Ligand Discrimination by TPR Domains

RELEVANCE AND SELECTIVITY OF EEVD-RECOGNITION IN Hsp70-Hop-Hsp90 COMPLEXES*

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Protein-protein interaction modules containing so-called tetratricopeptide repeats (TPRs) mediate the assembly of Hsp70/Hsp90 multi-chaperone complexes. The TPR1 and TPR2A domains of the Hsp70/Hsp90 adapter protein p60/Hop specifically bind to short peptides corresponding to the C-terminal tails of Hsp70 and Hsp90, respectively, both of which contain the highly conserved sequence motif EEVD-COOH. Here, we quantitatively assessed the contribution of TPR-mediated peptide recognition to Hsp70-Hop-Hsp90 complex formation. The interaction of TPR2A with the C-terminal pentapeptide of Hsp90 (MEEVD) is identified as the core contact for Hop binding to Hsp90. (In peptide sequences, italics are used to highlight residues specific for Hsp70 or Hsp90.) In contrast, formation of the Hsp70-Hop complex depends not only on recognition of the C-terminal Hsp70 heptapeptide (PTTVEED) by TPR1 but also on additional contacts between Hsp70 and Hop. The sequence motifs for TPR1 and TPR2A binding were defined by alanine scanning of the C-terminal octapeptides of Hsp70 and Hsp90 and by screening of combinatorial peptide libraries. Asp0 and Val-1 of the EEVD motif are identified as general anchor residues, but the highly conserved glutamates of the EEVD sequence, which are critical in Hsp90 binding by TPR2A, do not contribute appreciably to the interaction of Hsp70 with TPR1. Rather, TPR1 prefers hydrophobic amino acids in these positions. Moreover, the TPR domains display a pronounced tendency to interact preferentially with hydrophobic aliphatic and aromatic side chains in positions –4 and –6 of their respective peptide ligands. Ile-4 in Hsp70 and Met-4 in Hsp90 are most important in determining the specific binding of TPR1 and TPR2A, respectively.

The coordinated interaction of the Hsp70 and Hsp90 molecular chaperones is required for the folding and conformational regulation of a variety of signal transduction proteins and cell cycle regulators in the eukaryotic cytosol (reviewed in Refs. 1–5). To achieve the folding of proteins such as steroid hormone receptors and protein kinases, Hsp90 and Hsp70 cooperate with numerous cofactors containing so-called tetratricopeptide repeat (TPR)† domains. TPR domains are composed of loosely conserved 34-amino acid sequence motifs that are repeated 1–16 times per domain. Originally identified in components of the anaphase promoting complex (6, 7), TPR domains are now known to mediate specific protein interactions in numerous cellular contexts (8–10). Moreover, apart from serving mere anchoring functions, TPR domains of the chaperone cofactors Hip and p60/Hop also exert regulatory functions on the ATPase activities of Hsp70 and Hsp90, respectively (11, 12). The conserved architecture of the TPR fold is well established based on a number of x-ray structures of different TPR domains (13–18). Each 34-amino acid motif forms a pair of antiparallel α-helices. These motifs are arranged in a tandem array into a superhelical structure that encloses a central groove.

The TPR cofactors of the Hsp70/Hsp90 multi-chaperone system interact with the C-terminal domains of Hsp70 and Hsp90 (19–24). Deletion mutagenesis suggested that the C-terminal sequence motif EEVD-COOH, which is highly conserved in all Hsp70s and Hsp90s of the eukaryotic cytosol, has an important role in TPR-mediated cofactor binding (21, 25–27). Site-directed mutagenesis of the Hsp90 cofactor PP5 has suggested that the central groove of the TPR domain acts as the ligand binding site (26, 27). Hop serves as an adapter protein for Hsp70 and Hsp90 (28), optimizing their functional cooperation (29) without itself acting as a molecular chaperone (30). Hop contains three TPR domains, each composed of three TPR motifs (15) (Fig. 1A). The N-terminal TPR domain, TPR1, is implicated in the interaction with Hsp70 (25, 31, 32), whereas a fragment of Hop containing the middle and the C-terminal domains, TPR2A and TPR2B, mediates the interaction with Hsp90 (12, 25). TPR1 specifically recognizes the C-terminal seven amino acids of Hsp70 (PTTVEED), whereas TPR2A recognizes the C-terminal five residues of Hsp90 (MEEVD) (15) (Fig. 1B). An aspartate of three amino acids in the central grooves of TPR1 and TPR2A form the so-called “two-carboxylate clamp” structure that serves as a socket for the binding of Hsp70 or Hsp90.

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† The abbreviations used are: TPR, tetratricopeptide repeats; C70/C90, Hsp70/Hsp90 C-terminal domain; HPLC, high performance liquid chromatography; Hsp, heat shock protein; Hop, Hsp-organizing protein; ITC, isothermal titration calorimetry; O, defined amino acid position; OX-VD, combinatorial peptide library; RU, response units/relative units; X, randomized amino acid position; Fmoc, N-(9-fluorenylemethoxycarbonyl); ESI-MS, electrospray ionization mass spectrometry; CHIP, C-terminal Hsp70 interacting protein.
‡ In peptide sequences, italics are used to highlight residues for either Hsp70 or Hsp90.
Peptide Recognition Motifs of Hop TPR Domains

The two-carboxylate clamp interacts tightly with both carboxylate functions of the conserved C-terminal Asp residue (Asp0) of Hsp70 and Hsp90 and is thus a potential fingerprint motif for novel EEVD-binding chaperone cofactors.

