Asna1/TRC40-mediated membrane insertion of tail-anchored proteins

Vincenzo Favaloro, Fabio Vilardi, Rainer Schlecht, Matthias P. Mayer and Bernhard Dobberstein*

Zentrum für Molekulare Biologie der Universität Heidelberg (ZMBH), DKFZ – ZMBH Allianz, Im Neuenheimer Feld 282, 69120 Heidelberg, Germany

*Author for correspondence (b.dobberstein@zmbh.uni-heidelberg.de)

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Summary

Tail-anchored (TA) proteins insert post-translationally into the membrane of the endoplasmic reticulum (ER) and span the membrane by their C-terminal transmembrane domain. We have reconstituted membrane insertion of TA proteins from recombinant Asna1/TA protein complexes and ER-derived membranes. Our data show that Asna1 can mediate membrane insertion of RAMP4 and Sec61β without the participation of other cytosolic proteins by a mechanism that depends on the presence of ATP or ADP and a protease-sensitive receptor in the ER membrane. By contrast, membrane insertion of cytochrome b5 can proceed independently of Asna1 and nucleotides.

Key words: Tail-anchored protein, Asna1/TRC40, RAMP4, Sec61β, Cytochrome b5, Endoplasmic reticulum

Introduction

Tail-anchored (TA) proteins belong to a special class of membrane proteins that insert post-translationally into the membrane of the endoplasmic reticulum (ER) but also into the mitochondrial outer membrane (Borgese et al., 2003a). They participate in cellular processes such as protein translocation, apoptosis, vesicular traffic or lipid biosynthesis (Borgese et al., 2003a; Kutay et al., 1993; Wattenberg and Lithgow, 2001). TA proteins are anchored in the lipid bilayer by a single transmembrane domain (TMD) located near their C termini. This TMD also functions as a targeting signal for membrane insertion. The C-terminal location of this signal poses a unique challenge to membrane targeting of the completed protein, as hydrophobic TMDs are prone to aggregation. To cope with this problem, cells have evolved diverse mechanisms for chaperoning the newly synthesized membrane protein and targeting it to its destination membrane (Borgese et al., 2007; Rabu et al., 2009).

TA proteins are found in the organelles of the secretory pathway, peroxisomes and mitochondria. Within the secretory pathway, TA proteins play diverse roles such as supporting protein translocation and promoting vesicular fusion. TA proteins of the secretory pathway are initially inserted into the ER membrane and from there sorted to their ultimate destination. Mitochondrial TA proteins are inserted directly into the mitochondrial outer membrane (Borgese et al., 2007). Among the TA proteins of the secretory pathway are the β subunit of the Sec61 translocon complex, the ribosome-associated membrane protein 4 (RAMP4) (Rapoport et al., 1996), cytochrome b5 (Cytb5) (Borgese and Gaetani, 1983) and the SNAREs, which mediate fusion of secretory vesicles (Borgese et al., 2007; Borgese et al., 2003b; Kutay et al., 1993; Wattenberg and Lithgow, 2001; Yamaguchi et al., 1999). Membrane insertion of TA proteins in vitro can require ATP or GTP (Abell et al., 2007; Kutay et al., 1995) but in some cases can proceed even in the absence of nucleotides (Annis et al., 2004; Brambillasca et al., 2006; Colombo et al., 2009; Janiak et al., 1994; Kim et al., 1997). Nucleotide dependence could reflect the involvement of molecular chaperones, such as members of the Hsp70 family and SRP (Abell et al., 2007; Rabu et al., 2008). More recently a distinct pathway for the membrane insertion of a subset of TA proteins was discovered in mammalian cells, which involves the ATPase Asna1, also called transmembrane domain recognition complex (TRC) 40 (Favaloro et al., 2008; Stefanovic and Hegde, 2007). Asna1/TRC40 is the mammalian ortholog of Arr4/Get3 in yeast (Rabu et al., 2009; Schuldiner et al., 2008). Sec61β and RAMP4 are examples of TA proteins that are proposed to be inserted into the ER membrane by the ATP-dependent Asna1/TRC40 pathway (Favaloro et al., 2008; Stefanovic and Hegde, 2007). By contrast, Cytb5 can insert unassisted into the ER membrane (Brambillasca et al., 2006; Colombo et al., 2009; Kim et al., 1997). This has been shown in a cell-free system, in which the TA proteins were synthesized and associated with Asna1 prior to membrane insertion. In yeast it has been shown that the homolog of Asna1, Get3, and its receptor Get1/Get2 can mediate membrane insertion of a subset of TA proteins (Schuldiner et al., 2008). Furthermore it was shown that two other proteins, Get4 and Mdy2/Get5 associate in yeast cytosol with Get3 and are important for TA protein insertion into the ER membrane (Jonikas et al., 2009).

