A novel HSP90 chaperone complex regulates intracellular vesicle transport

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Summary

Heat shock protein 90 (HSP90) is considered a specialized molecular chaperone that controls the folding of cell-regulatory proteins such as steroid receptors and kinases. However, its high abundance is suggestive of a more general function in other fundamental processes. Here, we show that HSP90 is required for vesicular protein transport in the cell. We have identified a novel chaperone complex comprising HSP90 and TPR1 that is recruited to the membrane protein VAP-33. Depletion of the TPR1 protein in mammalian cells inhibits transport of vesicular stomatitis virus glycoprotein (VSVG) and leads to accumulation of this cargo protein in the Golgi apparatus. Furthermore, trafficking of VSVG between Golgi stacks is dependent on the ATPase function of HSP90 and can be inhibited by drugs specific for HSP90. Our results identify a new role for HSP90 in protein sorting, pointing to a central role for this molecular chaperone in the cell.

Key words: HSP90, Molecular chaperones, Protein folding, Radicicol

Introduction

Heat shock protein 90 kDa (hereafter termed HSP90) works in an ATP-dependent manner to regulate the activity of a limited subset of medically relevant signalling proteins such as steroid hormone receptors and several kinases (Obermann et al., 1998; Panaretou et al., 1998; Pratt and Toft, 2003). In order to control the conformation of its target proteins, HSP90 cooperates with a variety of co-chaperones such as Aha1, Hop, p23, p50, Tpr2 and others. The underlying mechanism of nucleotide-dependent HSP90 function is beginning to emerge from recent structures of this molecular chaperone together with p23, p50 and target proteins (Ali et al., 2006; Shiau et al., 2006; Vaughan et al., 2006). Furthermore, we are beginning to understand how HSP90 discriminates between different types of target proteins (Hawle et al., 2006). The dependence of oncogenic kinases on HSP90 and its vulnerability to drugs have made HSP90 a promising target for cancer therapy (Kamal et al., 2003; Whitesell and Lindquist, 2005). In contrast to the more specialized HSP90, heat shock 70 kDa protein (HSP70) regulates the conformation of a broad range of target proteins in the cell. Importantly, the chaperone activity of HSP70 is also required for intracellular vesicle transport processes such as clathrin-dependent endocytosis and exocytosis of synaptic vesicles, a hallmark of neurotransmitter release (Tobaben et al., 2001; Ungewickell et al., 1995). In order to affect these membrane-dependent processes, HSP70 is recruited by dedicated cofactors such as auxillin for uncoating of endocytic vesicles or by cysteine string protein (CSP) for exocytic release of neurotransmitter. Its high abundance in the cytosol led to the hypothesis that HSP90 has other roles in addition to its established function in the activation of signal proteins. New findings have reported that HSP90 has localized functions in the cell and is involved, together with HSP70, in the import of pre-proteins into mitochondria (Young et al., 2003a; Young et al., 2003b), suggesting that the spectrum of HSP90 action is broader than previously assumed. In this study, we demonstrate that HSP90 forms a novel complex with the co-chaperone tetratricopeptide repeat domain 1 (TTC1, hereafter referred to as TPR1) that associates with the membrane-bound protein VAPA (hereafter referred to as VAP-33). Depletion of TPR1 or the inhibition of the ATPase activity of HSP90 by specific drugs inhibits transport of the VSVG cargo protein. These observations suggest that HSP90 plays a role in the regulation of intracellular vesicle transport.

Results

The co-chaperone TPR1 acts as an adaptor that brings HSP90 to the membrane bound protein VAP-33

The 12-kDa C-terminal domain of HSP90 (Hsp90C) is sufficient to interact with proteins bearing tetratricopeptide repeat (TPR) domains such as the immunophilins, hTOM34p, Tpr2 or Hop (Brychzy et al., 2003; Scheufler et al., 2000; Young et al., 1998). The presence of TPR sequences in various other proteins, revealed by inspection of human databases, suggested that previous yeast two-hybrid screens were not saturating and further putative binding partners of HSP90 might yet be identified. Using Hsp90C in the pAS2-1 vector as a bait, we have identified TPR1 (Swiss-Prot accession number Q99614) as a novel HSP90-interacting protein in a yeast two-hybrid screen against a human brain cDNA library. In a reciprocal experiment, TPR1 was employed as a bait and returned positive isolates encoding HSP90 as well as the A and B isoforms of VAP-33 (accession numbers Q9P0L0 and O95292). The TPR1 protein consists of a unique sequence ('Tpr1unique') without any homology to other sequences in the protein databases, followed by a TPR domain (Tpr1TPR) (Fig. 1A). VAP-33 contains a major sperm protein (MSP) domain followed by a coiled-coil region that