Although the recent structures of TPR-peptide complexes revealed the general nature of TPR-mediated ligand binding (15), the extent to which recognition of the EEVD motif contributes to the interaction of the full-length proteins in the Hsp70-Hop-Hsp90 complex remains to be determined. Moreover, the relative significance of individual amino acid residues for peptide binding cannot easily be deduced from crystallographic data. In the present study we analyzed in quantitative terms the contribution of TPR-peptide interactions to the Hsp70/Hsp90 adapter function of Hop. The potential of short EEVD peptides to compete the interactions of the full-length proteins was evaluated, and the ligand recognition motifs of TPR1 and TPR2A were defined. We show that TPR-mediated binding to N-terminally extended EEVD peptides is necessary for complex formation of both full-length Hsp70 and Hsp90 with Hop. The TPR2A-EEVD interaction represents the core contact between Hsp90 and Hop. However, additional binding sites outside the TPR1-P1EVEEV interaction appear to play an important role in stabilizing the Hsp70-Hop complex. Although electrostatic interactions of Asp0 at the C terminus of Hsp70 and Hsp90 with the two-carboxylate clamp structure are indeed necessary for TPR domain binding, hydrophobic interactions contribute substantially to complex formation. Whereas the small hydrophobic residue Val-1 is critical for the general anchoring function of the Hsp70 and Hsp90 EEVD sequence, the highly conserved glutamate residues are not generally required and are recognized differentially by TPR1 and TPR2A. The hydrophobic residues Ile-4 in Hsp70 and Met-4 in Hsp90 are not only necessary for the high affinity of TPR interactions but are also the primary determinants of binding specificity. On the basis of these results, the development of small molecule inhibitors can be envisioned that specifically inhibit individual TPR-chaperone interactions. Such compounds would be useful in further dissecting the complex reaction mechanisms of Hsp70 and Hsp90 in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Protein Preparation—Human Hsp90α (codons 1–731) was cloned in-frame into the NcoI/KpnI sites of plasmid pPROEX. Human Hsp70 as well as its 25-kDa C-terminal domain C70 (codons 392–641) were cloned into the EcoRI/Xhol sites of the same plasmid. All proteins were expressed in Escherichia coli as fusion proteins with cleavable, N-terminal hexahistidine tags using BL21(DE3) pLysS cells and terrific phase; and Rmax, the saturated equilibrium response (in RU)). In those terms the contribution of TPR-peptide interactions to the EEVD interface appear to play an

PEPTIDE SYNTHESIS—Synthetic heptapeptide collections Ac-X6/OVD-OH and Ac-X7-VD-OH were prepared by fully automated solid phase peptide synthesis using Fmoc chemistry and Wang resins (35, 36). Briefly, introduction of randomized sequence positions (O), a 5-fold molar excess of single Fmoc-L-amino acids was added. An optimized diisopropylcarbodiimide/1-hydroxybenzotriazole method was used for coupling (37). N-acetylation was performed by reacting the N-terminally deprotected resin-bound peptide with a 1:1 mixture of diisopropylethylamine and acetic anhydride (both 1.5 M in dichloromethane/dimethylformamide (1:1)) for 30 min. The amino acid composition in the defined positions and the random sequence positions of the peptide mixtures were determined by peptide sequencing (38) and by electrospray ionization mass spectrometry (ESI-MS) (39). Deviations from an equimolar representation of the amino acids in randomized sequence positions were found to be within the error limits of the analytical method.

Defined peptides were synthesized with acetylated N termini using solid phase peptide synthesis. After purification by HPLC they were analyzed by ESI-MS. The purity was >95% as determined by HPLC (214 nm). Defined peptides were dissolved directly in buffer G (25 mM Hepes pH 7.5, 100 mM KAc, 5 mM MgCl2). Combinatorial peptide collections were dissolved in Me2SO and diluted into the final assay mixture.

Surface Plasmon Resonance—SPR measurements were performed on a BiAcore 2000 instrument at 25 °C. Hsp70, Hsp90, C70, and C90 were chemically biotinylated and loaded onto streptavidin derivatized CM5 biosensor chips (Biosensor). The biotinylation reaction was carried out in 50 mM NaHCO3, pH 8.5, for 10 min on ice. The final protein concentration was 20 μM, and the biotinylation reagent (EZ-Link Sulfo-NHS (N-hydroxysuccinimide)-LC-LC-Biotin, Pierce) was used at a 3-fold substoichiometric concentration. Biotinylated peptide was expressed on an AP5 column. ~5,000 RU streptavidin (Sigma, affinity-purified) was immobilized via standard amine coupling procedures (40) using HBS (10 mM Hepes pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.05% Tween 20) as the running buffer. 1500–3000 RU of biotinylated Hsp70 or Hsp90 and 300–600 RU of C70 and C90 were loaded onto the chips for binding and competition experiments. Hop as well as its TPR fragments were inactivated by this immobilization procedure, probably because of modification of critical carboxylic clamp residues (see “Results”). Alternatively, the cysteine derivatized dodecameric Hsp70 or Hsp90 peptides were generated by injection of ethylenediamine hydrochloride (1 M, pH 6.0) for 10 min. Maleimido groups were introduced via the heterobifunctional cross-linker Sulfo-GMBS (Pierce, 50 μM in HBS, 10 min). Finally, Cys-70C-12 and Cys-90C-12 (1 μM in HBS) were exposed to the modified surface for 10 min and unreacted maleimido groups were inactivated by a 2-min pulse of 0.1 M NaOH.

