently, tight binding of Tah1 to Hsp90 is determined within the conserved MEEVD motif, and upstream amino acids appear to be involved in mainly main chain interactions. However, since the EEVD motif is common to both Ssa1 and Hsp90 it follows that the conserved methionine residue of the MEEVD motif is responsible for affecting tight binding to Tah1. The results of the present study show that a decapeptide containing an alanine (PADTE*A*EEVD) or valine (PADTE*V*EEVD) residue in place of the conserved methionine residue in the MEEVD motif significantly reduced the affinity for Tah1 ($K_d = 3.3 \pm 0.2$ and $9.8 \pm 0.7\ \mu$M respectively; Figures 5b and 5c) relative to the wild-type decapeptide ($K_d = 0.73 \pm 0.04\ \mu$M; Figure 3c). Therefore the results suggest that ligand discrimination for the MEEVD peptide by Tah1 and the TPR2a domain of Hop [21] is similar in that both favour a methionine residue at position −4 of the MEEVD motif of Hsp90.

Tah1 positively discriminates against the valine residue at position −4 in the AEGPTVEEVD motif of Ssa1

The results so far suggest that the first valine residue in the VEEVD motif of Ssa1 prevents tight association with Tah1. Thus introducing an alanine or methionine residue at position −4 of the Ssa1 decapeptide should increase the binding affinity relative to a decapeptide containing a valine residue at this position. Our results show that this is indeed the case, where the decapeptides AEGPT*A*EEVD and AEGPT*M*EEVD were found to bind with increasing affinity ($K_d = 14.8 \pm 0.7\ \mu$M and $K_d = 4.2 \pm 0.5\ \mu$M respectively; Figures 5d and 5e) relative to the wild-type sequence decapeptide ($K_d = 28.9 \pm 3.1\ \mu$M; Figure 4c).

The M705V mutation of Hsp90 disrupts Tah1 binding

Having established with decapeptides that the methionine residue in the conserved MEEVD motif of Hsp90 confers specificity for Tah1 binding we investigated whether this was also true for the intact yeast Hsp90. We therefore made the yeast Hsp90 M705V mutation to see whether it would bind Tah1 significantly less tightly. First we tested the M705V mutation with CHIP, known to bind both Hsp90 and Hsp70, and then with the C-terminal domain of Sti1 (cSti1), which favours Hsp90 binding. As expected, the M705V mutation bound CHIP with more or less equal affinity ($K_d = 6.7 \pm 0.62\ \mu$M) relative to wild-type Hsp90 ($K_d = 4.2 \pm 0.63\ \mu$M; Figures 6a and 6b). A previous estimate for the CHIP and human Hsp90β interaction by ITC ($K_d = 4.9\ \mu$M) is consistent with the value obtained in the present study with yeast protein [24]. For cSti1, the M705V mutation bound less tightly ($K_d = 0.20 \pm 0.03\ \mu$M) than the wild-type protein ($K_d = 0.04 \pm 0.01\ \mu$M; Figures 6c and 6d). It is noted that previous estimates with full-length Sti1 appear to be slightly lower in affinity ($K_d = 0.24 \pm 0.07\ \mu$M and $0.33 \pm 0.03\ \mu$M; [35,47]) against the wild-type Hsp90, indicating that TPR1 may have a negative effect on the binding of the TPR2a domain. However, having established that the M705V mutation affected the binding of both CHIP and the cSti1 in a predictable manner, we next tested the binding of Tah1 and, as expected, we found that the M705V mutation inhibited the binding of Tah1 ($K_d = 13 \pm 1.7\ \mu$M) relative to wild-type protein ($K_d = 0.78 \pm 0.06\ \mu$M; Figures 1a and 6e).