To rigorously test the contribution and the requirements of Asna1 for ER membrane insertion of TA proteins, we reconstituted the Asna1-mediated membrane insertion of TA proteins from purified soluble Asna1/TA protein complexes and rough microsomal (RM) membranes.

Results

Purification of a soluble Asna1/TA protein complex and membrane insertion of the TA protein

It has previously been shown that the mammalian ATPase Asna1 associates with the newly synthesized TA proteins RAMP4op and Sec61βop before they are inserted into membranes derived from the rough ER, rough microsomes (RMs) (Favaloro et al., 2008; Stefanovic and Hegde, 2007). In this system a TA protein is synthesized in a cellular lysate lacking RMs and associates with Asna1. When RMs are added to the lysate, the interaction of the

TA protein with Asna1 is broken and the TA protein inserts into the RM membranes (Favaloro et al., 2008; Stefanovic and Hegde, 2007). This can be verified by the *N*-glycosylation of the TA protein (Brambillasca et al., 2005; Favaloro et al., 2008) In order to further characterize the role of Asna1 in membrane insertion of TA proteins we expressed TA proteins in *Escherichia coli* either alone or together with Asna1. After purification of the soluble TA protein or Asna1/TA protein complexes, we analyzed the insertion of the cargo TA protein into RM membranes. Membrane insertion was monitored by the glycosylation of the opsin (op)-tagged TA protein on the lumenal side of the ER membrane.

RAMP4op (R4op) was tagged N-terminally with ten His residues (H) and two IgG binding domains of Protein A (ZZ) followed by a TEV-protease recognition site resulting in HZZ-R4op. To the N-terminus of Asna1, maltose-binding protein was added followed by a TEV protease recognition site (MBP-Asna1) (supplementary material Fig. S1). cDNAs coding for these recombinant proteins were cloned in an expression vector placed behind an arabinose (ARA) or IPTG inducible promoter, respectively, allowing for independent inducible expression of the two cDNAs.

After the induction by ARA, the 23 kDa HZZ-R4op could be seen as major protein that was not present in non-induced cells (Fig. 1A, cf. lanes 1 and 2). After separation of the cell lysates in a supernatant (cytosol) and pellet (membranes and aggregates), the TA protein was found nearly exclusively in the pellet fraction (Fig. 1A, cf. lanes 3 and 4). To allow the newly synthesized HZZ-R4op to bind to MBP-Asna we first induced the expression of MBP-Asna1 and after 1 hour we induced the expression of HZZ-R4op for 4 hours. MBP-Asna1 was then seen as a major 80 kDa protein (Fig. 1A, cf. lanes 1 and 5) and HZZ-R4op as a 23 kDa protein (Fig. 1A, lane 6). Also in this case HZZ-R4op was mainly found in the pellet fraction (lane 8) but the amount of soluble HZZ-R4op was significantly increased compared with the expression in the absence of MBP-Asna1 (Fig. 1A, cf. lanes 7 and 3). HZZ-R4op was identified by western blot analysis using an anti opsin (α-op) antibody (Fig. 1A, lanes 3, 4, 7, 8).

Next we purified soluble HZZ-R4op, expressed in the absence of MBP-Asna1 (Fig. 1A, lane 3), by Ni-NTA affinity chromatography. The eluted material was separated by SDS-PAGE and analyzed by Coomassie Blue staining and western blotting (WB) with an α-op antibody (Fig. 1B, lanes 1 and 2). This analysis showed that HZZ-R4op, when expressed alone, is partially degraded during the purification (Fig. 1B, lane 2). Several bacterial proteins including the chaperone of the Hsp70 family DnaK were present in the eluted fraction (supplementary material Fig. S2, lanes 16-18).

When MBP-Asna1 and HZZ-R4op were coexpressed and the supernatant fraction (Fig. 1A, lane 7) was sequentially purified on Ni-NTA and amylose columns, these proteins copurified in both steps, indicating that the two proteins were associated with each other (Fig. 1B, lanes 3 and 4). In the final purified complex, HZZ-R4op was not degraded and the bacterial chaperones DnaK and DnaJ were not detectable in the HZZ-R4op/MBP-Asna1 complex fraction (supplementary material Fig. S2, lane 3).