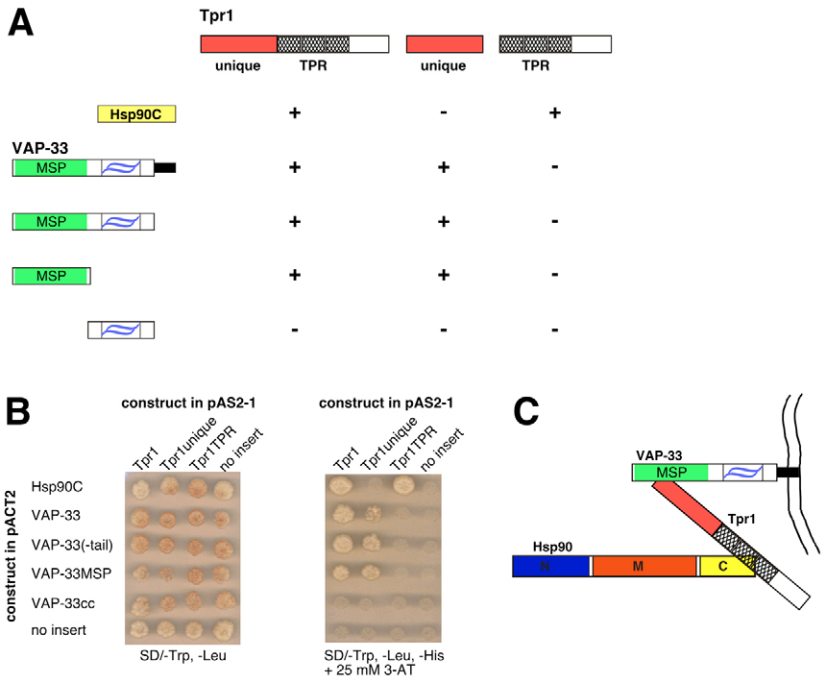

Fig. 1. The co-chaperone TPR1 binds to HSP90 and VAP-33. (A) Representation of the HSP90, TPR1 and VAP-33 constructs used for the two-hybrid experiments. The top row shows, from left to right, full-length TPR1, Tpr1unique and the fragment containing the TPR domain. The column on the left shows, from top to bottom, Hsp90C, full-length VAP-33, VAP-33 lacking its membrane anchor tail, the MSP domain of VAP-33 and the fragment containing the coiled-coil region (represented by blue double-tilde). The plus (+) and minus (–) symbols indicate the presence or absence of an interaction when various constructs were co-transformed in yeast cells and tested for growth as shown in panel B. (B) Constructs in the pAS2-1 vector were co-transformed with pACT2 constructs into *S. cerevisiae* Y190 as indicated, with empty vectors serving as a control. After growth on SD/–Trp, –Leu plates, cells were re-plated on the same medium to confirm the presence of the pAS2-1 and pACT vectors (left panel) and on SD/–Trp, –Leu, –His plates containing 25 mM 3-amino-1,2,4-triazole (right panel) to select for protein interactions. TPR1 and Tpr1TPR interacted with Hsp90C, whereas TPR1 and Tpr1unique interacted with VAP-33, VAP-33(–tail) and VAP-33MSP. (C) Illustration of the complex comprising HSP90, TPR1 and the membrane-anchored protein VAP-33 based on our yeast two-hybrid data.

mediates dimerization of the protein and a C-terminal membrane-anchor sequence (Fig. 1A). We have used full-length TPR1 as well as the fragments Tpr1unique and Tpr1TPR in pAS2-1 in combination with Hsp90C or VAP-33 and various truncations thereof in pACT2 and tested their interactions in yeast cells (Fig. 1A,B). This analysis confirms the interaction of TPR1 with Hsp90C and VAP-33 and identifies the MSP domain of VAP-33 as being sufficient to mediate the two-hybrid interaction with Tpr1unique (Fig. 1A,B). Thus, our two-hybrid data suggest the existence of a trimeric complex comprising the membrane protein VAP-33, TPR1 and HSP90 (Fig. 1C).

Next, we used purified full-length HSP90, TPR1 and VAP-33, lacking its membrane anchor to prevent aggregation of the protein due to hydrophobic interactions, to confirm the formation of a trimeric complex by gel filtration analysis. Analysis of the human TPR1 sequence with the SMART algorithm (http://smart.embl-heidelberg.de) predicted a putative coiled-coil motif in the amino acid sequence EEYLIELEKNMSDEEKQKRREESTRLKEE preceding the TPR domain. This motif might mediate oligomerization and contribute to the broad elution profile observed for TPR1 when run individually (Fig. 2A). Upon mixing together, HSP90 interacted with TPR1 in the presence or absence of the nonhydrolysable nucleotide ATPγS, as indicated by co-elution of TPR1 with HSP90 compared with the profiles of the individual proteins (Fig. 2A). VAP-33 co-migrated with TPR1 but not with HSP90. When all three proteins were combined, a further shift to the left of the VAP-33 elution peak together with TPR1 and HSP90 was observed, consistent with the formation of a trimeric complex (Fig. 2A). This complex was not affected by ATPγS.

Next, we analyzed the interaction of TPR1 and VAP-33 further at the level of individual domains. When Tpr1unique was combined with either VAP-33 or VAP-33MSP, co-migration of the two respective fragments indicated the formation of a stable complex between the two proteins (Fig. 2B). Next, the HSP90-TPR1 interaction was corroborated by surface plasmon resonance experiments. Dodecamer peptides containing the C-terminal sequence of HSP90 (90C-12), sufficient for domain-specific binding to the TPR domains of Hop and Tpr2, were coupled to the chip. TPR1 showed binding to 90C-12 (Fig. 2C) with a dissociation constant K_d=3.4 μM, similar to those measured for Hop (K_d=6.0 μM) and Tpr2 (K_d=2.7 μM) (Brinker et al., 2002; Brychzy et al., 2003). Binding of TPR1 was specific, as shown by competition by free peptides from HSP90 (90C-12) but not by the control peptide SKL (Fig. 2D). Finally, we found that VAP-33 coprecipitated with HSP90 from brain extracts, supporting the view that the proteins exist together in a complex (Fig. 2E). Remarkably, purified TPR1 did not show any effect on the ATPase or refolding activity of HSP90 (data not shown), in contrast to other cofactors such as Aha1, p50, Hop or p23, suggesting that TPR1 might function primarily as an adaptor molecule that makes the connection between HSP90 and VAP-33.

