The assembly and intermolecular properties of the Hsp70-Tomm34-Hsp90 molecular chaperone complex*

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*Running title: Tomm34 scaffolds Hsp70 and Hsp90

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Background: Hsp70 and Hsp90 molecular chaperones associate with the network of co-chaperone proteins.

Results: Tomm34 protein interacts with both Hsp70 and Hsp90 chaperones.

Conclusion: Tomm34 scaffolds Hsp70/Hsp90 chaperones by their simultaneous binding.

Significance: Tomm34 represents a novel Hsp70/Hsp90 co-chaperone.

ABSTRACT

Maintenance of protein homeostasis by molecular chaperones Hsp70 and Hsp90 requires their spatial and functional coordination. The cooperation of Hsp70 and Hsp90 is influenced by their interaction with the network of co-chaperone proteins, some of which contain tetratricopeptide repeat (TPR) domains. Critical to these interactions are TPR domains that target co-chaperone binding to the EEVD-COOH motif that terminates Hsp70/Hsp90. Recently, the two TPR domain containing protein, Tomm34, was reported to bind both Hsp70 and Hsp90. Here we characterize the structural basis of Tomm34-Hsp70/Hsp90 interactions. Using multiple methods including pull-down assays, fluorescence polarization, hydrogen/deuterium exchange and site-directed mutagenesis we defined the binding activities and specificities of Tomm34 TPR domains towards Hsp70 and Hsp90. We found that Tomm34 TPR1 domain specifically binds Hsp70. This interaction is partly mediated by a non-canonical TPR1 two-carboxylate clamp and is strengthened by so far unidentified additional intermolecular contacts.

The two-carboxylate clamp of the isolated TPR2 domain has affinity for both chaperones, but as part of the full-length Tomm34 protein, the TPR2 domain binds specifically Hsp90. These binding properties of Tomm34 TPR domains thus enable simultaneous binding of Hsp70 and Hsp90. Importantly, we provide evidence for the existence of Hsp70-Tomm34-Hsp90 tripartite complex. In addition, we defined the basic conformational demands of the Tomm34-Hsp90 interaction. These results suggest that Tomm34 represents a novel scaffolding co-chaperone of Hsp70 and Hsp90 which may facilitate Hsp70/Hsp90 cooperation during protein folding.

INTRODUCTION

The processes of folding and conformational surveillance of proteins are mainly accomplished by two major cytosolic molecular chaperones, Hsp70 and Hsp90. To fulfill the task of folding diverse client proteins, Hsp70 and Hsp90 associate with a variety of co-chaperones that participate in the regulation of Hsp70/Hsp90 assembly, Hsp90 ATPase activity and client protein recruitment (1). Although different interaction interfaces between Hsps and co-chaperones exist (2-3), the largest group of co-chaperones interacts with the highly conserved EEVD-COOH motif present in the C-terminal domains of both Hsp70 and Hsp90 (4-7). These co-chaperones contain so-called tetratricopeptide repeat (TPR) domains (8). TPR domains are present in a variety of proteins and serve as a versatile protein-protein interaction module (4). TPR domains consist of tandem repeats of a
degenerate 34-amino acid motif that folds into two anti-parallel α helices and tandem arrays of TPR motifs form a saddle-like groove which is able to accommodate polypeptides from other proteins (7-8). Apart from loosely conserved residues in the TPR motif responsible for its helicity, TPR domains of co-chaperones that bind to the C-terminal domain of Hsps contain five highly conserved basic residues that are involved in electrostatic interactions with the EEVD-COOH motif (7). These five residues form a so-called two-carboxylate clamp that tightly interacts with the ultimate Asp residue of the EEVD-COOH motif. Electrostatic interactions between the EEVD-COOH motif and residues forming the two-carboxylate clamp represent a general binding anchor for both Hsp70 and Hsp90 co-chaperones (9). Binding specificity to Hsp70 or Hsp90 is determined by the ability of co-chaperone TPR domains to accommodate Hsp70/Hsp90-specific hydrophobic residues located N-terminally to the EEVD-COOH motif (7,10-11).

Cooperation of Hsp70 and Hsp90 is facilitated by the scaffolding function of HOP (Hsp70/Hsp90 organizing protein) co-chaperone (12). HOP bears three TPR domains: TPR1, TPR2A and TPR2B. While the TPR1 domain exhibits preferential binding to Hsp70, TPR2A binds with high affinity to Hsp90. Binding of TPR2B to Hsp70/Hsp90 is weaker and less selective. The interaction of full-length HOP with Hsp90 is mediated mainly by TPR2A-EEVD contacts (9). On the contrary, the interaction of full-length HOP with Hsp70 through the TPR1 domain seems to be more complex, involving TPR1-EEVD-independent contact sites and in some experimental setups was shown to be EEVD-independent (9,13).

Tomm34 (34-kDa translocase of the outer mitochondrial membrane) was originally identified as a component of the mitochondrial import machinery for nuclear-encoded mitochondrial proteins and is found in both the cytosol and mitochondrial membranes (14-15). Recently, Tomm34 was reported to form a complex with both Hsp70 and Hsp90 (16). Unlike HOP, Tomm34 contains only two TPR domains, both of which have conserved amino acid residues able to participate in the formation of a two-carboxylate clamp (Fig. 1AB). The aim of this work was to study the ability of individual Tomm34 TPR domains to interact with Hsp70 and Hsp90 as well as to determine the biochemical and structural properties of this interaction. We confirmed the presence of Hsp70-Tomm34-Hsp90 complex in vitro. Our results indicate that a canonical Tomm34 TPR2 domain two-carboxylate clamp is both necessary and sufficient for interaction of Tomm34 and Hsp90. On the contrary, a newly identified non-canonical two-carboxylate clamp of the Tomm34 TPR1 domain may be only partly functional and does not fully mediate the Tomm34-Hsp70 interaction, which seems to be more complex. Additionally, we provided initial insight into the conformational preferences of Tomm34-Hsp90 interaction. Taken together, we described Tomm34 as a novel Hsp70/Hsp90 scaffolding co-chaperone.
EXPERIMENTAL PROCEDURES

Cloning and protein preparation—All coding sequences were cloned by Gateway recombination technology (Invitrogen). The full coding sequence of human TOMM34 gene (NM_006809) and sequences coding for TPR1 (1-188aa) and TPR2 (188-309aa) domains of Tomm34 protein were cloned into a vector containing N-terminal Hisα-GST tag cleavable by TEV protease; the full coding sequences of human HSP90AA1 (Hsp90α; NM_001017963) and HSPA1A (Hsp70; NM_005345) genes were cloned into a vector containing N-terminal 6xHis tag. All cloned genes were expressed in BL21 (DE3) RPL cells for production of eukaryotic proteins.

The cells were grown in LB medium at 37 °C up to OD600 of 0.5. Induction of gene expression was achieved by adding isopropyl β-D-thiogalactopyranoside (IPTG) to the culture (final concentration 1mM). The bacterial culture was grown at 30 °C for another 2 h (for each TPR domain of Tomm34) or 3 h (full-length Tomm34, Hsp70 and Hsp90α constructs). Cells were harvested by centrifugation for 10 min at 4600g, the pellet resuspended in buffer containing 50 mM Tris, pH 8.0, 0.5 M NaCl, 1 mM PMSF, 1 mg/ml lysozyme (FL Tomm34, TPR1 and TPR2 domain of Tomm34) or 50 mM Hepes, pH 7.4, 0.3 M KCl, 1 mM PMSF, 1 mg/ml lysozyme (Hsp70) or 50 mM Tris, pH 8.0, 0.2 M NaCl, 5 mM MgCl2, 10% glycerol, 1 mM PMSF, 1 mg/ml lysozyme (Hsp90α) and disrupted by sonication. Bacterial lysates were obtained by centrifugation for 30 min at 12000g. Over-expressed recombinant fusion proteins from cell lysates expressing Hisα-GST-Tomm34, Hisα-GST-TPR1 and Hisα-GST-TPR2 were captured on GSTrap glutathione-agarose column (GE Healthcare). Bound proteins were eluted with 20 mM glutathione. Purified Hisα-GST-tagged protein solutions were either loaded on 7K MWCO Zeba Spin Desalting column (Thermo Scientific) to remove glutathione for performing protein pull-down assays, or cleaved with Hisα-TEV protease to remove Hisα-GST tags. N-terminal Hisα-GST tag of Tomm34/TPR1/TPR2 with Hisα-TEV protease was then captured using immobilized metal affinity chromatography (IMAC) on HisTrap column (GE Healthcare), whereas purified recombinant proteins were present in the flow-through fractions. Recombinant proteins Hisα-Hsp70 and Hisα-Hsp90α were purified on HisTrap column (GE Healthcare). The purified proteins were further processed by preparative gel filtration using HiPrep 16/60 Sephacryl S-300 HR column (GE Healthcare). The purity of all isolated proteins was confirmed by SDS-PAGE/Coomassie staining (data not shown).