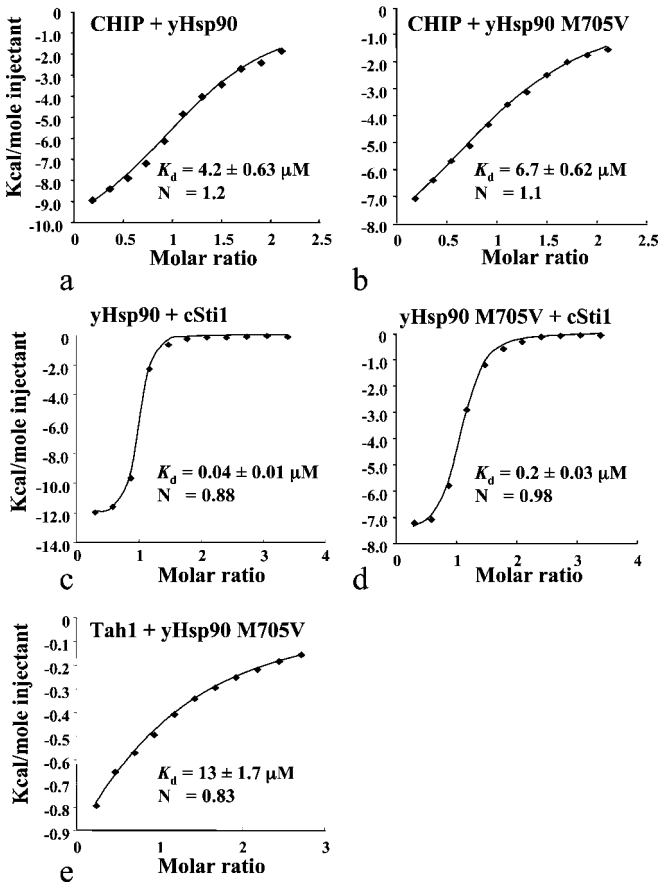

Figure 6 ITC of TPR-domain-containing proteins and the Hsp90 M705V mutation

ITC by injecting CHIP into (**a**) yeast Hsp90 (yHsp90) or (**b**) M705V mutant, or by injecting (**c**) yeast Hsp90 or (**d**) M705V mutant into cSti1, or by injecting (**e**) Tah1 into the M705V mutant. The results show that the M705V mutation of Hsp90 does not affect the binding of CHIP, but, in contrast, the binding of the cSti1 and Tah1 is significantly weaker.

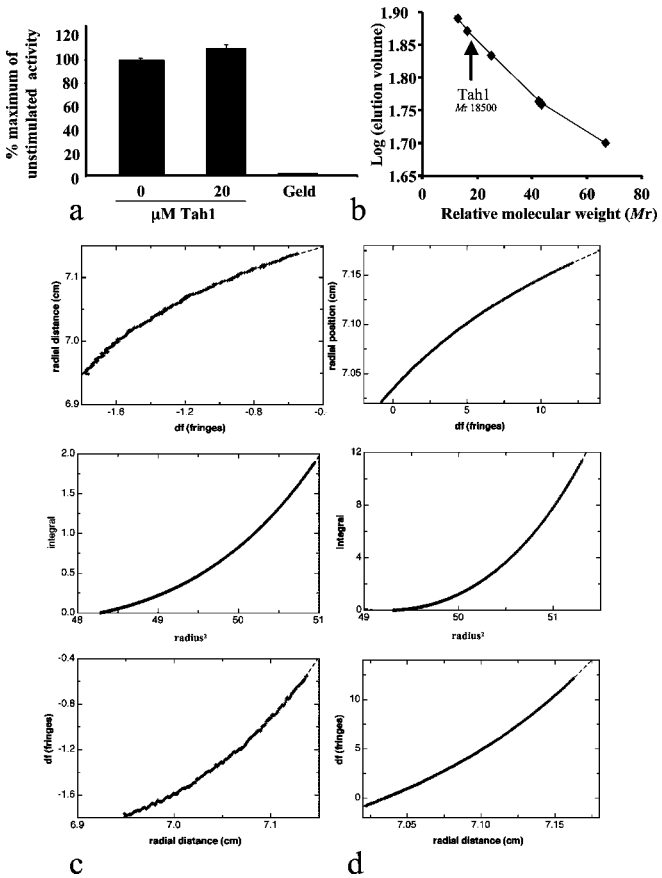

Figure 7 Tah1 activation of Hsp90 and determination of the relative molecular mass of Tah1

(**a**) ATPase activation of yeast Hsp90 by PreScission-cleaved Tah1; (**b**) gel-filtration chromatography of PreScission-cleaved Tah1; (**c** and **d**) analytical equilibrium sedimentation ultracentrifugation analyses (INVEQ, Mfit and NONLIN fits, top to bottom panels) of PreScission-cleaved Tah1 at 7.0 mg · ml^{-1} (**c**) and (**d**) 0.5 mg · ml^{-1}. The broken line represents the fit to the data points. The results show that PreScission-cleaved Tah1 can weakly activate the ATPase activity of Hsp90, elutes with a M_r of 18500 on gel filtration, which is close to the values estimated by analytical centrifugation [M(z) by Mfit = 15968 and 16106]. The results indicate that Tah1 is mostly a monomer under the conditions used.

Table 2 Summary of values for parameters estimated via sedimentation equilibrium analysis for Tah1 at two cell loading concentrations

Protein concentration (mg · ml^{-1})	K_d by INVEQ (mM)	M(z) by Mfit	M(z) by NONLIN′ (kDa)	$K_{d'}$ by NONLIN′ (mM)
7.0	3.9	15.968	16.062	1.80
0.5	0.51	16.106	15.845	0.15

Tah1 weakly stimulates the ATPase activity of Hsp90

TPR-domain-containing proteins have been implicated in the regulation of the ATPase activity of Hsp90. The first report of such regulation was for Sti1, which was shown to be a potent inhibitor of the ATPase activity of Hsp90 [35]. In contrast, the TPR-domain-containing co-chaperone, Cpr6 (cyclosporin-sensitive proline rotamase 6), has been shown to have a weak stimulatory affect on the ATPase activity of Hsp90 [45]. Using a 10-fold excess of Tah1 we were able to show in the present study a very weak stimulation of the ATPase activity of Hsp90 (Figure 7a).