Knockdown of TPR1 obstructs vesicular transport of the VSVG^{ts045}-GFP cargo protein in vivo

VAP-33 was discovered as an integral plasma membrane protein required for release of neurotransmitter in the central nervous system of *Aplysia* (Skehel et al., 1995) and later found to be expressed ubiquitously and associated with mammalian ER, Golgi membranes and microtubules (Kaiser et al., 2005; Skehel et al., 2000). It was suggested that VAP-33 might function in the transport of secretory vesicles (Soussan et al., 1999). The organization of TPR1 and HSP90 in a complex together with VAP-33 was suggestive of a role for the molecular chaperone HSP90 in vesicle-dependent transport events. To test this hypothesis, we decided to impede HSP90 function and analyse the subsequent effects on the transport of cargo protein. Despite the fact that geldanamycin and radicicol are readily available drugs for inhibiting the ATPase activity of HSP90, their application in vivo would at the same time adversely affect normal cellular functions, as many signalling pathways are dependent on the molecular chaperone. Thereby, it might be difficult to determine whether an effect results directly from HSP90 inhibition or is caused indirectly as a result of general HSP90 failure.

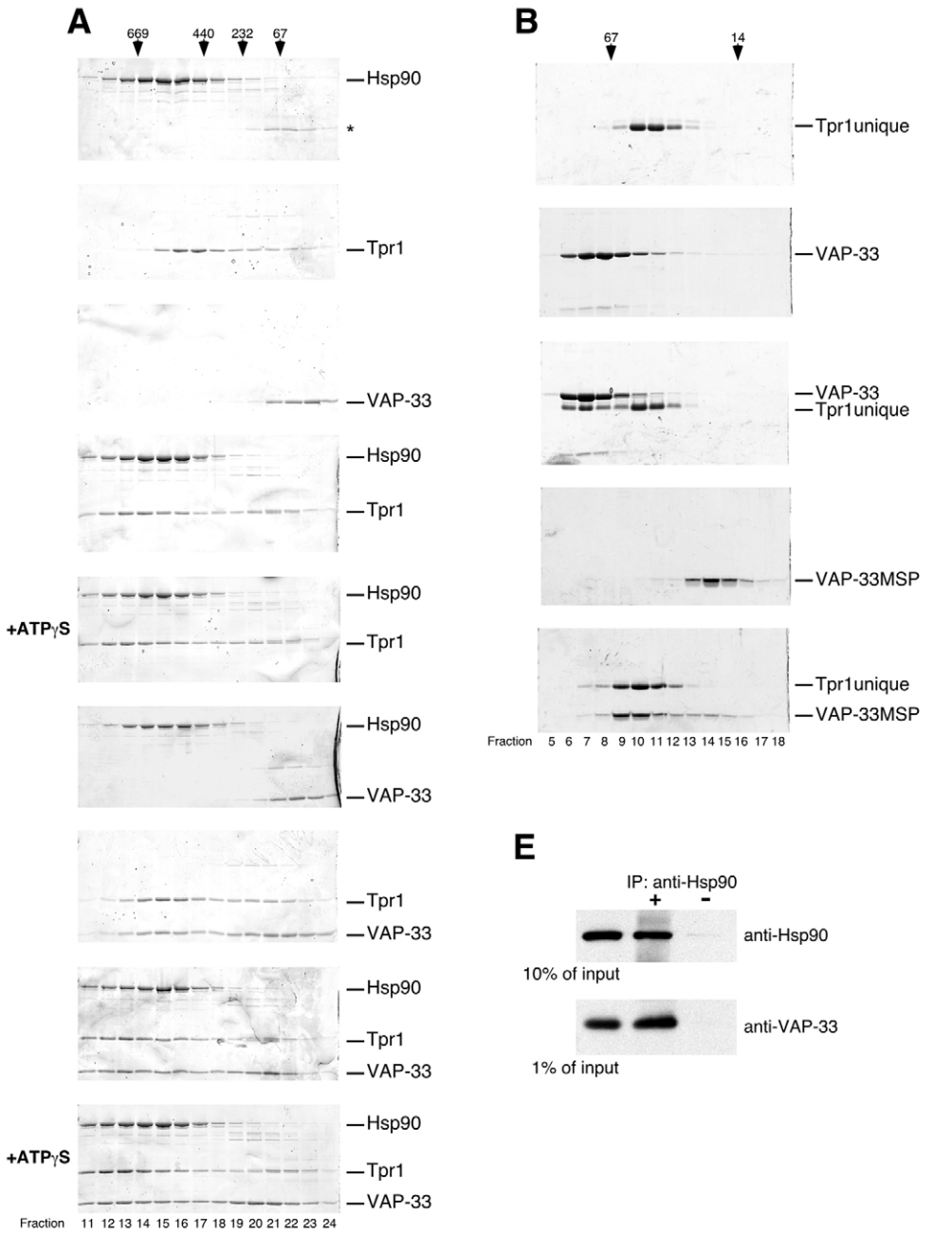

Fig. 2. Analysis of the TPR1 interaction with HSP90 and VAP-33 by gel filtration chromatography and surface plasmon resonance. (A) Purified HSP90, TPR1 and VAP-33, lacking its membrane anchor, were incubated as indicated in the presence or absence of ATPγS, separated by gel filtration chromatography on a Superose 6 column and fractions analyzed by SDS-PAGE. Elution profiles of marker proteins are shown on top (thyroglobulin, 669 kDa; ferritin, 440 kDa; catalase 232 kDa; bovine serum albumin, 67 kDa). *, a degradation product of the HSP90 preparation. (B) Tpr1unique binds to VAP-33MSP. Tpr1unique was incubated with either VAP-33 lacking its membrane anchor, or VAP-33MSP, and the mixtures were run on a Superdex 75 column. Co-migration indicated complex formation between Tpr1unique and VAP-33 or VAP-33MSP. Marker proteins are shown on top (bovine serum albumin, 67 kDa; ribonuclease A, 14 kDa). (C) 12-mer peptides containing the C-terminal sequence of HSP90 (90C-12) were covalently coupled to a BiaCore chip. The binding kinetics of TPR1 in the concentration range of 0.1 to 60 μM were tested, and the relative response units during the equilibrium phase of binding to 90C-12 were plotted against TPR1 concentrations. (D) Increasing concentrations (0.1 to 100 μM) of 90C-12 in solution were used to compete for binding of TPR1 to immobilized 90C-12. Binding of TPR1 to HSP90 peptides could be competed using free 12mer peptides from HSP90 (90C-12) but not the control peptide SKL. (E) Cow brain lysate was incubated with or without an HSP90-specific antibody. After pulldown with ProteinG beads, immunoprecipitates were analyzed with antibodies against HSP90 and VAP-33. Antibody reactivity using 1% or 10% of the input (brain lysate) is shown as a standard.

Thus, we reasoned that depletion of the adaptor protein TPR1 would interfere with localizing HSP90 to VAP-33 but at the same time leave other HSP90-dependent processes untouched. Accordingly, we diminished cellular levels of TPR1 protein by small interfering RNA (siRNA) targeting in HEK293 cells (Fig. 3A). In order to monitor the consequences of this treatment on intracellular protein transport, we analysed the fate of a temperature-sensitive mutant of VSVG fused to enhanced green fluorescent protein (VSVG^{ts045}-