Binding and competition experiments were performed in buffer G (25 mM Hepes, pH 7.5, 100 mM KAc, 5 mM MgCl2) at a flow rate of 20 μl/min. For binding studies, 30 μl of protein solutions containing either Hop or its isolated TPR domains were passed over immobilized Hsp70 and Hsp90 proteins or the respective protein and peptide fragments. After each injection of protein complexes were followed for 10 min. Complete regeneration of the chip surfaces was subsequently achieved by two 30-s pulses with 0.5 M NaCl. For binding experiments Hop or its isolated TPR domains were injected as serial dilutions in the concentration range of 200 nM to 100 μM to determine the thermodynamic dissociation constants (Kd), the average equilibrium response values (Rmax) were plotted versus the protein concentrations applied, and the resulting titration curve was fitted to a simple 1:1 steady state binding model using BIAREF evaluation 3.0 software.

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R_{equ} = (K_d \times C \times R_{max})/(1 + K_d \times C)
\]

where \(R_{equ}\) is equilibrium response (in relative units (RU)); \(K_d\), the thermodynamic affinity constant; \(C\), protein concentration in mobile phase; and \(R_{max}\) the saturated equilibrium response (in RU). In those cases where binding reactions did not reach equilibrium levels during the injection period, the response units obtained over the last 15 s of sample injection were averaged and plotted as an approximation of the true equilibrium responses, \(R_{equ}\). Longer injection times resulted in the accumulation of material on the chip surface that could not be regenerated by mild salt washes. Thus, reliable titration experiments could not be performed when allowing for longer contact times.

Competition experiments were performed by preincubating Hop or its isolated TPR domains with short defined peptides or combinatorial peptide mixtures. Protein-peptide mixtures were passed over immobilized Hsp70 and Hsp90 proteins, and binding of the TPR constructs to the heat shock proteins was followed. SPR signals obtained in the
absence of competing peptides were used as a reference (100% binding) to normalize the values obtained in the presence of peptides. For competition experiments involving defined peptides, the concentration of TPR proteins was kept constant, whereas the peptide concentrations of the protein-peptide mixtures were systematically increased as specified in the figure legends. To determine the IC$_{50}$ values for the defined peptides the normalized binding signals of protein-peptide mixtures were plotted versus the peptide concentrations, and a competitive inhibition model was fitted to the data using the KaleidaGraph 3.0 software.

$$r = \frac{[A] + (K_A \times (1 + (B/K_B)))}{[A] + K_A}$$

where $r$ is the normalized SPR binding signal; $A$, immobilized Hsp70/ Hsp90; $K_A$, the thermodynamic affinity constant for the Hsp-TPR interaction; $B$, the concentration of competing peptide in protein-peptide mixture; and $K_B$, the thermodynamic affinity constant for the TPR- peptide interaction. The competition potential of the partially defined Ac-X$_1$-VD-OH mixtures was expressed relative to the competition potential of the maximally randomized Ac-X$_N$-VD-OH mixture. Isothermal Titration Calorimetry—ITC experiments were performed at 25 °C in buffer G using a VP-ITC titration calorimeter (MicroCal Inc., Northampton, MA) (42). 30 to 60 aliquots of 5–10 μl peptide solution (1–10 mM) were titrated by injection into 1.36 ml of TPR domain solution (0.1–1 mM) in the chamber. Peptides were dissolved in protein solutions dialyzed against buffer G. Injections were continued beyond saturation levels to allow for the determination of the heat of the ligand dilution. After subtraction of dilution heat, calorimetric data were analyzed using the evaluation software provided by the manufacturer (Origin 5.0 adapted by MicroCal Software, Inc.).

RESULTS

Analysis of Hop-Hsp70 and Hop-Hsp90 Interactions—To evaluate the contribution of TPR-mediated peptide interactions to the formation of the Hop/Hsp70-Hsp90 complex (Fig. 1), bimolecular interactions were analyzed using a surface plasmon resonance (SPR)-based binding assay. Full-length Hsp70 or Hsp90 or C-terminal domains and peptide fragments thereof were immobilized on sensor chips. Subsequently, either full-length Hop or its TPR domains were passed over the derivatized chip surfaces, and protein interactions were monitored (Fig. 2). Association as well as dissociation phases of all TPR-mediated protein interactions investigated were dominated by very rapid kinetics, prohibiting the determination of kinetic constants ($K_{off}$, $k_{off}$). However, concentration-dependent SPR signals were recorded for specific protein interactions, and thermodynamic dissociation constants ($K_D$ values) could be determined by titration of equilibrium binding signals ($R_{eq}$) (Fig. 2, C and D; Table I).

Whereas some of the interactions analyzed exhibited appar-
The SPR signal in the absence of competing peptides was used as a reference (100\%) or EEVD (\(1\text{ mM}\)) were normalized and plotted. The SPR signal in the absence of competing peptides (MEEVD (\(1\text{ mM}\)), IEEVD (\(1\text{ mM}\)), or EEVD (\(1\text{ mM}\)) were normalized and plotted versus the peptide concentrations. Error bars reflect standard deviations of three independent experiments. IC\(_{50}\) values were determined for each peptide after fitting the data using a competitive inhibition model. C and D are analogous to A and B except that binding of full-length Hop (0.54 \(\mu M\)) to Hsp90 was competed by short EEVD peptides.