To obtain bacterially expressed SBP-tagged Tomm34 protein, the BL21 (DE3) RPL cells containing pT7-N-SBP-Tomm34 construct were treated with 1mM IPTG for 2 h, lysed in 50 mM Tris, pH 8.0, 0.5 M NaCl, 1 mM PMSF, 1 mg/ml lysozyme by sonication and cleared lysates incubated with streptavidin-agarose beads (Thermo Scientific) to immobilize the SBP-Tomm34 protein. Washed (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Nonidet P-40) streptavidin beads were then used in further experiments.

Site-directed mutagenesis—The full-length Hsp70 and/or Hsp90α pCMV-N-SBP expression vectors were mutated to encode premature stop codons using QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies) according to manufacturer instructions. Primers used for individual mutagenesis reactions were: Hsp70 5ggagggtctgggtcatgacccaccattgag3 and Hsp90α 5ggagggtctgggtcatgacccaccattgag3

Size exclusion chromatography (SEC)—Separations by SEC were carried out using ProSEC 300S columns (300 x 7.5 mm, Agilent Technologies) pre-equilibrated with 50 mM Hepes, 100 mM KAc, pH 7.4. Tomm34, Hisα-Hsp70 and Hisα-Hsp90α as single proteins or protein mixtures were loaded and isocratically eluted at 0.8 ml/min. The injection volume was 25 µl. Proteins were mixed in equimolar ratios (250 µM each protein). The eluted fractions were read at 280 nm. From each run, 0.6 ml fractions between 8th and 16th minute were collected and analyzed by gel electrophoresis followed by colloidal Coomassie staining (Invitrogen).

Affinity precipitation using tosyl-activated beads—Hisα-Hsp70 protein was incubated with tosyl-activated magnetic beads (Invitrogen) for 1
agarose beads with immobilized peptides were pre-incubated with 2 mM biotin. Unbound peptides were removed by washing the beads three times with washing buffer. Streptavidin-peptides were specifically eluted by 20 mM glutathione and analyzed on SDS-PAGE followed by Coomasie staining. For SBP-tagged protein detection, we used peroxidase-conjugated streptavidin (Sigma-Aldrich) diluted in 5% milk in the presence of avidin at final concentration of 10 ng/µl (avidin was added to avoid streptavidin binding to biotinylated proteins present on a membrane after blocking with milk). The blots were developed with polyclonal anti-mouse rabbit IgG or anti-rabbit swine IgG secondary antibodies conjugated with horseradish peroxidase (Dako).

Fluorescence polarization assay—Full-length Tomm34 and single TPR domains of Tomm34 in various concentrations were titrated against 30 nM fluorescein-labeled C-terminal Hsp70 (GGSGSPTIEEVD) or Hsp90α (GDDDTSRMEEVD) peptide in assay buffer (25 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM DTT, 0.05% Tween-20). To distinguish nonspecific binding, we used bovine serum albumin with the same concentration as analyzed protein. All reactions were carried out in a total volume of 60 µl per well of a 96-well black Nunc Plate (Thermo Scientific). The plate was incubated for 1 h at room temperature with shaking. Fluorescence polarization was measured at 21 °C using FilterMax™ F5 Multimode Microplate Reader (Molecular Devices) with excitation and emission wavelengths of 485 nm and 535 nm, respectively. The equilibrium dissociation constants \( K_d \) and Hill slope values were determined by fitting the fluorescence polarization data to the equation:

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FP = B_{max} \frac{C^h}{(K_d)^h + C^h}
\]

where FP is fluorescence polarization that reflects specific FITC-peptide binding to analyzed protein, C is the concentration of analyzed protein, \( B_{max} \) is the maximum specific binding in the same units as FP, \( K_d \) is the dissociation constant, and \( h \) is the Hill slope. The
analysis was performed using GraphPad Prism version 5.03 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

Cell culture and transfection–HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and 300 mg/l L-glutamine in a humidified atmosphere of 5% CO₂ at 37 °C. Transient transfection of cells with the plasmids pCMV-N-HA-TPR1, pCMV-N-HA-TPR2, pCMV-N-HA-Tomm34fl, pCMV-N-SBP-TPR1, pCMV-N-SBP-TPR2, pCMV-N-SBP-Tomm34fl, pCMV-N-SBP-HSP70, pCMV-N-SBP-HSP90α, pCMV-N-SBP-HSP70ΔPTIEEVD and pCMV-N-SBP-HSP90αΔRMEEVD, was performed by calcium phosphate precipitation according to standard protocols (18). The sequences of SBP-tag and HA-tag are MDEKTTGWRGHSVVEGLAGELEQLRARLEHEHPQGQREPS and YPYDVPDYA, respectively.

Immunoprecipitation and affinity precipitation from HEK293 cell lysates– HEK293 cells grown up to 90% confluency in three 140 mm diameter tissue culture dishes were collected and lysed by sonication in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40) supplemented with protease inhibitor mixture (Sigma-Aldrich). The lysates were then incubated with Protein G agarose beads (GE Healthcare) pre-coated with 4 µg of either specific anti-Tomm34 antibody or anti-HA antibody for 2 h at 4 °C and washed four times with washing buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Nonidet P-40). Following the last washing step, the beads were transferred to new tubes and bound proteins were non-specifically eluted by boiling in NuPAGE LDS Sample Buffer (Invitrogen) and analyzed by western blotting using anti-Hsp70, anti-Hsp90 and anti-Tomm34 antibody. For expression of SBP-tagged proteins, HEK293 cells were grown to 70% confluency in 140 mm diameter tissue culture dishes and transfected by constructs encoding SBP-fusion genes. Cells were collected 24 h after transfection and lysed by sonication in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40) supplemented with protease inhibitor mixture (Sigma-Aldrich) and avidin (Sigma-Aldrich) to a final concentration of 10 ng/µl. The cell lysates were then incubated with high capacity streptavidin-agarose beads (Thermo Scientific) for 1 h at 4 °C. Lysates incubated with streptavidin beads pre-incubated with 2mM biotin diluted in lysis buffer served as control of non-specific binding in each reaction. After incubation the resin was washed four times with washing buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Nonidet P-40). Bound SBP-tagged proteins were specifically eluted by 2 mM biotin diluted in washing buffer and analyzed by gel electrophoresis followed by Coomassie staining. Affinity precipitation from non-transfected HEK293 cells using bacterially expressed SBP-Tomm34 protein was done similarly as described above.

In competitive binding assays, Hsp70 and Hsp90α peptides (GGSGSGPTIEEVD and GDDDTSRMEEVD, respectively) were added to affinity precipitation reactions at the indicated concentrations. Instead of streptavidin-agarose, streptavidin magnetic beads were used (Invitrogen) and bound proteins were finally analyzed by western blotting.

Cell lysates containing HA-tagged proteins for peptide pull-down assay were prepared similarly as described above. Streptavidin-agarose was pre-incubated either with 2 mM biotinylated Hsp70/Hsp90α peptides (biotin-GGSGSGPTIEEVD/biotin-GDDDTSRMEEVD) or with 2 mM biotin for 30 min and then extensively washed with lysis buffer. Next, cell lysates were incubated with peptide pre-coated beads for 1 h at 4 °C and washed four times with washing buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Nonidet P-40). The beads were then transferred to new tubes and bound proteins were non-specifically eluted by boiling the beads in NuPAGE LDS Sample Buffer (Invitrogen) and analyzed by western blotting using anti-HA antibody.