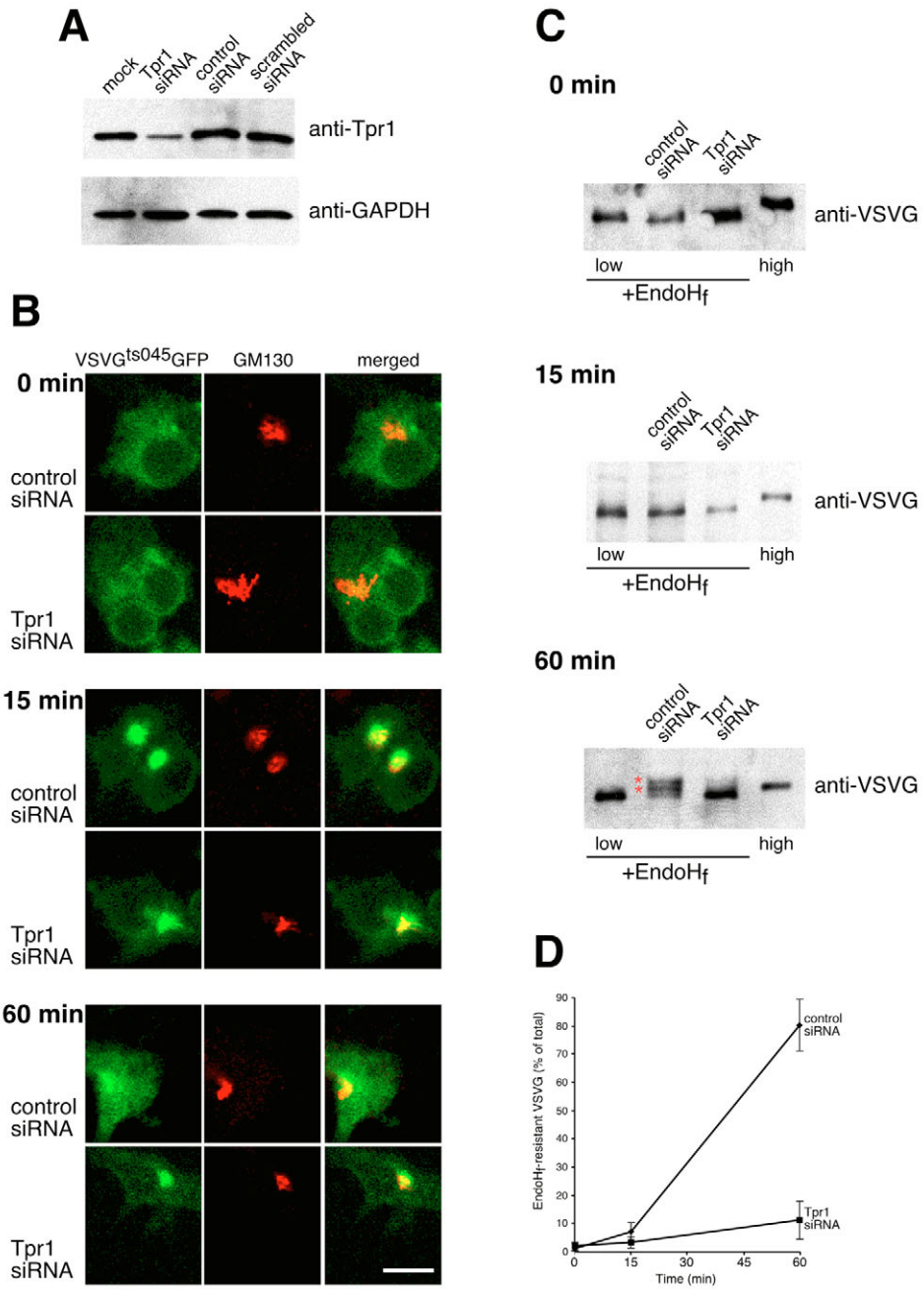

Fig. 3. TPR1 downregulation obstructs vesicular transport of the VSVG^{ts045}-GFP cargo protein in vivo. (A) Decrease of TPR1 expression levels in HEK293 cells by specific siRNA. Cells were mock transfected, treated with siRNA targeting human TPR1 or with mutated or control siRNA. Equal protein loading is shown by an antibody specific for GAPDH. (B) Time-course of intracellular VSVG^{ts045}-GFP protein trafficking in cells treated with control siRNA or siRNA targeted against human TPR1. Localization of VSVG^{ts045}-GFP and the Golgi marker GM130 was assessed after 0, 15 and 60 minutes. Bar, 10 μm. (C) Resistance of VSVG^{ts045}-GFP protein against EndoH$_f$ digestion in cells treated with control siRNA or siRNA specific for TPR1 after 0, 15 and 60 minutes at 32°C. The markers 'low' and 'high' refer to the endoplasmic reticulum form of VSVG^{ts045}-GFP from cells that have been kept at 40°C after and before EndoH$_f$ treatment (Balch and Keller, 1986). Asterisks indicate two EndoH$_f$-resistant bands, a hallmark of arrival in the medial-Golgi compartment (Schwaninger et al., 1992). Presented is a typical result of an experiment performed three times. (D) EndoH$_f$ resistancy of VSVG after 0, 15 and 60 minutes in cells treated with control siRNA or siRNA specific for TPR1. Quantification was based on the areas in each blot that correspond to the two bands marked with asterisks in C and is represented as a percentage of the total signal measured. Bars, ± s.e.m.

GFP) (Presley et al., 1997) in those cells. At 40°C, VSVG^{ts045}-GFP is retained in the ER owing to the temperature-dependent missense folding phenotype of the viral glycoprotein (Fig. 3B, 0 minutes). When the temperature is lowered to 32°C, VSVG^{ts045}-GFP that has accumulated in the ER redistributes to the juxtanuclear Golgi complex after 15 minutes in control cells (Presley et al., 