**Fig. 3. Competition of TPR2A binding to Hsp90.** A, TPR2A (1.8 \(\mu M\)) was passed over immobilized Hsp90 (~2500 RU) in the absence or presence of increasing concentrations of the tetrapeptide EEVD (100 nM, 200 nM, 1 \(\mu M\), 2 \(\mu M\), 10 \(\mu M\), 20 \(\mu M\), 100 \(\mu M\), 200 \(\mu M\), 1 mM, and 2 mM). The SPR signal in the absence of competing peptides was used as a reference (100\% binding). B, equilibrium response levels obtained in the presence of competing peptides (MEEVD (\(1\text{ mM}\)), DVEEM (\(1\text{ mM}\)), EEVD (\(1\text{ mM}\)), or EEVD (\(1\text{ mM}\)) were normalized and plotted versus the peptide concentrations. Error bars reflect standard deviations of three independent experiments. IC\(_{50}\) values were determined for each peptide after fitting the data using a competitive inhibition model. C and D are analogous to A and B except that binding of full-length Hop (0.54 \(\mu M\)) to Hsp90 was competed by short EEVD peptides.

**Fig. 4. Competition of TPR1 binding to Hsp70.** A, TPR1 (5.4 \(\mu M\)) was passed over immobilized Hsp70 (~2000 RU) in the absence or presence of increasing concentrations of the tetrapeptide EEVD (1 \(\mu M\), 2 \(\mu M\), 10 \(\mu M\), 20 \(\mu M\), 100 \(\mu M\), 200 \(\mu M\), 1 mM, and 2 mM). The SPR signal in the absence of competing peptides was used as a reference (100\% binding). B, equilibrium response levels obtained in the presence of competing peptides (GPTIEEVD (\(1\text{ mM}\)), DVEE1 (\(1\text{ mM}\)), TSRMEEVD (\(1\text{ mM}\)), or EEVD (\(1\text{ mM}\)) were normalized and plotted versus the peptide concentrations. Error bars reflect standard deviations of three independent experiments. IC\(_{50}\) values were determined for each peptide after fitting the data using a competitive inhibition model. C and D are analogous to A and B except that binding of full-length Hop (1.8 \(\mu M\)) to Hsp70 was competed by short EEVD peptides.

**Table I** Binary protein interactions in the Hsp70·Hop·Hsp90 complex

| Protein Interaction | \(K_d\) (\(\mu M\)) | Hop FL | TPR1 | TPR2A | TPR2B | TPR2L |
|--------------------|---------------------|--------|------|-------|-------|-------|
| Hsp70 FL           | 2 ± 1\*             | 18 ± 8 | NSB  | 3 ± 2 | 3 ± 2 | 3 ± 2 |
| Hsp90 FL           | 1 ± 1\*             | 0 ± 1 | NSB  | 0 ± 1 | 0 ± 1 | 0 ± 1 |
| C70                | 2 ± 1\*             | 22 ± 2 | NSB  | 5 ± 1 | 6 ± 2 | 6 ± 2 |
| C90                | 7 ± 4\*             | 33 ± 2 | NSB  | 7 ± 2 | 6 ± 3 | 6 ± 3 |
| 70C-12             | 8 ± 3\*             | 33 ± 2 | NSB  | 7 ± 2 | 6 ± 3 | 6 ± 3 |

* Multiphasic binding curves where no equilibrium response levels were reached during sample injection. In these cases the maximal responses obtained at the end of sample injections (R\(_{max}\)) were plotted to approximate the equilibrium response levels (R\(_{eq}\)). The application of larger sample volumes and longer contact times was not feasible in titration experiments because of the buildup of protein complexes that could not be regenerated under mild conditions.
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TABLE II
Inhibitory activity of short EEVD-peptides

| Competitors | TPR1-Hsp70 interaction | TPR2A-Hsp70 interaction |
|-------------|------------------------|-------------------------|
| **IC<sub>50</sub>** | **μM** | **μM** |
| EEVD | 221 ± 18 | 142 ± 10 |
| DVEEI | >2000 | >2000 |
| GPTI EEVD | 37 ± 3 | 14 ± 2 |
| TSRM EEVD | 157 ± 3 | 44 ± 4 |

The reverse of MEEVD, was without effect on these protein interactions when analyzed at up to millimolar concentrations (Fig. 3, B and D).

The TPR1-Hsp70 and Hop-Hsp70 interactions were also specifically competed by the tetrapeptide EEVD (Fig. 4, Table II). The control peptide DVEEI in the micromolar concentration range had no appreciable effect on the TPR1-Hsp70 interaction and was 10-fold less efficient than EEVD in competing the Hop-Hsp70 interaction (Fig. 4, B and D; Table II). The Hsp70-derived octapeptide GPTI EEVD, on the other hand, was 10-fold more active than EEVD in inhibiting the TPR1-Hsp70 and Hop-Hsp70 interactions (Fig. 4, B and D; Table II). Compared with GPTI EEVD, the corresponding Hsp90-derived octapeptide TSRM EEVD was 9-fold less efficient in competing the TPR1-Hsp70 interaction and 3-fold less efficient in competing the Hop-Hsp70 interaction.

We conclude from these results that the interactions between the TPR domains of Hop and the extended EEVD peptides of Hsp70 and Hsp90 are a necessary requirement for complex formation between the full-length proteins. The predicted additional contact sites are not sufficient to ensure efficient binding. However, the EEVD peptides competed complex formation between the full-length proteins with reduced specificity (Figs. 3D and 4D; Table II) as compared with their effects on the interactions of Hsp70 or Hsp90 with the TPR domains (Figs. 3B and 4B; Table II). This tendency was more pronounced for Hsp70 binding to Hop, supporting the conclusion drawn above that in this case EEVD-independent contacts contribute substantially to complex formation (see Table I).