H/D Exchange–Deuteration of Tomm34, Hsp70 and Hsp90α proteins was followed on peptides generated after pepsin digestion. Tomm34 protein was analyzed either free or with biotinylated Hsp70/Hsp90α (biotin-GGSGSGPTIEEVD/biotin-GDDDTSRMEEVD, respectively) C-terminal peptides or with full-length Hsp70/Hsp90α proteins. Tomm34 was incubated with ligands for 30 min prior to the exchange. The exchange started by a 10-fold dilution into a deuterated buffer (25 mM Hapes, pH 7.5 and 150 mM NaCl). The molar ratios were 1:5 between Tomm34 and Hsp70/Hsp90α peptides and 1:2.5 between Tomm34 and...
Hsp70/Hsp90α full-length proteins. Tomm34 concentration during H/D exchange was kept at 20 µM. Aliquots (10 µl) were taken after 20 sec, 2 min, 5 min, 10 min, 1 h, 3 h and 5 h. The exchange was performed at 21 °C and was quenched by the addition of 10 µl of 1 M glycine (pH 2.3) and rapid freezing in liquid nitrogen. Deuteration of Hsp70 and Hsp90α proteins was followed in the presence of 1:2.5 molar excess of Tomm34 protein in the same experimental setup. Chaperone concentration during H/D exchange was 20 µM.

**Digestion and HPLC separation**—Each sample was quickly thawed and injected onto an immobilized pepsin column (66 µl bed volume). Digestion was driven by a flow of 0.4% formic acid in water and the rate was 100 µl/min. Peptides were trapped and desalted online on a peptide microtrap (Michrom Bioresources), eluted onto an analytical column (Jupiter C18, 0.5 x 50 mm, 5 µm, 300 Å, Phenomenex) and separated by a linear gradient elution 10-35% B in 12 minutes at a flow rate 15 µl/min. Solvents were: A – 0.4% formic acid in water, B – 95% acetonitrile/0.4% formic acid. In all analyses, injection and switching valves, immobilized pepsin column, trap cartridge and the analytical column were kept at 0 °C (immersed into an ice-water bath) to minimize back-exchange. Outlet of the LC system was interfaced to ESI source of a mass spectrometer.

**Mass Spectrometry and Data Analysis**—Mass spectrometric analysis used Electrospray Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (ESI-FT-ICR MS, 9.4T Apex-Qe, Bruker Daltonics). For peptide mapping (HPLC-MS/MS) the instrument was operated in a data-dependent mode. Each MS scan was followed by MS/MS scans of the top three most intense ions. Tandem mass spectra were searched using MASCOT against a database containing sequences of Tomm34, Hsp70 and Hsp90α. Sequence coverage was visualized using Draw Map script - http://ms.biomed.cas.cz/MSTools (19). Analysis of deuterated samples was done in HPLC-MS mode and the data were processed by ExPro script provided by Gary H. Kruppa (personal communication) and transferred to DataAnalysis. Deuteration percentages were calculated as follows: % D = 100*((Mpd – Mnd)/N) where Mpd is the mass of partially deuterated peptide, Mnd is the mass of non-deuterated and N is the total number of exchangeable amide hydrogens within the peptide. No correction for back-exchange was done. Average deuteration per amino acid was calculated as follows: Deuteration percentage $D_j$ of j-th amino acid was calculated as weighted mean of peptide deuteration percentages of all peptides containing this particular amino acid, weights were inversely proportional to peptide lengths:

$$D_j = \frac{\sum_{i=1}^{n} \frac{L_i}{L_j} D_{ji}}{\sum_{i=1}^{n} \frac{L_i}{L_j}}$$

where $j$ is amino acid index, $n$ is the number of peptides containing j-th amino acid, $i$ is the peptide index within all $n$ peptides containing j-th amino acid, $D_{ji}$ is deuteration percentage of i-th peptide containing j-th amino acid, $L_i$ is length of i-th peptide containing j-th amino acid, $L_j$ is SUM($L_i$).

**Sequence analysis**—The program TPRpred (20), http://toolkit.tuebingen.mpg.de/tprpred) was used to detect TPR repeats in the Tomm34 protein sequence. Multiple sequence alignment was done using the T-Coffee method (21).
RESULTS

Tomm34 simultaneously binds Hsp70 and Hsp90 chaperones—Tomm34 protein was previously described as an Hsp90 interacting protein (22-23). Recently, Faou et al. (16) reported Tomm34 protein as a co-chaperone able to bind both Hsp70 and Hsp90 chaperones. However, the presence of tripartite Hsp70-Tomm34-Hsp90 complex has not yet been experimentally confirmed. In order to reproduce the results of Faou et al. (16), we performed in vitro GST pull-down assays with purified full-length Tomm34 and Hsp70 and Hsp90α proteins (Fig. 2A). In this assay, Hsp70 and Hsp90α were pulled-down by GST-tagged Tomm34. The ability of Tomm34 to bind Hsp70 and Hsp90 chaperones in complex biological samples was confirmed by co-precipitation of endogenous Hsp70 and Hsp90 proteins with affinity precipitated SBP-tagged Tomm34 from HEK293 cell lysates (Fig. 2B). Additionally, the interaction of Hsp70 and Hsp90 proteins with Tomm34 in non-transfected cell lysates was demonstrated by co-immunoprecipitation (Fig. 2C) and pull-down with bacterially expressed SBP-Tomm34 protein (Fig. 2D). These results confirmed the ability of Tomm34 protein to interact with both Hsp70 and Hsp90 chaperones. To determine whether Tomm34 is also able to form a complex with both Hsp70 and Hsp90 simultaneously, we analyzed Tomm34/Hsp70/Hsp90α protein mixtures by size-exclusion chromatography (Fig. 3A) and Coomassie staining of selected elution fractions separated by gel electrophoresis (Fig. 3B). First, we analyzed the elution profiles of single purified Tomm34, Hsp70 and Hsp90α proteins. Tomm34 protein was eluted in one predominant elution peak indicating its uniform distribution in the sample. The majority of the Hsp90α protein was eluted in an early elution peak. Since Hsp90α predominantly forms dimers (24), we suggest that this peak corresponds to Hsp90α dimer. Hsp70 protein was eluted in two distinct elution peaks. The observed elution profile of Hsp70 protein may reflect the recently described concentration-dependent ability of human Hsp70 to form oligomers (25). Next, we analyzed the elution profile of Tomm34 in the presence of single or combined chaperones. We observed shifts of the Tomm34 elution peak and a gradual reduction of the Tomm34 peak height in the presence of Hsp70, Hsp90α and both, respectively. Gel electrophoresis and Coomassie staining of the collected elution fractions revealed (a) a weak shift of Tomm34 protein to the earlier fractions induced by the presence of Hsp70 protein, (b) a strong association of Tomm34 protein with Hsp90α and (c) a shift of Hsp70 protein to the earlier fractions in the simultaneous presence of Tomm34 and Hsp90α proteins (Fig. 3B). Correspondingly, the absorbance $\text{(A}_{280}\text{)}$ of the Hsp90α elution peak is substantially increased in the presence of both Tomm34 and Hsp70 (Fig. 3A). These results indicate the presence of a tripartite Hsp70-Tomm34-Hsp90α complex. To further support the previous observation, the ability of Tomm34 to connect both Hsp70 and Hsp90 simultaneously was tested by affinity precipitation of Tomm34, Hsp90α, or both, on tosyl-activated beads with immobilized Hsp70 (Fig. 3C). While Tomm34 co-precipitated with immobilized Hsp70, Hsp90α was co-precipitated only in the presence of Tomm34 in the reaction mixture. Interestingly, the level of Tomm34 co-precipitated with Hsp70 decreased in the presence of Hsp90. This may be caused by sterical obstacles during binding of the Hsp90-Tomm34 complex to immobilized Hsp70. Together these results imply the existence of a tripartite Hsp70-Tomm34-Hsp90α complex in vitro.