1997) as well as in cells treated with siRNA directed against TPR1 (Fig. 3B, 15 minutes). In control cells, after 60 minutes, VSVG^{ts045}-GFP moves out of the Golgi complex towards the cell surface (Presley et al., 1997) (Fig. 3B, 60 minutes) and, owing to passage through the medial-Golgi compartment, becomes resistant to digestion with EndoH$_f$, consistent with the appearance of bands of higher molecular mass (Fig. 3C, 60 minutes). In TPR1 siRNA-treated cells, VSVG^{ts045}-GFP remains in the Golgi compartment (Fig. 3B, 60 minutes), hardly distinguishable from the 15 minute time point, and is sensitive to EndoH$_f$ treatment (Fig. 3C, 60 minutes compared with 0 minutes and 15 minutes; and Fig. 3D). Thus, the TPR1 protein is required for the efficient transport through the Golgi apparatus, which leads to trimming of sugar chains of VSVG^{ts045}-GFP and finally to exit out of this organelle.

Transport of the VSVG protein in a cell-free system is susceptible to HSP90-specific drugs and dependent on the interaction between the HSP90-TPR1 chaperone complex with VAP-33

To get a more detailed insight into the role of HSP90 in intracellular protein trafficking, we reconstituted intra-Golgi transport of wild-type VSVG protein in an established cell-free system (Balch et al.,

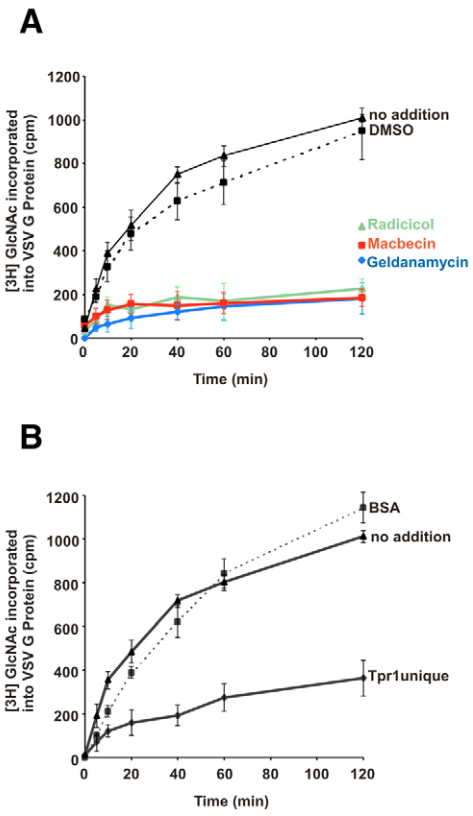

Fig. 4. Transport of the VSVG protein in a cell-free system is susceptible to HSP90-specific drugs and dependent on the interaction between the HSP90-TPR1 chaperone complex and VAP-33. (A) Intra-Golgi transport of VSVG protein is inhibited by geldanamycin, radicicol and macbecin compared with control assays. (B) Intra-Golgi transport can be inhibited by addition of the Tpr1unique fragment but not by bovine serum albumin (BSA) serving as a control. Quantified data are averages of three independent experiments. cpm, counts per minute; GlcNAc, *N*-acetylglucosamine. Bars, ± s.e.m.