Tah1 appears to be primarily monomeric

TPR-domain-containing proteins have been shown to be both monomeric [immunophillins such as, FKBP51 (FK506-binding protein 51) and Cpr6] and dimeric (Hop/Sti1 and CHIP) in nature [24,35,48]. We were interested in determining the oligomeric state of Tah1. We employed gel-filtration chromatography to determine the relative molecular mass of Tah1. Tah1 eluted, as a single peak, with a M_r of 18500 (Figure 7b). As Tah1 is basically a TPR domain it is reasonable to expect this protein to be slightly elongated, rather than globular, in common with other TPR-domain motifs. Consequently, on gel filtration it would appear slightly larger than M_r 12800 (cleaved Tah1). Our gel-filtration results therefore suggest that Tah1 is most probably monomeric. To support this we conducted an analytical sedimentation equilibrium analysis on Tah1. Good results with high-quality fits were obtained from both 7 and 0.5 mg · ml^{-1} samples, and these are shown in Figures 7(c) and 7(d). The Tah1 sample clearly cannot be simply monomeric, as the average molecular mass (see Table 2) by two independent methods confirms this. The excellent fit obtained with the INVEQ approach is essentially conclusive evidence that a dimeric species is present (estimated to be approx. 19 %), but probably not higher oligomers, at least in significant quantity. However, the single

experiment at 7 mg · ml^{-1} is not by itself capable of distinguishing between the presence of a dimer in reversible equilibrium with monomer, and an irreversible dimer being present. To make this distinction we must compare the two, in this case widely separated, solute concentrations. The predictions for the effect of dilution are that, if a reversible dimer is present, the K_d value estimated will be the same, but the average molecular mass will decrease markedly. However, if an irreversible dimer is present, the K_d value estimated will increase markedly, but the average molecular mass will remain the same. Results shown in Table 2 indicate immediately that it is the second hypothesis that is supported.

The results of the present study show that Tah1 is primarily monomeric under the conditions used, but that some dimeric species is also present. The fact that the monomeric form is not in equilibrium with the dimeric species suggests that the dimer may not be a biologically native species, but rather aggregated molecules of Tah1. TPR domains are known to self-associate [20] and we have in the present study observed irreversible aggregation and precipitation of Tah1 on prolonged storage and during handling of the protein. Even though our samples were spun to remove such aggregates small soluble species might not be removed by centrifugation. We therefore conclude that Tah1 is most probably monomeric as a biologically active species and that the dimeric species is most probably an irreversibly aggregated dimer.

DISCUSSION

The present study has shown that the TPR domain of Tah1 is similar to that of the TPR2b domain of Hop. Tah1 was shown to selectively favour the binding of a methionine residue over a valine residue at position −4, and therefore selectively binds Hsp90 over Ssa1, in common with the TPR2a domain of Hop. However, although tight binding of ligand by Tah1 and the TPR2a domain of Hop is dependent on a methionine residue at this position, the overall interactions that these domains make with the MEEVD motif are actually different since the sequence conservation between the two domains is significantly diverged. This is even more remarkable as many of the upstream contacts from the MEEVD motif and contacts made by the TPR2a domain with MEEVD appear to be main chain contacts to their respective peptides.

The orientation of peptide binding in different TPR domains has been shown to vary. For example the peptide in the TPR2a–MEEVD complex is bound in an antiparallel orientation to α-helices 1, 3, 5 and 7 of the domain. However, in the Cyp40 and FKBP51 complexes the EEVD is bound in the opposite orientation [21–23,25]. Our amino-acid-sequence alignment showed that for residues involved in contacting the MEEVD motif by the TPR2a domain, seven residues are identical and eight residues are different at equivalent positions in the Tah1 domain. Consequently, the orientation in which the MEEVD peptide binds to Tah1 cannot be assumed to be the same as seen in the TPR2a–MEEVD complex, and must await structural determination to be resolved.

A high-affinity-binding motif for the TPR2b domain of Hop has not been identified [21,29], but surprisingly Tah1 was shown to be most similar to the TPR2b domain of Hop. This suggests that differences between these two TPR domains direct their selectivity towards different binding sites. It is interesting to note that the TPR2b domain has been shown to be critical for the interaction of the TPR2a domain of yeast Sti1 with Hsp90 [49].

It has also been reported that binding of TPR domain containing co-chaperones to Hsp70 and Hsp90 can influence the ATPase activity of the chaperone [32–36]. Under the assay conditions that we applied in the present study we were able to detect a weak stimulation of the ATPase activity of Hsp90 with a 10-fold excess of Tah1. Although this activation is very weak, the ATPase activity of Hsp90 is critical to its proper functioning [46]. However, how TPR domain proteins bind the extreme C-terminus of Hsp90 and influence its ATPase activity is still not understood and may have to wait for structural studies to shed light on the precise mechanism.

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