Tomm34 TPR domains mediate the interaction with Hsp70 and Hsp90—According to amino acid sequence analysis using TPRpred Software (20), Tomm34 contains two TPR domains, N-terminal TPR1 and C-terminal TPR2, each consisting of three TPR repeats (Fig. 1A). The TPR domains are connected with a linker. To determine binding activity and specificity of these Tomm34 TPR domains towards Hsp70/Hsp90α proteins in vitro, we performed GST pull-down assay with purified TPR1 and TPR2 domains (Fig. 4A). This experiment revealed specific binding of Hsp90α to the TPR2 domain of Tomm34, whereas Hsp70 protein bound to both TPR1 and TPR2. Although the Hsp70 interaction with both constructs may point to non-specific binding, there is significant difference in Hsp70 protein level pulled-down by TPR constructs and by GST as a negative control. When analyzing cell lysates, endogenous Hsp70 protein was efficiently co-precipitated with SBP-tagged TPR1 domain and weakly with TPR2 (Fig. 4B). Endogenous Hsp90 protein was specifically co-precipitated by TPR2 domain only. These results indicate that Tomm34 TPR domains represent interaction interfaces for Hsp70 and Hsp90 binding. While the Tomm34 TPR1 domain
C-terminal peptides make αHsp70 and Hsp90 preference towards Hsp90. is less selective and binds both chaperones with exhibiting specificity to Hsp70, the TPR2 domain is less selective and binds both chaperones with preference towards Hsp90.

Hsp70 and Hsp90α C-terminal peptides make contact with Tomm34 TPR domains—Results of multiple sequence alignment of several Hsp70/Hsp90 binding co-chaperones containing TPR domains with Tomm34 revealed the presence of a canonical two-carboxylate clamp (7) in the Tomm34 TPR2 domain (Fig. 1B). Interestingly, all residues of a canonical two-carboxylate clamp are conserved in the Tomm34 TPR1 domain with the exception of lysine in helix 1A, which is substituted to arginine at position 13. Since both lysine and arginine are basic residues, we speculated that this substitution may not disrupt electrostatic interaction of the TPR1 two-carboxylate clamp with the terminal aspartate of the chaperone C-terminal EEVD motif.

To study whether the interaction of Hsp70/Hsp90 with TPR1 and TPR2 domains is mediated through the C-terminal EEVD peptide motif of Hsp70/Hsp90, we evaluated the ability of biotinylated C-terminal Hsp70 tridecapeptide (biotin-GGSGSGPTIEEVD) and/or Hsp90α dodecapeptide (biotin-GDDDTSRMEEVD) to pull-down TPR1, TPR2 and full-length Tomm34 in vitro (Fig. 5A) and in complex biological samples (Fig. 5B). Both experiments showed similar results. The Tomm34 TPR2 domain and full-length Tomm34 protein associate with both Hsp70 and Hsp90α C-terminal peptides, with a binding preference towards the Hsp90α peptide. Interestingly, the Tomm34 TPR1 domain is specifically but weakly pulled-down only by the Hsp70 C-terminal peptide. These results support our previous observations (Fig. 4AB) and suggest that while the TPR2 domain is able to accommodate both Hsp70/Hsp90α C-terminal peptides, the TPR1 domain of Tomm34 is only partly capable of accommodating Hsp70 C-terminal peptide.

To further verify and localize the interaction site between Tomm34 TPR domains and Hsp70/Hsp90α C-terminal peptides, we employed the H/D exchange method (Fig. 6). Pepsin digestion yielded complete peptide coverage of Tomm34 protein (data not shown). After data processing, the level of average deuteration of single amino acids over time was calculated (Fig. 6A, see Experimental procedures). We observed several regions of Tomm34 that are specifically protected from deuteration by the presence of Hsp70 or Hsp90α peptides. Namely, the N-terminal part of Tomm34 sequence forming the TPR1 domain was protected upon binding of both Hsp70 and Hsp90α peptides, with preferential protection induced by Hsp70 peptide. Importantly, the protected regions corresponded to the predicted TPR1 two-carboxylate clamp residues R13, N17 and N56 (Fig. 6B). However, K86 and R90 residues representing the remaining two clamp residues are buried in solvent inaccessible region of Tomm34 independently of Hsp peptides presence. Compared to protection of the N-terminal part of Tomm34, the C-terminal region that forms the TPR2 domain is more extensively protected upon binding of Hsp peptides. Protection in the TPR2 region is preferentially mediated by Hsp90α peptide, but considerable protection is also induced by Hsp70 peptide. The level of protection of TPR2 domain deuteration in the presence of Hsp peptides corresponds well with the positions of the two-carboxylate clamp residues K197, N201, N232, K262 and R266 (Fig. 6B). These results indicate that the TPR1 domain preferentially accommodates Hsp70 C-terminal peptide. Nevertheless, K86 and R90 residues of TPR1 are not involved in Hsp70 peptide induced Tomm34 structural changes under these experimental conditions. On the contrary, the TPR2 domain is likely to possess a two-carboxylate clamp able to fully accommodate both Hsp peptides, with preference for Hsp90α. These results are in good agreement with our peptide pull-down results (Fig. 5). H/D exchange data also distinguished a highly solvent exposed linker region connecting the TPR1 and TPR2 domains, which is not differentially deuterated in the presence of Hsp peptides. Additionally, monitoring of deuteration of selected peptides corresponding to predicted TPR1 and TPR2 two-carboxylate clamp residues over time revealed differential deuteration kinetics for the TPR1 and TPR2 domains in the presence of Hsp peptides (Fig. 6B). While deuteration kinetics plots for TPR1 clamp peptides implicate transient structural changes induced by Hsp peptides, structural changes induced in the TPR2 clamp region are more stable. This observation may explain the weak binding of TPR1 domain to Hsp70 C-terminal peptide as examined by peptide pull-down experiments (Fig. 5).
Hsp70 and Hsp90α C-terminal peptides bind Tomm34 with micromolar affinities—Based on H/D exchange results, we proceeded to a more precise characterization of the interaction between Hsp70/Hsp90α C-terminal peptides and Tomm34 protein using fluorescence polarization measurements of equilibrium dissociation constant $K_d$ (Fig. 7, Table 1). For this assay we used Hsp70 (fluorescein-GGSGSGPTIEEVD) and Hsp90α (fluorescein-GDDDTSRMEEVD) peptides labeled with FITC. Since results of peptide pull-down assays and H/D exchange measurement indicated cross-specificity of Hsp peptides binding to Tomm34 TPR domains, we determined the $K_d$ of Hsp70/Hsp90α C-terminal peptide interactions with Tomm34 TPR domains and full-length protein (Table 1). We were not able to measure a $K_d$ for Hsp70/Hsp90α peptides binding to the TPR1 domain (Fig. 7A). The $K_d$ of this interaction is probably too low to be determined under our experimental setup. The Tomm34 TPR2 domain interacts with Hsp70 and Hsp90α C-terminal peptides with a $K_d$ of 32.9 μM and 1.5 μM, respectively (Fig. 7B). These $K_d$ values well reflect the binding preference of the TPR2 domain towards Hsp90α C-terminal peptide (Fig. 5, 6). The $K_d$ values of full-length Tomm34 protein binding to Hsp70/Hsp90α peptides are 5.6 μM and 0.8 μM, respectively (Fig. 7C). These results show that full-length Tomm34 has higher affinity for Hsp70/Hsp90α peptides.

Next, we determined Hill coefficients of the binding curves (Table 1). Importantly, the Hill coefficient of full-length protein interactions with Hsp C-terminal peptides was greater than one (for Hsp70 1.5 ± 0.2, for Hsp90α 1.6 ± 0.2), indicating positive cooperativity during Hsp70/Hsp90α C-terminal peptide binding to full-length Tomm34 protein. Hill coefficient significantly greater than one was not observed for Hsp peptides binding to TPR2 domain (for Hsp70 0.9 ± 0.1, for Hsp90α 1.1 ± 0.1). We speculate that higher affinity of full-length Tomm34 protein for Hsp70/Hsp90α peptides may be caused by positive cooperativity effect. Analogous results were obtained for binding curves of full-length HOP protein and its TPR1 and TPR2A domains interaction with Hsp C-terminal peptides (9). The cooperative binding can be explained, at least partly, in principle by conformational flexibility of the interacting molecule and the existence of high- and low-affinity states in dynamic equilibrium. This explanation is supported by H/D exchange measurement of Tomm34 protein, revealing the presence of an unstructured linker separating the two Tomm34 TPR domains, which can enable flexible conformational changes of Tomm34 molecule (Fig. 6B).