1984) with some modifications (see Materials and Methods). As HSP90 is not required for viability in such an in vitro system, this allowed us to interfere with HSP90 function in several ways. Thus, we inhibited the intrinsic ATPase activity by addition of the HSP90-specific drugs geldanamycin, macbecin and radicicol. All three antibiotics reduced VSVG protein transport to ~20% of the control (Fig. 4A), indicating that the chaperone activity of HSP90 is crucial for effective intra-Golgi transport. To interfere specifically with the attachment of HSP90 to VAP-33, we added the recombinant Tpr1unique fragment, which lacks the HSP90-binding tetratricopeptide domain to the assay mix. This treatment decreased transport of VSVG protein to ~30% of the control (Fig. 4B). Furthermore, we could inhibit transport of VSVG protein to ~25% of the level of that of the control by addition of the TPR1-specific antibody mAb 292-200 (Fig. 5).

Rab8 is present in TPR1 immunoprecipitates together with HSP90 and VAP-33

Interestingly, several members of the Rab family of small GTPases, important regulators of various vesicle trafficking processes in the cell, are considered to be target proteins for the molecular chaperone HSP90. Ypt6, a yeast Rab protein, is proposed to interact with HSP90 (Zhao et al., 2005), whereas Rab1 and Rab3A activity in

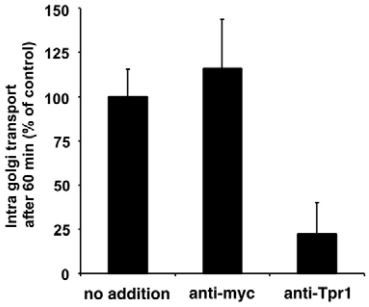

Fig. 5. The TPR1-specific antibody mAb292-200 inhibits intra-Golgi transport. 150 ng of antibody against TPR1 (mAb292-200) or an unrelated antibody against the Myc-tag (clone 9E10) serving as a control were added to 50 μl of the transport assay mixture and the transport reaction run for 1 hour, as described in Materials and Methods. The antibody against TPR1 but not the antibody against Myc results in decreased VSVG transport. Bars, ± s.e.m.

culture cells requires HSP90 (Chen and Balch, 2006). Rab8 is necessary for synaptic transport of glutamatergic receptors in the hippocampus, and this process was found to be sensitive to the HSP90-specific drug geldanamycin (Gerges et al., 2004). When we pulled down TPR1 from cell lysates, we detected Rab8 in addition to HSP90 and VAP-33 as a co-immunoprecipitated protein (Fig. 6). Other Rab proteins, the Golgi structural protein GM-130 and proteins of the Golgi membrane were not present in this material (Fig. 6).

Discussion

In summary, we have shown that a novel chaperone complex comprising HSP90 and TPR1 functions in the regulation of vesicle-dependent cargo transport in vivo and in vitro when bound to the membrane protein VAP-33. How the molecular chaperone HSP90 pushes vesicular trafficking forwards is currently poorly understood. However, the finding that the activity of some Rab proteins is dependent on HSP90 (Chen and Balch, 2006; Sakisaka et al., 2002; Zhao et al., 2005) suggests that HSP90 might function in docking and tethering, a course of events that occur between transport vesicles and their target membranes during vesicle movement and that are governed by members of the Rab protein family. Thus, HSP90 might provide chaperone activity for the components of the docking and tethering machinery, similar to the chaperone-like activity of NSF (*N*-ethylmaleimide-sensitive factor) for disassembly of SNARE complexes of the vesicle fusion machinery (Osten et al., 1998). Furthermore, HSP90 might act as a molecular chaperone for the arrangement of protein-protein interactions in multisubunit assemblies such as the conserved oligomeric Golgi (COG) complexes that are required to maintain the structure and function of the Golgi apparatus. The interesting finding that the glutamatergic receptor transport required for long-term potentiation in the hippocampus is sensitive to the HSP90-specific drug geldanamycin and dependent on Rab8 (Gerges et al., 2004), a component of the HSP90-TPR1 complex, suggests a possible role for the molecular chaperone HSP90 in synaptic activity and plasticity.

Materials and Methods

Antibodies

For immunoblotting and immunofluorescence, antibodies against GAPDH (clone 1D4, CSA-335, Stressgen), GM130 (clone 35, BD Bioscience), GS27 (clone 25, BD Bioscience), HSP90 (clone AC88, SPA-830, Stressgen), Rab1 (FL-205, Santa Cruz Biotechnologies), Rab5 (clone 15, BD Bioscience), Rab6 (C-19, Santa Cruz

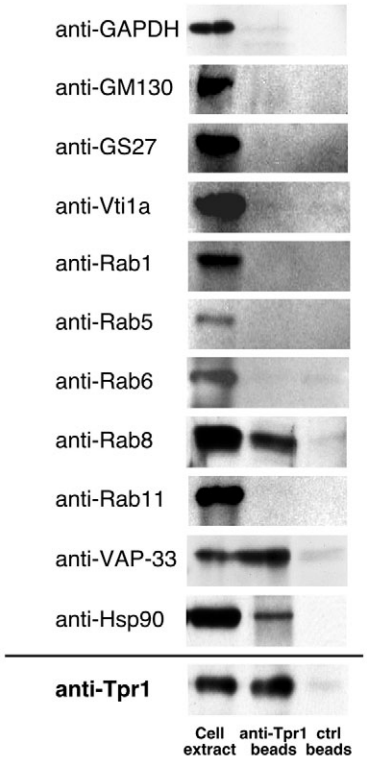

Fig. 6. TPR1 coprecipitates contain Rab8. TPR1 was isolated from cell extracts using the mAb292-200 monoclonal antibody against TPR1 coupled to beads. Bound material was separated by SDS-PAGE and analyzed with the antibodies indicated and compared with control (ctrl) beads.