**Significance of Individual Amino Acid Side Chains in Peptide Binding to TPR1 and TPR2A**—As determined previously, TPR1 recognizes the C-terminal heptapeptide sequence TEEVD of Hsp70 and TPR2A recognizes the C-terminal pentapeptide MEEVD of Hsp90 (Fig. 1B) (15). To evaluate the contribution to TPR binding of individual amino acid side chains in these sequences, we performed alanine scans on the octapeptides GPTI EEVD (Hsp70) and TSRM EEVD (Hsp90). In the following description of this analysis (Figs. 5-7), the C-terminal Asp residue of the peptides will be referred to as Asp0, and the preceding amino acid positions will be numbered in descending order, e.g. Val-1, Glu-2, Glu-3, Ile-4, Thr-5, Pro-6, and Gly-7 for the Hsp70 peptide. Affinities of the TPR1 and TPR2A domains for the alanine-substituted peptides were determined by ITC (Fig. 5, A and B).

Whereas electrostatic interactions centered on the conserved Asp0 residues were found to be an absolute requirement for efficient ligand binding to both TPR1 and TPR2A, the contri-
bution to binding of the adjacent, highly conserved Glu-2 and Glu-3 residues differed between the two TPR domains. Interestingly, neither of the two glutamates in the GPTIEEVD peptide of Hsp70 contributes to TPR1 binding, consistent with the structural analysis (15). By contrast, the exchange of these residues to alanine in the TPR2A ligand TSRMEEVD resulted in an increase of \(K_D\) values by factors of 3 and 10, respectively (Fig. 5, A and B). A surprising finding was the strong contribution of hydrophobic interactions to peptide binding by the TPR domains. Ala substitution of Val-1 and of the Hsp70- and Hsp90-specific residues in position -4 (Ile/Met) weakened peptide binding substantially. Substitution of Thr-5 and Pro-6 in GPTIEEVD-COOH resulted in a milder but still clearly detectable disruption of ligand recognition by TPR1. In contrast, substitutions upstream of position -6 in the TPR1 peptide ligand and upstream of position -4 in the TPR2A peptide ligand did not affect binding, consistent with the notion that these residues do not contact the TPR domains (15).

The significance of the two-carboxylate clamp structures for the interaction with the conserved C-terminal Asp0 residues of

**Fig. 5. Sequence dependence of peptide interaction with TPR domains.** A, alanine scan of the TPR1 peptide ligand Ac-GPTIEEVD-OH with respect to TPR1 binding. Single amino acid residues of the octapeptide Ac-GPTIEEVD-OH mimicking the C-terminal tail of Hsp70 were substituted for alanine and the affinities of the resulting ala-peptides for the TPR1 domain determined by ITC. In addition, the peptide amide Ac-GPTIEEVD-NH\(_2\) was tested. The inset shows the titration of TPR1 (450 \(\mu\)M) with the peptide Ac-AFTIEEVD-OH (7.5 \(\mu\)M). Fitting of the integrated titration curve to a 1:1 binding model yielded the following thermodynamic parameters: \(n = 0.8, K_D = 31 \mu\)M, \(\Delta H = -7.1\) kcal/mol, and \(\Delta S = -3.2\) cal/mol. B, alanine scan of the Hsp90 peptide Ac-TSRMEEVD-OH with respect to TPR2A binding. The affinities of Ala-peptides for TPR2A were determined by ITC. The inset shows the titration of TPR2A (350 \(\mu\)M) with the peptide Ac-ASRMEEVD-OH (7.5 \(\mu\)M). The thermodynamic parameters were: \(n = 0.9, K_D = 25 \mu\)M, \(\Delta H = -12.1\) kcal/mol, and \(\Delta S = -19.6\) cal/mol. Generally, stochiometries of TPR1- and TPR2A-peptide complexes ranged between 0.8 and 1.0. Whenever the binding enthalpies were too low to be reliably determined by calorimetry, the protein concentration in the chamber was taken to reflect the minimal \(K_D\)-values of the interactions. Representative replicate titration experiments yielded coefficients of variation (CV values) of 5–10%. C, SPR-based binding assay for two-carboxylate clamp mutants of TPR2A. TPR2A wild type (WT) (●), or the TPR2A point mutants K229A (○), N264A (△), K301A (○), or R305A (○) (10 \(\mu\)M) were passed over immobilized Hsp90 (~1500 RU).
Hsp70 and Hsp90 was analyzed in TPR2A by changing the clamp residues Lys-229, Asn-264, Lys-301, and Arg-305 to Ala. Binding of the mutant domains to full-length Hsp90 or the pentapeptide MEEVD was measured. Each point mutation of clamp residues in the isolated TPR2A domain severely impaired binding to Hsp90 (Fig. 5C). The affinity of the TPR2A clamp mutants to the pentapeptide MEEVD was reduced by at least two orders of magnitude (K_D ≥ 1 mM) as determined by ITC (data not shown). The mutant forms of TPR2A were indistinguishable from the wild-type domain by near and far UV-CD spectroscopy and had very similar thermal stability (data not shown), suggesting that the overall structure of the domains is preserved. Thus, each of the conserved clamp residues in the TPR domain is absolutely required for efficient peptide binding.