Hsp70-Tomm34 interaction is largely EEVD independent—We have determined specificities and affinities of Hsp70/Hsp90α C-terminal peptides towards Tomm34 TPR domains. Based on these results, we next asked whether the interaction of Tomm34 and Hsp70/Hsp90 chaperones is exclusively mediated through peptide-two-carboxylate clamp interfaces. To answer this question, we evaluated the stability of TPR1/TPR2-Hsp70/Hsp90 and Tomm34-Hsp70/Hsp90 complexes in vitro (Fig. 8A) and in cell lysates (Fig. 8B) after addition of molar excess of Hsp70/Hsp90α C-terminal peptides. The Tomm34 TPR1 domain was shown to bind selectively to Hsp70 protein (Fig. 4). Addition of the Hsp70 C-terminal peptide to pre-formed TPR1-Hsp70 complexes partly disassembled the complex only if added at 100 μM concentration (Fig. 8A). Conversely, in vitro TPR2-Hsp70 and TPR2-Hsp90α complexes were efficiently disrupted in the presence of increasing concentration of free Hsp70 and Hsp90α C-terminal peptides, respectively. While the Tomm34-Hsp70 complex was not affected at all by Hsp70 peptide addition in vitro, the Tomm34-Hsp90α complex was rapidly decomposed by excess of Hsp90α peptide. Peptide competition in cell lysates revealed similar results (Fig. 8B). SBP-Tomm34-Hsp90 complex was selectively disrupted by 100 μM Hsp90α peptide addition, while addition of Hsp70 peptide left this complex unaffected. The same specificity of Hsp peptides competing activity was observed for TPR2-Hsp90 complex. Interestingly, the TPR2-Hsp70 complex was unselectively abrogated by both Hsp C-terminal peptides. These results indicate that Hsp90’s interaction with Tomm34 is enabled by TPR2 two-carboxylate clamp contacts with the C-terminus of the chaperone. Interaction of the TPR2 domain with Hsp70 seems to be entirely two-carboxylate clamp dependent as well. The fact that Hsp70 C-terminal peptide is not able to out-compete TPR2 from complexing with Hsp90 while Hsp90α C-terminal peptide abrogates TPR2-Hsp70 binding supports the preference of TPR2 domain carboxylate clamp to Hsp90. On the contrary, Hsp70 binding to Tomm34 (both in vitro and in cell lysates) is not exclusively mediated through
two-carboxylate clamps, since it is affected by neither Hsp70 nor Hsp90α C-terminal peptide addition. Interestingly, 100 µM concentration of both Hsp peptides caused complete disassembly of the TPR1-Hsp70 complex in cell lysates (Fig. 8B). These results suggest the existence of an additional EEVD-independent interaction interface between Hsp70 and Tomm34.

In order to further address the dependence of Tomm34-Hsp70/Hsp90 protein-protein interactions on two-carboxylate clamp-EEVD motif contacts, we analyzed the binding activity of Hsp70 and Hsp90α ∆EEVD mutants towards HA-Tomm34 in cell lysates (Fig. 8C). The results of this experiment support the indispensability of C-terminal EEVD motif of Hsp90α for interaction with Tomm34, since a SBP-Hsp90α mutant lacking RMEEVD C-terminal amino acids completely lost its ability to co-precipitate with HA-Tomm34 protein compared to unmutated SBP-Hsp90α. On the contrary, HA-Tomm34 protein was co-precipitated with PTEEVD C-terminal peptide deficient SBP-Hsp70 protein, albeit the amount was reduced compared to unmutated SBP-Hsp70. Based on this result, we suggest that binding between Hsp70 chaperone and Tomm34 co-chaperone is not solely mediated through two-carboxylate clamp recognition of Hsp70 C-terminal EEVD motif, but involves other intermolecular contacts.

_Tomm34_ TPR2 domain of full-length Tomm34 is selectively accessible only for Hsp90α chaperone through its C-terminal EEVD motif. To gain more detailed insight into the protein-protein interaction interfaces between Tomm34 protein and Hsp70/Hsp90α chaperones, we performed H/D exchange experiments with full-length proteins (Fig. 9). The deuteration level was followed on all three proteins. Pepsin digestion yielded complete peptide coverage of Tomm34 protein and >90% peptide coverage of both Hsp70 and Hsp90α chaperones (data not shown). Similarly as in our previous H/D exchange experiment (Fig. 6A), we calculated the level of average deuteration of single amino acids (Fig. 9AC). Interestingly, we were not able to detect any significant protection of Tomm34 protein in the presence of 2.5 molar excess of Hsp70 chaperone and _vice versa_ (Fig. 9ABC). On the contrary, Tomm34 protein protection was significantly induced by excess of Hsp90α in the region corresponding to the TPR2 domain (Fig. 9AB). The absence of protection between Tomm34 TPR1 domain and Hsp70 protein may be explained by low affinity of Hsp70 C-terminal peptide towards TPR1 two-carboxylate clamp (Table 1) which is not sufficiently compensated by 2.5 molar excess of the protein ligand and thus the interaction is undetectable under this H/D exchange experimental setup. Importantly, since the TPR2 domain of full-length Tomm34 accommodates (Fig. 6) and significantly binds both Hsp70/Hsp90α C-terminal peptides (Table 1) and the isolated TPR2 domain interacts with both full-length chaperones (Fig. 4), we expected to observe Hsp70 induced TPR2 protection. This was not detected and instead we observed weak deprotection in peptides covering TPR2 two-carboxylate clamp amino acids (Fig. 9B). This result indicates that TPR2 domain in full-length Tomm34 protein is selectively accessible only for Hsp90α.

The strong protection of Tomm34 TPR2 domain induced by excess Hsp90α was not accompanied by any difference in deuteration of Hsp90α peptides in the presence of excess of Tomm34 protein (Fig. 9C). We have previously shown that the Tomm34-Hsp90α interaction is critically dependent on the Hsp90 C-terminal motif (Fig. 8). Even though we were not able to detect Hsp90α peptides covering the EEVD amino acids, the region preceding these amino acids was sufficiently covered (data not shown). The absence of the protection of the extreme Hsp90α C-terminus might be caused by the high flexibility of this region (26). It has been previously reported that within the time frame of the H/D experiment the fast deuteration kinetics of the flexible structures might abrogate the detection of the interaction event (27). Together, these data suggest that Tomm34 binding to Hsp90α is not assisted by any additional stable intermolecular contacts, but is mediated solely through the TPR2-two-carboxylate clamp interaction.

Additionally, the conformation of TPR1 domain is not affected by Hsp90α binding to TPR2 domain (Fig. 9A).

_Tomm34-Hsp90 interaction is affected by Hsp90 conformation._ Next, we decided to further analyze the Tomm34-Hsp90 interaction in the context of complex biological samples. Hsp90 is a flexible dimeric protein which adopts structurally distinct conformations upon ATP binding (28). While nucleotide-free Hsp90 exists in “open conformation”, nucleotide-bound Hsp90 adopts a more compact "closed
conformation”. The conformational changes of Hsp90 induced by ATP are assisted by co-chaperones and determine the spectrum of Hsp90-bound co-chaperones (29-30). In complex biological samples, the conformational status of Hsp90 can be influenced by addition of specific Hsp90 inhibitors: geldanamycin and molybdate. Geldanamycin or its derivate 17AAG prevents ATP binding and thus stabilizes the “open conformation” of Hsp90 (31). Conversely, molybdate stabilizes the nucleotide-bound “closed conformation” of Hsp90 (32). In order to determine the dependency of Tomm34-Hsp90 interaction on Hsp90 conformation in HEK293 cell lysates, we performed co-precipitation of endogenous Hsp90 protein by SBP-Tomm34 in the presence of either 4 µM 17AAG or 20 mM sodium molybdate (Fig. 10). The level of co-precipitated Hsp90 in the presence of 17AAG was not affected compared to the non-treated control. Conversely, the presence of molybdate significantly decreased the level of Hsp90 bound to SBP-Tomm34. This result implies that molybdate “locks” Hsp90 into a conformational status which is unfavourable for Tomm34 binding.
DISCUSSION
Tomm34 was originally described as a translocase localized on the surface of mitochondria (15). Later studies showed that Tomm34 is mainly localized in cytosol and functions as a component of large chaperone complex, which shuttles proteins from ribosomes to mitochondria (14,16). Recently, Tomm34 has been identified as an Hsp70 and Hsp90 co-chaperone (16,22-23) and, in addition to HOP (12), Tomm34 can serve as a scaffold protein by binding both Hsp70 and Hsp90. Therefore the presented work is focused on the analysis of complex formation between full-length Tomm34 and its individual domains with Hsp70 and Hsp90 to disclose the structural relations between these proteins. We showed the ability of full-length Tomm34 to interact with both Hsp70 and Hsp90α in vitro (Fig. 2A) and in cell lysates (Fig. 2BCD). The binding of both Hsp70 and Hsp90α chaperones to Tomm34 is not mutually exclusive as supported by size-exclusion chromatography of Tomm34/Hsp70/Hsp90α protein mixtures and by affinity precipitation of Hsp70-Tomm34-Hsp90 protein complexes (Fig. 3). These results suggest the ability of Tomm34 to enable spatial cooperation of Hsp70 and Hsp90α chaperones by their simultaneous binding. Multiple sequence alignment of Tomm34 with homologous TPR domains (Fig. 1B) indicated that the TPR2 domain of Tomm34 contains conserved amino acids able to form a positively charged clamp similar to the TPR domains of HOP that bind the C-terminal EEVD domain of Hsp70 or Hsp90 (7). In concordance with alignment results, we observed binding of the Tomm34 TPR2 domain to Hsp70 and particularly to Hsp90 (Fig. 4AB). These interactions were mediated through TPR2 two-carboxylate clamp accommodation of Hsp70/Hsp90α C-terminal peptides, since the peptides were shown to make contacts with entire TPR2 domain (Fig. 5AB, 6AB). No other interaction interfaces are expected between TPR2 and Hsp70/Hsp90 chaperones, considering that Hsp70/Hsp90α C-terminal peptides efficiently out-competed chaperone binding to TPR2 domain (Fig. 8AB). The preferential binding of TPR2 to Hsp90α C-terminal peptide was revealed by H/D exchange (Fig. 6AB) and quantified by fluorescence polarization $K_d$ measurement (Fig. 7B).