Biotechnologies), Rab8 (clone 4, BD Bioscience), Rab11 (clone 47, BD Bioscience), VSVG (P5D4, Roche) and Vti1a (clone 45 BD Bioscience) were used. The monoclonal antibody mAb292-200 was produced in mice that had been immunized with purified recombinant human TPR1 protein following standard procedures (Obermann et al., 1996). Alexa-Fluor-647-labeled mAb292-200 was used to identify TPR1 knockdown cells by immunofluorescence. The polyclonal antibody against VAP-33 (R683) had been elicited in rabbit and affinity purified using the recombinant protein. A Cy3-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories) was used for immunofluorescence detection of GM130.

Molecular biology

Yeast two-hybrid screening was performed as described previously (Young et al., 1998) using a human brain library based on the pACT2 vector (Clontech). Positive candidate cDNA inserts were analyzed by nucleotide sequencing. To confirm the results of the screens and map the protein interactions, genes encoding Hsp90C, TPR1, VAP-33 and their fragments were cloned into pAS2-1 and pACT vectors and inserted into *Saccharomyces cerevisiae* Y190 as indicated. Transformed yeast cells were grown on synthetic dropout (SD) medium/–Trp,–Leu at 30°C. To test for protein interactions, cells were resuspended in water and replated on SD/–Trp,–Leu,–His selection medium containing 25 mM 3-amino-1,2,4-triazole and analyzed for growth.

For expression of the proteins in *Escherichia coli,* TPR1 and VAP-33 cDNA sequences were amplified from human brain cDNA isolates in the pACT vector obtained by the two-hybrid screens. For interaction studies with purified proteins, VAP-33 was used without the membrane anchor to prevent nonspecific hydrophobic interactions. Full-length HSP90 was amplified from a cDNA construct in pUC19 (Obermann et al., 1998). PCR products were inserted into the pProExHTa expression vector and transformed into *E. coli* BL21(DE3)pLysS cells. Bacteria were grown at 37°C in LB medium supplemented with 100 mg/l ampicillin and 34 mg/l chloramphenicol to an optical density of 1.0 at 600 nm. Protein expression was induced for 5 hours with 0.25 mM IPTG (isopropyl-beta-D-thiogalactopyranoside) at 18°C and the cell pellets were frozen at –80°C after harvesting.

Protein interaction analysis

Proteins were enriched from bacterial cell pellets by nickel-nitrilotriacetic acid chromatography, as described previously (Obermann et al., 1998). Proteins were further purified by ion-exchange fast-performance liquid chromatography on MonoQ (HSP90, TPR1 and Tpr1unique) or on MonoS (VAP-33 and VAP-33MSP) columns (Amersham Biosciences). Before interaction analysis, proteins were transferred into 40 mM HEPES-KOH pH 7.4, 50 mM KCl, 2 mM $MgCl_2$ using PD-10 columns (Amersham Biosciences).

To analyse HSP90, TPR1, VAP-33 and fragments thereof for complex formation, purified proteins were incubated for 10 minutes at room temperature and for 10 minutes on ice in 40 mM HEPES-KOH pH 7.4, 50 mM KCl, 2 mM $MgCl_2$ and subsequently run on a Superose12 or Superdex75 column equilibrated in the same buffer as described previously (Harst et al., 2005; Lotz et al., 2003). Where indicated, ATPγS was added.

Surface plasmon resonance experiments were performed as described previously (Brychzy et al., 2003). Briefly, cysteine-derivatized 12-mer peptides of HSP90 (C90-12, Ac-C-GDDDTSRMEEVD-OH) were probed with recombinant TPR1 protein. To assess the thermodynamic dissociation constant (K_d), a concentration series (0.1 to 60 μM) of TPR1 was injected. The K_d was calculated based on a simple steady-state model for binding (Brinker et al., 2002) using KaleidaGraph software for curve fitting. For competition analysis, increasing amounts (0.1 to 100 μM) of 90C-12 or of SKL (Ac-TKRRESKL-OH), a control sequence that recognizes the TPR domain of Pex5p (Brychzy et al., 2003), peptides were preincubated for 5 minutes with 1 μM TPR1 on ice before injection. Equilibrium response units of three independent experiments were averaged for both types of analysis.

To confirm the presence of HSP90 in complex with VAP-33, we used the HSP90-specific monoclonal antibody SPA-830 coupled to UltraLinkProtein-G beads (Pierce) for pulldown from cow brain lysates that had been prepared in ice-cold 25 mM HEPES-KOH pH 7.4, 50 mM KCl, 2.5 mM $MgCl_2$, 0.5% Triton X-100 supplemented with a protease inhibitor cocktail (Complete, Roche). Precipitates were analyzed by immunoblotting with antibodies against HSP90 and VAP-33.