To test for the insertion of HZZ-R4op into RM membranes, we incubated the purified HZZ-R4op complexes with Mg-ATP in the presence or absence of RM membranes and rabbit reticulocyte lysate (RRL) for 30 minutes at 30°C. RMs were then pelleted by centrifugation and one aliquot was further incubated with endoglycosidase H (EndoH) which removes N-linked oligosaccharides. All fractions were then analyzed by SDS-PAGE and western blotting using α-Asna1 and α-op antibodies (Fig. 1C). In the pellet fractions, containing the RMs, a 3 kDa higher molecular weight glycosylated form of HZZ-R4op could be seen only when HZZ-R4op was in complex with MBP-Asna1, both in the presence and absence of rabbit reticulocyte lysate (cf. lanes 3 and 7 with lanes 11 and 15). Glycosylation was verified by the removal of oligosaccharide side-chains by EndoH (lanes 12 and 16). This shows that HZZ-R4op is inserted into RM membranes from MBP-Asna1/HZZ-R4op complexes, both in the presence and absence of other cytosolic proteins.

Endogenous Asna1, which is present in the RRL and in RMs, was found in our insertion assay with HZZ-R4op alone in the pellet as well as in the supernatant fraction (Fig. 1C, lanes 1 to 8). However, when MBP-Asna1/HZZ-R4op was used, endogenous Asna1 was exclusively found in the supernatant, implying that MBP-Asna1 complexes can displace Asna1 from a limiting number of membrane-binding sites (Fig. 1C, lanes 9-16). Although Asna1 is present in RRL and RMs, HZZ-R4op alone was not inserted into RM membranes. This indicates that endogenous Asna1 is not able to support the insertion of the TA protein. It could be argued that the concentration of endogenous Asna1 is too low. To see whether membrane insertion of HZZ-R4op can be restored by the addition of MBP-Asna1, we added increasing amounts of MBP-Asna1 to the insertion reaction. Again, no insertion of HZZ-R4op was observed (Fig. 1D). These data indicate that HZZ-R4op has to associate with MBP-Asna1 directly after translation in order to maintain TA protein competence for insertion into the membrane.

To investigate whether peripheral membrane proteins of the RMs are essential for the membrane insertion, we used RMs from which peripheral components were removed by treatment with puromycin and high salt. Such treated membranes, PKRMs, lack signal recognition particles (SRPs), Asna1, Hsc70 and Hsp40 (supplementary material Fig. S3). PKRM membranes were still competent for MBP-Asna1-mediated insertion of HZZ-R4op (Fig. 1E, cf. lanes 2 and 7). Addition of rabbit reticulocyte lysate (RRL), the high salt wash fraction of RMs (SE) or *E. coli* lysate (EcL) did not affect the membrane insertion into RMs (cf. lanes 2 to 5) or PKRMs (cf. lanes 7 to 10). Taken together these data indicate that once an Asna1/TA protein complex is formed, no other cytosolic proteins are required for the membrane insertion of a TA protein.

Membrane insertion occurred also when the MBP and HZZ tags were removed by TEV cleavage (supplementary material Fig. S4A,B) or after the first step of purification (supplementary material Fig. S4C).

Recombinant HZZ-R4op/MBP-Asna1 complexes are heterogeneous in size

In our membrane insertion assay, only a fraction of HZZ-R4op was inserted into RM membrane (Fig. 1C). To see whether HZZ-R4op/MBP-Asna1 complexes are heterogeneous, we analyzed them by size exclusion chromatography on a Superdex 200 column. The elution profile revealed a major peak close to the void volume fraction with protein complexes of about 700 kDa and a shoulder with complexes of about 400 kDa (Fig. 2A). Separating proteins of selected fractions by SDS-PAGE revealed that all contained MBP-Asna1 and HZZ-R4op as major constituents (Fig. 2A). The *E. coli* chaperone DnaJ was found to co-migrate with proteins in the 700 kDa complexes, whereas neither DnaJ nor DnaK were found in fractions containing the 400 kDa complexes (Fig. 2A). Fractions in the range of the 400 kDa complex were active in the insertion