Conversely, multiple sequence alignment of the Tomm34 TPR1 domain showed that its two-carboxylate clamp amino acid positions are conserved with the exception of the first lysine, which is substituted to arginine (R13). This non-canonical composition of the TPR1 two-carboxylate clamp is probably why the TPR1 domain of Tomm34 was omitted in similar TPR multiple sequence alignments reported previously (7,16). While the TPR1 domain exhibited specific and significant binding to Hsp70 protein (Fig. 4AB), its interaction with Hsp70 C-terminal peptide was specific but rather weak, with an undetectable $K_d$ value under our experimental conditions (Fig. 5AB, 7A). H/D exchange measurements showed that the region of the TPR1 domain corresponding to the first three two-carboxylate clamp residues was weakly protected upon Hsp70/Hsp90α C-terminal peptides addition, with Hsp70 peptide being more efficient, and that the remaining two clamp residues are not accessible for protection (Fig. 6AB). These observations indicate that non-canonical TPR1 two-carboxylate clamp recognition of Hsp70/Hsp90α C-terminus may be partly functional. This is supported by a study reporting that only simultaneous mutagenesis of the first two two-carboxylate clamp residues of the HOP TPR1 domain to alanines was sufficient to eliminate its interaction with Hsp70 (33).

Furthermore, participation of the last two clamp residues located in the third TPR repeat of TPR domains on accommodation of Hsp peptides might not be necessary, since efficient binding to Hsp90 EEVD motif was reported for Tah1 (TPR-containing protein associated with Hsp90) protein lacking the third TPR repeat in its TPR domain (34).

Peptide competition assays for TPR1-Hsp70 complex revealed contradictory results in vitro and in cell lysates (Fig. 8AB). This discrepancy between the ability of Hsp70/Hsp90α peptides to compete for binding to TPR1 domain with Hsp70 protein could be explained by high molar excess of these peptides present in cell lysate experiments compared to in vitro conditions. Moreover, TPR1-Hsp70 complex formed under controlled conditions in vitro was markedly more resistant to Hsp70 peptide than TPR2-Hsp70 complex. Since we showed that the TPR2-Hsp70 binding is mediated by clamp-Hsp70 EEVD motif interaction, we suggest that TPR1 interaction with Hsp70 is not solely dependent on EEVD motif recognition but is accompanied by additional molecular contacts. This effect was even more pronounced for full-length Tomm34-Hsp70 interaction (Fig. 8AB), which was only
partly decreased by deletion of Hsp70 EEVD motif (Fig. 8C). Similar results have been reported previously for HOP’s interaction with C-terminally truncated Hsp70 protein (13). Several other studies of the HOP-Hsp70 complex suggested the existence of secondary contacts strengthening the clamp-EEVD interaction between these proteins (9,35). Additionally, a model has been proposed in which the TPR domain is initially recognized by multiple Hsp70/Hsp90 sites and this interaction is then secured by two-carboxylate clamp-EEVD motif interaction (11). This model may also explain the observed EEVD-independence of the Tomm34-Hsp70 interaction.

Unfortunately, we were not able to reveal the interaction interface between full-length Tomm34 and Hsp70 proteins using H/D exchange (Fig. 9). This was probably caused by the weak \textit{in vitro} affinity of Tomm34 towards Hsp70 which is undetectable by H/D exchange method in our experimental setup. Nevertheless, the experiment indicated that the TPR2 domain of full-length Tomm34 is not accessible for Hsp70. This implies that Hsp70 and Hsp90 are not competing for binding to Tomm34 through the TPR2 domain. Such exclusivity of chaperone binding to TPR domains of full-length Tomm34 further supports the role of Tomm34 as an Hsp70/Hsp90 scaffolding co-chaperone.

Interestingly, we pulled-down Hsp70 and Hsp90 from the cell lysates by SBP-Tomm34 with high efficiency (detected by Coomassie staining, Fig. 2B). On the contrary, the interaction of Tomm34 and Hsp70 was considerably weaker \textit{in vitro} (Fig. 3) and even undetectable by H/D exchange method (Fig. 9). We speculate that additional low-molecular weight or protein factors present in the cells may strengthen the Tomm34-Hsp70 interaction. Moreover, it has been reported that the binding of Hsp90 to the TPR2A domain of HOP influenced the conformation of its TPR1 domain to enhance the binding of Hsp70 (36). We do not expect this mechanism for Hsp70-Tomm34-Hsp90 tripartite complex because the binding of Hsp90α to Tomm34 TPR2 domain did not change the conformation of the TPR1 domain (Fig. 9A).

The full-length Tomm34-Hsp90 complex exhibited similar behavior during peptide competition assay as the TPR2-Hsp90 complex (Fig. 8AB). Moreover, the \( K_d \) of full-length Tomm34-Hsp90α C-terminal peptide interaction (\( K_d \) 0.8 µM) is comparable to the TPR2-Hsp90α C-terminal peptide interaction (\( K_d \) 1.5 µM). These observations indicated that most of the affinity of full-length Tomm34 for Hsp90α resides in the TPR2 two-carboxylate clamp-Hsp90α C-terminal peptide interaction. The dependence of the Hsp90-Tomm34 interaction on clamp-EEVD recognition was demonstrated by the loss of binding between Hsp90α and Hsp90 protein and Tomm34 (Fig. 8C). Indeed, Tomm34 TPR2 domain protection was strongly induced by flexible C-terminal motif of full-length Hsp90α protein (Fig. 9AB). Importantly, we did not observe any other regions of Hsp90α protein protected by Tomm34 binding (Fig. 9C). Recently, Lee \textit{et al.} (26) has shown using a very similar H/D exchange experimental setup that Sti1 (yeast HOP homolog) extensively contacts Hsp90 and reduces its conformational flexibility, stabilizing Hsp90 in an “open conformation”.