**Screening of the Combinatorial Peptide Library O/X/V**

To obtain a more general and unbiased description of the TPR1 and TPR2A ligand recognition motifs, a binding screen was performed with the anchored heptapeptide library O/X/V-OH. This peptide library is composed of 96 heptapeptide collections arrayed in a positional scanning format. The peptide collections can be organized into five subsets (OXXXX-VD, OXXXX-XD, ... , XXXX-OV) containing 19 partially defined mixtures each (e.g. for OXXXX-VD: AXXX-VD, DXXX-XV, DXXX-VD, ... , XXXX-XD) plus the maximally diverse mixture XXXX-VD (X-VD) where bold letters highlight the defined sequence positions. The defined sequence positions “**O**” contain one of 19 proteinogenic amino acids with cysteine being excluded. The randomized “**X**”-positions contain an equimolar mixture of the same 19 amino acids. As a consequence, each peptide mixture represents a subcollection of 19^4 = 130,321 theoretical peptide sequences. Each O/X/V-VD subcollection contains 19 individual mixtures resulting in a total number of theoretical peptide sequences of ~2.5 × 10^6. To achieve a homogenous orientation of the diverse heptapeptides relative to the protein surfaces of the TPR domains, the C-terminal two positions were fixed (O/X/V-VD). Based on the results of the alanine scans (Fig. 5, A and B), Asp90 and Val-1 were expected to act as binding anchors in the sequence positions 0 and 1. The O/X/V-VD subcollections were screened for binding to TPR1 and TPR2A using the SPR-based competition assay. The defined peptide ligands C70–12 or C90–12 were immobilized on sensor chips and protein-peptide mixtures containing the TPR domains and individual peptide collections (e.g. AXXX-VD, DXXX-VD, ... , XXXX-OV) were passed over it. The SPR signal resulting from TPR1 or TPR2A binding to C70–12 or C90–12 in the absence of peptide mixtures was used as a reference (100% binding) (Fig. 6A). The relative concentrations of TPR1 domains and peptide collections were adjusted such that the maximally randomized peptide mixture X-VD competed 50% of the TPR1-C70–12 or TPR2A-C90–12 interaction.

The binding preference of TPR1 and TPR2A for the defined amino acids in positions –2 to –6 of the O/X/V-VD peptide collections were then assessed by comparing the inhibition potential of O/X/V-VD mixtures with the inhibition potential of the X-VD mixture (Fig. 6A). Defined O-residues that promote TPR binding relative to the effect of an equimolar amino acid mixture X increase the competition potential of O/X/V-VD peptides relative to X-VD. The results of this analysis are shown in Fig. 6. Amino acid residues favoring TPR binding to immobilized C70–12 or C90–12 are represented by positive values (Fig. 6, A–F), whereas amino acid residues that compete TPR binding to the immobilized ligands score negative values. The peptide mixture XXXX-XV competed the interaction of TPR2A with 90C-12 much more efficiently than XXXX-VX (Fig. 5A), consistent with the importance of the glutamate residue at the corresponding position of the C-terminal sequence of Hsp90 (Fig. 5B). The Glu-3 residue of the MEEVD peptide forms a specific salt bridge with Arg-305 in the TPR2A domain (15). Thus, the complex peptide subcollection XXXX-XV adopts the same binding register relative to the TPR2A domain as the defined peptide ligand MEEVD.

Interestingly, screening of the peptide library for binding to TPR1 revealed a strong preference for the hydrophobic amino acids Phe, Ile, Leu, Met, Val, and Tyr over all five of the ligand positions analyzed (Fig. 6, B–F). This tendency also includes positions –2 and –3 that correspond to the conserved glutamate residues in the Hsp70 C terminus. Importantly, the relative as well as the absolute contribution to binding of any of these favorable residues was strongly dependent on the sequence position. For example, isoleucine in position –4 was clearly conducive to peptide binding by TPR1, whereas it behaved neutrally in position –2. Surprisingly, acidic amino acids have no pronounced effects on ligand binding to TPR1, although the C terminus of Hsp70 carries four negative charges. Basic residues, however, are strongly disfavored at all sequence positions. In contrast to TPR1, TPR2A has an overall tendency to interact with negatively charged side chains, even in ligand positions –4 to –6 where the Hsp90 C terminus features neutral and basic residues (Fig. 6). The strongest interaction of TPR2A with a negatively charged amino acid is seen at position –3, in support of the significance for binding of a specific salt bridge (see above) (15). Hydrophobic and small neutral residues are preferred by TPR2A in ligand positions –4 and –6. In position –4, Met, Phe, Leu, and Ile are preferred, whereas Pro, Ser, and Tyr were found to support the binding of O/X/V-VD mixtures at position –6. Basic side chains strongly impair ligand binding to TPR2A at all sequence positions.

Amino acids increasing ligand binding to TPR1 and TPR2A in specific positions are listed in Table III and are ordered according to decreasing efficiency. Unlike other aromatic and bulky hydrophobic amino acids such as Phe or Ile, Trp was identified in 9 of 10 cases as a favorable amino acid irrespective of the TPR domain tested and the sequence position analyzed. Presumably as a result of the pronounced hydrophobicity of Trp, which substantially reduces the solubility of short peptides, this residue often causes nonspecific effects in peptide library screens and is not considered in Table I.

Notably, the sequence YYILD-VD, predicted to represent the optimal TPR1 ligand, is more hydrophobic than the authentic Hsp70 C terminus PTEIEVD, with Leu replacing the conserved Glu in position –3 and two aromatic amino acids replacing an aliphatic and a small neutral side chain. Interestingly, Ile was identified by the screen as the most favored amino acid in position –4. Ile is present in position –4 of the Hsp70 C terminus and was shown by alanine scanning to represent an important anchor residue for TPR1 binding (Fig. 5A). Compared with the authentic Hsp70 C terminus PTEIEVD, the sequence YYILD has a 2-fold higher affinity for TPR1 (Table III). The same increase in affinity can also be achieved by substituting only Glu-3 in PTEIEVD for leucine, resulting in the peptide PTLEVD (K_D = 10 μM, data not shown). Apparently, leucine but not glutamate in position –3 can make additional hydrophobic contacts with the TPR1 binding groove.