Analysis of VSVG^{ts045}-GFP transport in culture cells

For siRNA experiments, a double-stranded synthetic RNA oligomer (sense sequence 5′-GGGAGAAGAGGAGUGUUUUtt and antisense sequence 5′-AAAACAC-UCCUCUUCUCCCtg, Ambion) against the sequence GGGAGAAGAGGAGTG-TTTT of the human TPR1 cDNA was used. As a control, the mutated oligomer (sense sequence 5′-GGGAGAAUAGGAGGGCUUUtt and antisense sequence 5′-AAA-GCCCUCCUAUUCUCCCtg, mutations underlined, Ambion) and the *Silencer* negative control #1 siRNA (Ambion) were used. HEK293 cells were grown in six-well plates and transfected with 100 nM siRNA oligomers with Oligofectamine (Invitrogen). To monitor TPR1 protein levels, cell lysates were analysed by immunoblotting with mAb292-200 and antibody against GAPDH to confirm equal protein loading. For analysis of cargo protein transport, cells were grown on coverslips and VSVG^{ts045}-GFP was introduced into cells using Lipofectamine (Invitrogen) 48 hours after siRNA transfection. Cells were kept for 7 hours at 37°C and then shifted to 40°C for 20 hours to accumulate VSVG^{ts045}-GFP in the endoplasmic reticulum. Export of cargo protein was initiated by lowering the temperature to 32°C for the time period, as indicted. After fixation, TPR1-downregulated cells were identified by lack of an epifluorescent signal from the Alexa-Fluor-647-labeled antibody against TPR1. In order to monitor transport of VSVG^{ts045}-GFP, cells were analysed on a Zeiss Meta 510 confocal microscope equipped with a P/LS_V1.10 scan unit and operated by LSM 5 version 3.2 Sf2 acquisition and LSM 5 Image Examiner software. The 488 nm line of the argon laser and the 543 nm line of the helium neon laser were used to detect GFP (505-530 nm filter) and Cy3 (563-584 nm filter) fluorescence, respectively. All images were recorded using a Zeiss Plan-Apochromat 63×/1.4 oil (∞/0.17) objective with a pixel time of 3.2 μseconds.

To probe the sensitivity of VSVG^{ts045}-GFP protein to EndoH_f digestion, cell lysates were denatured in 0.5% SDS, 1% β-mercaptoethanol for 10 minutes at 100°C, Na-citrate was added to yield a final concentration of 50 mM before addition of 300 units of EndoH_f (NEB), separated on 4-10% gradient SDS gels and analysed by immunoblotting with the VSVG-specific antibody P5D4. Quantification of the immunoblots was performed using AlphaEase FC software (AlphaInnotech).

Immunoprecipitation from culture cells

Cells were grown in 10 cm tissue-culture dishes, resuspended in ice-cold 25 mM HEPES-KOH pH 7.4, 50 mM KCl, 2.5 mM $MgCl_2$, 0.5% Triton X-100 supplemented with a protease inhibitor mixture (Complete, Roche) and homogenized with a ball-bearing homogenizer. After centrifugation, the cell lysate was incubated for 60 minutes at 4°C with the monoclonal antibody specific to TPR1 immobilized on UltraLinkProtein-G beads (Pierce) that had been blocked with 3% (w/v) bovine serum albumin and 3% fish skin gelatine to minimize nonspecific protein interactions. As a control, we used beads alone or beads coupled to an unrelated antibody against Myc (clone 9E10). After incubation, the immunoprecipitates were washed three times with 25 mM HEPES-KOH pH 7.4, 50 mM KCl, 2.5 mM $MgCl_2$, resuspended in SDS loading buffer and analysed by immunoblotting with the indicated antibodies.

VSVG transport reconstituted in a cell-free system

Transport assays were performed as described previously (Balch et al., 1984). For reasons of biosafety, we used a lentiviral system to introduce wild-type VSVG protein in CHO-15B cells. Lentiviral particles expressing VSVG protein were generated by triple transfection of the VSVG gene construct in the pHR′SIN vector together with the packaging construct pCMVΔR8.91 and the envelope plasmid pMD.G, as

described previously (Zufferey et al., 1998). Vector DNA was transiently introduced into 293T cells by the calcium phosphate coprecipitation method. Viral supernatant was used to transduce CHO-15B cells at a confluency of 70%. Twelve hours later, cells were harvested and frozen in liquid nitrogen. Donor Golgi membranes were prepared from lentiviral-infected CHO-15B and acceptor membranes from CHO wild-type cells. The assay mixture consisted of donor membranes (6 μg), acceptor membranes (6 μg) and bovine brain cytosol (70 μg), with an ATP-regeneration system, in a total volume of 50 μl. Where indicated, geldanamycin (Sigma), radicicol (Sigma) and macbecin (all at 80 μM), purified proteins [Tpr1unique or bovine serum albumin (BSA), at 2 μM], or the TPR1-specific antibody mAb292-200 (150 ng/50 μl) were added before the start of the transport reaction. Transport was initiated at 37°C by addition of 0.5 μCi UDP-[^{3}H]*N*-acetylglucosamine (BioTrend) and stopped at the times indicated by returning the samples to ice. Samples were run on 10% PAGE gels and VSVG protein identified by immunoblotting with the monoclonal antibody P5D4, and incorporation of radioactivity into this band was quantified by scintillation counting.

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