These results indicate that HOP binding, compared to Tomm34, has a profound effect on the conformational status of Hsp90. According to these differences between HOP and Tomm34 effects on Hsp90 conformation, we propose that each co-chaperone assists Hsp90 in different folding processes, or at different stages of a multi-step protein folding process. Conversely, we observed that the conformational and/or functional status of Hsp90 influences Tomm34 binding in cell lysates. The addition of molybdate destabilized Hsp90-Tomm34 complexes (Fig. 10). Correspondingly, molybdate has been previously shown to stabilize Hsp90 complexes lacking HOP (30). Since molybdate “locks” Hsp90 in a “closed conformation”, we propose that, similarly to HOP, Tomm34 binds preferably to “open” Hsp90 dimers and participates during the loading of client proteins from Hsp70 (37). In conclusion, we show in this study that Tomm34 represents a novel Hsp70/Hsp90 co-chaperone. Since the interaction with both Hsp70 and Hsp90 is not mutually exclusive, Tomm34 may serve in a similar manner as the co-chaperone HOP and facilitate the functional cooperation of Hsp70 and Hsp90 chaperones by their simultaneous scaffolding. Our results also identify the structural basis for specific binding of Tomm34 TPR domains to Hsp70 and Hsp90, a pre-requisite to understand the mechanism of tripartite Hsp70-Tomm34-Hsp90 complex assembly and function.
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FOOTNOTES:

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FIGURE LEGENDS:

Figure 1:
Tomm34 protein sequence analysis.
(A) Amino acid sequence analysis of Tomm34 according to TPRpred software (20). TPRpred detects 6 TPR repeats forming two TPR domains connected with a linker.
(B) Multiple sequence alignment of several Hsp70/Hsp90 binding co-chaperones containing TPR domains. Highly conserved residues of the two-carboxylate clamp involved in electrostatic interactions with EEVD motif of Hsps are highlighted in black, polar residues in the conserved position of two-carboxylate clamp are highlighted in grey. Sequences were aligned and visualized using T-Coffee software (21).

Figure 2:
Tomm34 interacts with Hsp70 and Hsp90.
(A) His$_6$-GST-Tomm34 or His$_6$-GST was mixed with His$_6$-Hsp70 and/or His$_6$-Hsp90α and incubated with glutathione-agarose beads. After washing out the unbound proteins, bound chaperones were specifically eluted by glutathione and analyzed by western blotting. (B) HEK293 cell lysate with over-expressed SBP-Tomm34 was incubated with streptavidin-agarose beads. As a control of nonspecific binding, streptavidin beads were pre-blocked with biotin. After washing, bound proteins were eluted by biotin and analyzed by gel electrophoresis followed by Coomassie staining. (C) HEK293 cell lysate was incubated with Protein G agarose beads pre-coated with anti-Tomm34 or with anti-HA antibodies as a control for non-specific binding. After washing, bound proteins were eluted by boiling the beads in Sample Buffer and analyzed by western blotting using specific anti-Tomm34, anti-Hsp70 and anti-Hsp90 antibodies. TCL, total cell lysate. (D) HEK293 cell lysate was incubated with streptavidin-agarose beads pre-coated with bacterially expressed SBP-Tomm34 protein or with beads pre-blocked by biotin. After washing, the biotin eluted complexes were analyzed by western blotting.

Figure 3:
Analysis of Hsp70-Tomm34-Hsp90α tripartite complex.
(A) Size exclusion chromatography analysis. Superposition of chromatograms from SEC of Tomm34, His$_6$-Hsp70 or His$_6$-Hsp90α as single proteins (upper part) or protein mixtures (lower part) with emphasis on Tomm34 peak. 25 µl of sample was loaded on ProSEC 300S column (Agilent Technologies) and isocratically eluted at 0.8 ml/min. Proteins were mixed in equimolar ratio, the concentration of each protein in the sample was 250 µM. (B) 0.6 ml fractions between 8th and 16th minute were analyzed by gel electrophoresis followed by colloidal Coomassie staining (Invitrogen); Purified proteins were run as positive controls. (C) Affinity precipitation on tosyl-activated beads. After covalent linking of His$_6$-Hsp70 protein to tosyl-activated bead surface (see Experimental procedures), beads were mixed with Tomm34 and/or His$_6$-Hsp90α (line 2-4). Beads with covalently bound His$_6$-Hsp70 mixed with neither Tomm34 nor His$_6$-Hsp90α were used as a negative control (lane 1). After washing, bound proteins were eluted by boiling the beads in Sample Buffer and analyzed by gel electrophoresis followed by Coomassie staining (Tomm34, lower gel) or western blotting (His$_6$-Hsp90α, upper gel). * Nonspecific band present in all fractions.
Figure 4: Tomm34 TPR domains binding specificity towards Hsp70/Hsp90 proteins.
(A) His$_6$-GST tagged TPR domains or His$_6$-GST was mixed with His$_6$-Hsp70 and/or His$_6$-Hsp90α and pull-down experiments performed similarly as described in Figure 2A.
(B) Affinity precipitation from HEK293 cell lysates with over-expressed SBP-tagged TPR domains performed similarly as described in Figure 2B.

Figure 5: Tomm34 TPR domains accommodate Hsp70/Hsp90α C-terminal peptides.
(A) Peptide pull-down assay. After immobilization of biotinylated Hsp70/Hsp90α C-terminal peptides on streptavidin-agarose beads, His$_6$-GST-tagged TPR1, TPR2 or full-length Tomm34 protein were added and incubated for 2 h at 4 °C. After washing, bound complexes were eluted by boiling the beads in Sample Buffer and analyzed by gel electrophoresis followed by Coomassie staining. (B) HEK293 cell lysates with over-expressed HA-tagged TPR1, TPR2 or Tomm34 were used in this assay. Streptavidin-agarose beads were pre-coated with either biotinylated Hsp70/Hsp90α peptides or biotin and incubated with cell lysates. After 1 h incubation at 4 °C and extensive washing, bound proteins were non-specifically eluted by boiling the beads in Sample Buffer and analyzed by western blotting using anti-HA antibody. (C) The same film as in (B) developed after longer exposition; 70 pept – biotin-GGSGSGPTIEEVD, 90 pept – biotin-GDDDTSRMEEVD.

Figure 6: H/D exchange analysis of Tomm34 interaction with C-terminal Hsp70/Hsp90 peptides.
(A) The level of average deuteration of single amino acids at particular times (5 min, 1 h) computed according to the equation defined in Experimental procedures. TPR repeats predicted by TPRpred software are illustrated as gray boxes under the corresponding amino acid sequence (x axis). (B) Time course of Tomm34 protein deuteration; graphs summarizing deuteration kinetics of Tomm34 at three different states – apo (without peptides, blue) or in complex with Hsp70/Hsp90α C-terminal biotinylated peptides (Hsp70: GGSGSGPTIEEVD, green; Hsp90α: GDDDTSRMEEVD, red). Original spectra at particular times are depicted under each plot.

Figure 7: Binding of Tomm34 and its TPR domains to Hsp70/Hsp90α peptides analyzed by fluorescence polarization (FP).
TPR1 (A), TPR2 (B) and full-length Tomm34 (C) were titrated against 30nM fluorescein-labeled C-terminal Hsp70 (GGSGSGPTIEEVD, continuous line) or Hsp90α (GDDDTSRMEEVD, dashed line) peptide and FP values were measured. Specific binding was calculated by removing nonspecific peptide binding to BSA from total peptide binding to analyzed protein. The fluorescence polarization values were plotted versus the protein concentration, and binding curves were obtained by fitting the equation defined in Experimental Procedures to the titration data.

Figure 8: Competition of Tomm34 and its TPR domains binding to Hsp70/Hsp90.
(A) His-GST tagged proteins (TPR1/TPR2/Tomm34) were mixed with their binding partners (His-Hsp70/Hsp90α chaperones) and increasing concentration of C-terminal Hsp70/Hsp90α peptides. Protein complexes were then captured on glutathione-agarose beads and after washing steps specifically eluted by glutathione and analyzed by western blotting using specific antibodies. (B) HEK293 cell lysates with over-expressed SBP-tagged proteins (TPR1/TPR2/Tomm34) were incubated with streptavidin magnetic beads alone or in the presence of Hsp70 or Hsp90α C-terminal peptides in final concentration of 100 µM. As a control of nonspecific binding, streptavidin beads were pre-blocked with biotin. After washing, bound proteins were eluted by biotin and analyzed by western blotting using specific antibodies. (C) HEK293 cell lysates with over-expressed HA-tagged Tomm34 and SBP-tagged proteins (FL Hsp70, Hsp90α or truncated mutants Hsp70APTIEEVD and Hsp90αARMEEVD) were incubated with streptavidin-agarose beads. After washing, bound proteins
Tomm34 scaffolds Hsp70 and Hsp90

were eluted by biotin and analyzed by western blotting using peroxidase-conjugated streptavidin and anti-HA antibody.