Contrary to the results obtained for TPR1, in the case of TPR2A the binding screening identified the entire peptide sequence of the natural peptide ligand MEEVD in the optimal amino acid sequence PEMEVEVD (Table III). Accordingly, the affinity of PEMEVEVD for TPR2A (K_D = 11 μM) is identical to the affinity of the MEEVD peptide for TPR2A (K_D = 11 μM). Interestingly, the C-terminal tail of Hsp90 contains the basic amino acid Arg at position –5, which reduces the binding af-
finity for TPR2A (K_D (TPR2A-SRM EEVD)/H11005 27/H9262 M (15)). Substitution of Arg-5 for glutamate restores the binding affinity to the value measured for MEEVD (K_D (TPR2A-S.RM EEVD)) = 12 μM, data not shown). The inhibitory effect of Arg-5 in Hsp90 peptides is consistent with the results of the peptide library screen (Fig. 6C). Arg-5 may be masked in the context of the three-dimensional structure of Hsp90 by intramolecular salt bridges.

In summary, the peptide library screen revealed overall binding preferences of TPR1 and TPR2A for hydrophobic and acidic residues, respectively (Fig. 6). In addition, both TPR domains display a pronounced tendency to interact preferentially with hydrophobic aliphatic and aromatic side chains in positions –4 and –6.

DISCUSSION

As demonstrated in a recent structure-based approach, the isolated TPR1 and TPR2A domains of the Hsp70/Hsp90 adapter protein, Hop, specifically recognize 5–7-mer amino acid sequences in the C-terminal tails of Hsp70 and Hsp90 (15). The peptide ligands of these TPR domains contain the motif EEVD, a C-terminal sequence highly conserved in all eukary-
Peptide Recognition Motifs of Hop TPR Domains

Amino acids in a defined O-position of OX,VD mixtures leading to at least 60% inhibition of binding of TPR1 to 70C-12 or TPR2A to 90C-12 (Fig. 5) are regarded as favorable building blocks of TPR peptide ligands. Favorable amino acids are listed for each TPR domain and position in the heptapeptide ligands according to their decreasing efficiency. For comparison, the peptide sequences of the Hsp70 and Hsp90 C termini are given. Affinities (K_D) of peptide ligands composed of only the most preferred amino acid residues for TPR1 and TPR2A were determined by ITC and compared with the affinities of the respective Hsp70 and Hsp90 peptide ligands, PTIEEVD and SRMEEVD. The affinity of the TPR2A pentapeptide ligand MEEVD is given in parentheses.

| TPR1 | OxxxVD | OxxOVD | xxxOVD | xxxxxOVD | K_D μM |
|------|--------|--------|--------|----------|---------|
| Hsp70: | Pro | Thr | Ile | Glu | Val-Asp |
| Tyr | Tyr | Ile | Leu | Asp |
| Val | Phe | Leu | Glu |
| Phe | Ile |

| TPR2A | OxxxVD | OxOVD | xxOVD | xxxOVD | K_D μM |
|-------|--------|--------|--------|----------|---------|
| Hsp90: | Ser | Arg | Met | Glu | Val-Asp |
| Pro | Glu | Met | Glu | Glu |
| Ser | Phe | Asn | Leu |
| Tyr | Leu |
| Asp | Glu |
| Glu | Ile |

a Ref. 15.

Although the TPR-mediated recognition of extended EEVD sequences is a major determinant for the formation of Hsp70/Hop/Hsp90 complexes, the presence in vivo of substrate proteins, nucleotides and a plethora of competing TPR cofactors and non-TPR co-chaperones is likely to impose an additional level of complexity and regulation on these interactions. Indeed, the proposed role of the conserved EEVD sequences as general docking sites for TPR cofactors is consistent with these sequences having additional regulatory functions that may or may not depend on TPR cofactor binding. Evidence has been presented that deletion of the EEVD sequence of Hsp70 affects the ATPase activity and the ability of Hsp70 to interact with substrates and Hsp40 cofactors (44). A regulatory effect of the EEVD-binding protein StI1, the yeast homolog of Hop, on the ATPase of yeast Hsp90 has also been reported (12).

Our analysis of the TPR1 and TPR2A peptide binding motifs (Fig. 7) revealed the following basic features of TPR-mediated ligand recognition in Hsp70/Hsp90 multi-chaperone complexes.

First, electrostatic interactions between the two-carboxylate clamp structures of the TPR domains of Hop and the conserved Asp0 residues in Hsp70 and Hsp90 are essential requirements for ligand binding. Removal of either the main-chain or side-chain carboxylate function of Asp0 or any single substitution of two-carboxylate clamp residues in the isolated TPR2A domain severely disrupts ligand binding. However, interactions mediated by the two-carboxylate clamp structure alone are insufficient to produce physiologically relevant ligand affinities. The tetrapeptide EEVD alone binds only very weakly to TPR1, TPR2A, and TPR2B. Additional interactions with adjacent amino acids are required to yield tightly binding peptide ligands for TPR1 and TPR2A (15). TPR2B, however, displays only a very low affinity for full-length Hsp70 and Hsp90, despite the presence of a two-carboxylate clamp, and the role of this TPR domain remains unclear. It is possible that TPR2B functions as a nonselective low-affinity binding site for Hsp70 or Hsp90, increasing the kinetics of Hsp70/Hop/Hsp90 complex formation. Thus, although the two-carboxylate clamp structure can be used as a fingerprint motif for the identification of novel TPR factors interacting with Hsp70 or Hsp90, predictions must be confirmed experimentally, and the actual binding preferences for Hsp70 or Hsp90 must be determined.

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4 C. Patterson, personal communication.
Hsp70  P T I E E V D -COOH
Conservation 100 66 100 98 100 100 100 %

TPR1 motif  Hph-Hph-Ile-Leu-Hph-Val-Asp-COOH

Hsp90  S R M E E V D -COOH
Conservation 91 68 100 100 91 100 100 %

TPR2A motif  Hph-Hsp-Met-Glu-Neg-Val-Asp-COOH

Fig. 7. Ligand recognition motifs of TPR1 (A) and TPR2A (B). The letter size reflects the relative importance of side-chain residues. Side chains of critical significance for binding are highlighted by bold print. Hph, hydrophobic interactions; Neg, electrostatic interactions with acidic side chains. For comparison the sequences of the Hsp70 and Hsp90 C termini are given, and the sequence conservation of individual residues among eukaryotic cytosolic Hsp70 and Hsp90 proteins is indicated.

Second, the other acidic residues of the EEVD anchor sequence are differentially recognized by TPR1 and TPR2A. TPR2A depends on both glutamate residues for efficient ligand binding. Of particular significance is residue Glu-3, shown to be involved in a network of electrostatic interactions with TPR1. Moreover, a binding screen of the combinatorial peptide library OX_{4-VD} revealed a general preference of TPR1 for hydrophobic residues in position -3, with Leu clearly being favored. Apparently, the C terminus of Hsp70 is not evolutionarily optimized for high-affinity binding to the TPR1 domain of Hop, perhaps because additional contacts with other regions of Hsp70 contribute substantially to the interaction or because stronger binding would be functionally detrimental. In any case, the ligand recognition motif of TPR1 cannot explain the almost 100% conservation of Hsp70 sequences in positions Glu-2 and Glu-3. We therefore suggest that other Hsp70 cofactors may recognize different aspects of the extended Hsp70 EEVD motif and depend more strongly on its conserved glutamate residues.

Finally, a third important aspect of TPR-mediated ligand recognition is the strong contribution of hydrophobic residues in the N-terminally extended EEVD ligands to both high affinity and specificity of binding. Whereas Val-1 is important in the peptide interactions with both TPR1 and TPR2A, supporting the general anchor function of the EEVD motif, Ile-4 and Met-4 determine primarily the specificity of Hsp70 and Hsp90 peptide ligands, respectively. Pro-6 in the Hsp70 C terminus does not contribute substantially to TPR1 binding and therefore might be regarded as a secondary determinant of specificity.

Although it is clear from the present study and from additional data (18, 21, 28, 27) that the principles of ligand recognition by TPR domains in the Hsp70-Hsp90 complex apply more generally to TPR-mediated protein interactions, a number of interesting issues remain to be addressed. For example, recent work suggests that CHIP, a cofactor of both Hsp70 and Hsp90, contains a TPR domain that binds EEVD peptides with high affinity but with a broader specificity, enabling CHIP to interact with both Hsp70 and Hsp90 (Refs. 45–47 and data not shown). How this is achieved is not yet understood. TPR proteins operating in entirely different functional contexts have also been found to recognize the C-terminal tails of target proteins. Two clusters of three TPR motifs each cooperate in the human peroxisomal targeting receptor PEX5 to mediate the high-affinity binding of peroxisomal targeting sequences ~SKL-COOH (17). It will be interesting to compare structurally the TPR motifs in PEX5 to the six TPR motifs in domain TPR2L of Hop (Fig. 1). In both cases the TPR motifs are clustered in two pairs of three repeats. However, whereas in PEX5 these clusters form one single binding site for peptide ligand (17), our results suggest that domains TPR2A and TPR2B in TPR2L do not act cooperatively but rather form independent ligand binding sites.

An alternative mode of interaction of TPR modules has recently been described for the TPR protein p67phox, a component of the NADPH oxidase multi-protein enzyme. p67phox contains a β-hairpin insertion in its otherwise classical TPR scaffold. This insertion mediates the interaction of p67phox with the small GTPase Rac (16). An additional intramolecular interaction between a C-terminal extension of the p67phox TPR domain and the ligand binding groove of the domain (16, 48) may be involved in regulation. An alternative binding mode for TPR domains is also expected for Hip, a TPR cofactor of Hsp70. Unlike the TPR domains of Hop and CHIP, Hip does not interact with the EEVD tail of Hsp70 but rather binds to an as yet unidentified internal sequence of the N-terminal ATPase domain of Hsp70 (49, 50).

In summary, protein domains containing TPR motifs represent a highly versatile class of protein interaction modules. The recognition of N-terminally extended EEVD sequences represents the predominant mode of interaction for TPR cofactors of Hsp70/Hsp90 multi-chaperone complexes. The insights now gained into these interactions at the molecular level may lead to the development of specific inhibitors for TPR cofactor binding to Hsp70 or Hsp90. Such compounds would be valuable tools for the functional dissection of the Hsp70/Hsp90 chaperone mechanism in vitro and in vivo.

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Ligand Discrimination by TPR Domains: RELEVANCE AND SELECTIVITY OF EEVD-RECOGNITION IN Hsp70·Hop·Hsp90 COMPLEXES
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