**Figure 9:**
**H/D exchange analysis of Tomm34 interaction with full-length Hsp70/Hsp90α proteins.**
(A) The level of average deuteration of individual Tomm34 amino acids at 1 h computed according to the equation defined in *Experimental procedures*. TPR repeats predicted by TPRpred software are illustrated as gray boxes under the corresponding amino acid sequence (x axis). (B) Time course of Tomm34 protein deuteration; graphs summarizing deuteration kinetics of Tomm34 at three different states – apo (without proteins, blue) or in complex with Hsp70/Hsp90α proteins (Hsp70, green; Hsp90α, red). Original spectra at 1 h of deuteration are depicted under each plot. (Note: Some of the selected peptides covering the same region differ between Fig.6 and Fig.9. This is caused by technical difficulties to obtain the same peptides from more complex digest and separation.). (C) The level of average deuteration of individual Hsp70 and Hsp90α amino acids at 1 h.

**Figure 10:**
**Hsp90 conformational status affects the Hsp90-Tomm34 interaction.**
HEK293 cells expressing SBP-Tomm34 or SBP-GFP were lysed in lysis buffer alone or supplemented with either 4 μM 17AAG or 20 mM sodium molybdate. Next, the cell lysates were incubated with streptavidin-agarose beads. After washing in buffer containing 4 μM 17AAG or 20 mM sodium molybdate, bound proteins were eluted by biotin and analyzed by western blotting using peroxidase-conjugated streptavidin and anti-Hsp90 antibody. SBP-GFP samples served as a control for non-specific binding. TCL, total cell lysate.
Table 1

| Hep70 peptide | Hep90α peptide |
|---------------|----------------|
|               | $K_d$ (μM)     | h   | $K_d$ (μM) | h   |
| Tomm34        | 5.6            | 0.6 | 1.5        | 0.2 | 0.8 | 0.1 | 1.6 | 0.2 |
| TPR1          | NSB            | -   | -          | -   | NSB | -   | -   | -   |
| TPR2          | 32.9           | 6.2 | 0.9        | 0.1 | 1.5 | 0.2 | 1.1 | 0.1 |

$K_d$ values (μM) for Tomm34, TPR1 and TPR2 ligand binding (fluorescein-Hsp70/Hsp90α peptides). Data were obtained by analyzing binding curves from fluorescence polarization from three independent measurements (see Figure 7) using GraphPad Prism version 5.03; NSB, Non-Saturating Binding; SEM, Standard Error of the Mean; h, Hill slope.
Figure 1

A

| Repeat | Begin | End |
|--------|-------|-----|
| TPR 9  | VEELRAGNESFRNGQYAESAALYGLRALRVLQQ | 42  |
| TPR 51 | SVLYSNRAACHLKDGCNRCDICKDTSALALVPFS | 84  |
| TPR 85 | IKPLLRRASAYEALKEPMAYVDYKTVLQIDDNV | 118 |
| TPR 193| ARVLKEEGNELVKKGNHKKAIAEKYSESSLNLE | 226 |
| TPR 227| SATYSNRLCYVLKQYTEAVKDCTEALKLDGKN | 260 |
| TPR 261| VKAFYRRAQAHKLKDYKSSFADISNLLQIEPRN | 294 |

B

| Helix 1A | Helix 1B |
|---------|---------|
| Tomm34-TPR1| VEELAAGNESFRNGQYAESAALYGRALRVLQQ | 42 |
| Tomm34-TPR2| ARVLKEEGNELVKKGNHKKAIAEKYSESSLCC | 238 |
| CHIP | AQELKEEGNELVKKGNHKKAIAEKYSESSLCC | 238 |
| HOP-TPR1 | VNELKKEKALKSVGNIDDALQCYSEAIAL | 263 |
| HOP-TPR2A | AKIKKELGLDYKKFDLTALKHYDKEAL | 298 |
| FKBP5 | SSTLFEVFEFKGYKOADYI | 333 |
| PPS5 | AEELTGQKDYKDYENAIKFYSQALEL | 368 |

| Helix 2A | Helix 2B |
|---------|---------|
| Tomm34-TPR1 | SSDPEEEVLYNHRKAAACHLKDGNCRCDICKDTSALALVPFS | 4 |
| Tomm34-TPR2 | SSNLETSATYSNRALCYLVKQYTEAVKDCTEALKLDGKN | 23 |
| CHIP | NS- | 23 |
| HOP-TPR1 | DPNLVAAYNYKALCYLMKQHSEQALADCRRALEDGQ3 | 23 |
| HOP-TPR2A | DPKHKLYLYKASTAYRKGDYQKAYDCKTVDLKDPW | 23 |
| FKBP5 | QAQALRLASHIAMCBLKLQAFSAAIESCNALELDSNN | 23 |
| PPS5 | NNPN | 23 |

| Helix 3A | Helix 3B |
|---------|---------|
| Tomm34-TPR1 | --- | --- |
| Tomm34-TPR2 | --- | --- |
| CHIP | --- | --- |
| HOP-TPR1 | DPKHKLYLYK | 23 |
| HOP-TPR2A | DPKHKLYLYK | 23 |
| FKBP5 | QAQALRLASHIAMCBLKLQAFSAAIESCNALELDSNN | 23 |
| PPS5 | NNPN | 23 |
Figure 2

Tomm34 scaffolds Hsp70 and Hsp90

A

B

C

D
Figure 3

A

B

C

Tomm34 scaffolds Hsp70 and Hsp90
Figure 4

A

Hsp90
Hsp70
GST-TPR1
GST-TPR2
GST

B

Hsp90
Hsp70

SBP-TPR1
SBP-TPR2
Figure 5

A

+ TPR1 + TPR2 + Tomm34

Bio-Hsp70 prep
Bio-Hsps prep
Biotin blocked
Bio-Hsp70 prep
Bio-Hsps prep
Biotin blocked
Bio-Hsp70 prep
Bio-Hsps prep
Biotin blocked
Bio-Hsp70 prep
Bio-Hsps prep
Biotin blocked
Bio-Hsp70 prep
Bio-Hsps prep
Biotin blocked

190 125 80 50 40

Tomm34 TPR1 TPR2

B

TCL TPR1 TPR2 Tomm34

Tomm34 TPR1 TPR2

C

TPR1
Tomm34 scaffolds Hsp70 and Hsp90

Figure 6

A

5 min

1 hr

Average deuteration [%]

AA residue

TPR TPR TPR TPR TPR TPR

Tomm34  Tomm34+Hsp70pept  Tomm34+Hsp90pept

B

5 min

1 hr

1 hr

Deuteration [%]

log(time) [s]

Non-deuterated
Tomm34
Tomm34+Hsp70pept
Tomm34+Hsp90pept

1 hr

log(time) [s]

Non-deuterated
Tomm34
Tomm34+Hsp70pept
Tomm34+Hsp90pept

1 hr

log(time) [s]

Non-deuterated
Tomm34
Tomm34+Hsp70pept
Tomm34+Hsp90pept

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Figure 7

A

TPR1

FP [mP]

Protein concentration [μM]

Hsp70
Hsp90

B

TPR2

FP [mP]

Protein concentration [μM]

Hsp70
Hsp90

C

TOMM34

FP [mP]

Protein concentration [μM]

Hsp70
Hsp90
Tomm34 scaffolds Hsp70 and Hsp90

Figure 8

A

TPR1 + Hsp70
Hsp70 pept [μM] 0 10 50 100 C-
Hsp70
GST-TPR1

TPR2 + Hsp70
Hsp70 pept [μM] 0 10 50 100 C-
Hsp70
GST-TPR2

Tomm34 + Hsp70
Hsp70 pept [μM] 0 10 50 100 C-
Hsp70
GST-Tomm34

TPR2 + Hsp90
Hsp90 pept [μM] 0 10 50 100 C-
Hsp90
GST-TPR2

Tomm34 + Hsp90
Hsp90 pept [μM] 0 10 50 100 C-
Hsp90
GST-Tomm34

B

--- Biotin blocked
100μM Hsp70 peptide
100μM Hsp90 peptide

Hsp90
Hsp70
SBP-TPR1
SBP-TPR2
SBP-Tomm34

C

TCL Pull-down
SBP-Hsp70/Hsp70Δ
Tomm34

TCL Pull-down
SBP-Hsp90/Hsp90Δ
Tomm34
Figure 9

A

B

C

Tomm34 scaffolds Hsp70 and Hsp90
Figure 10

Tomm34 scaffolds Hsp70 and Hsp90
The assembly and intermolecular properties of the Hsp70-Tomm34-Hsp90 molecular chaperone complex.
Filip Trcka, Michal Durech, Petr Man, Lenka Hernychova, Petr Muller and Borivoj Vojtesek

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