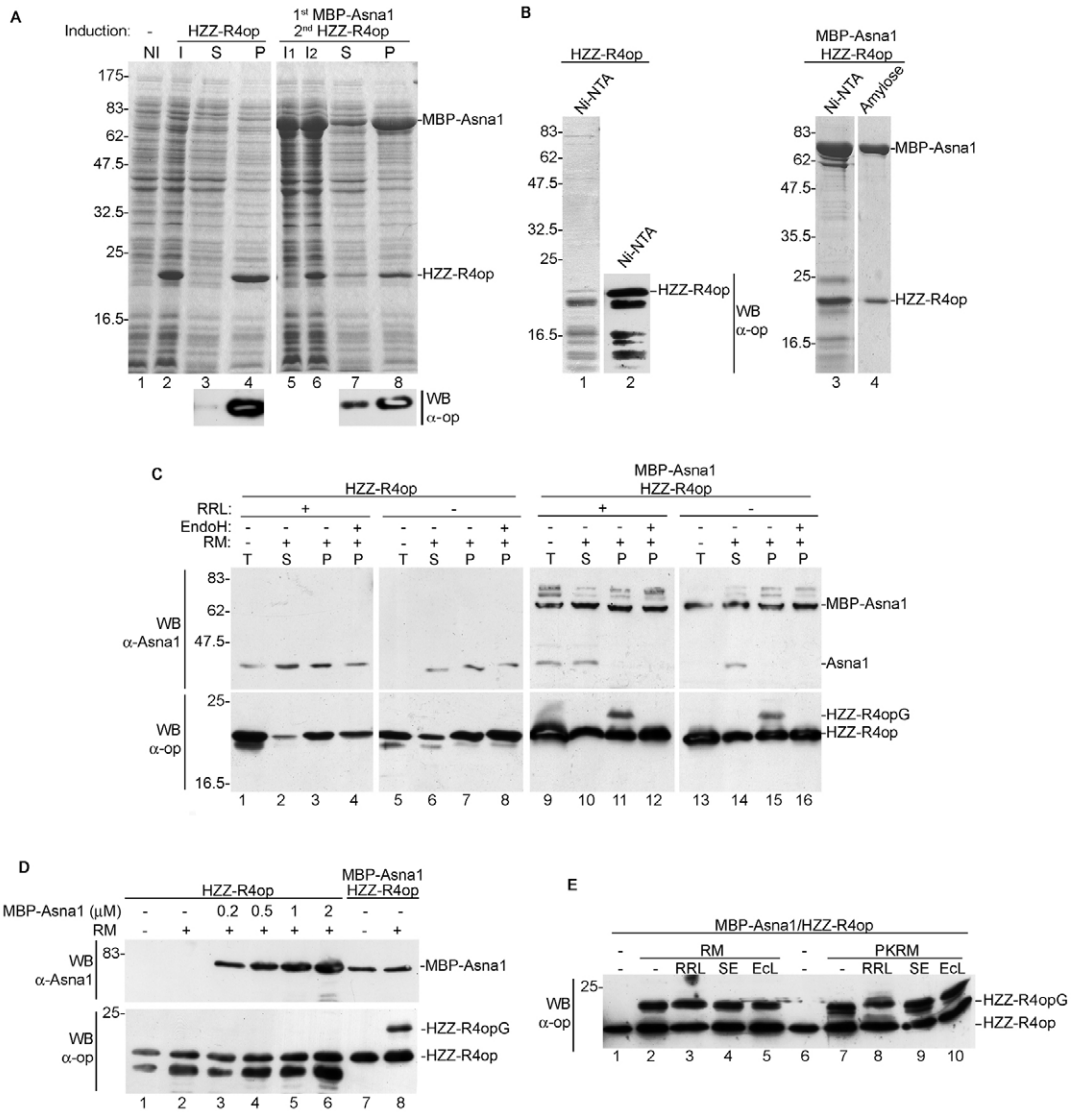

Fig. 1. Membrane insertion of recombinant HZZ-R4op from an Asna1 complex. (**A**) Expression of HZZ-R4op was induced (I) in *E. coli* cells by arabinose (ARA) for 4 hours (lane 2) or cells were not induced (NI) (lane 1). Alternatively, MBP-Asna1 was induced by IPTG for 1 hour (lane 5) and HZZ-R4op by ARA for 4 hours (lane 6). Cell homogenates were then separated by ultracentrifugation into a supernatant (S) and pellet (P) fraction. Proteins were separated by SDS-PAGE and stained by Coomassie Blue. HZZ-R4op was identified by western blotting (WB) using an anti-op (α-op) antibody. (**B**) Purification of soluble HZZ-R4op complexes. The supernatant fractions containing HZZ-R4op (Fig. 1A, lane 3) or MBP-Asna1/HZZ-R4op (Fig. 1A, lane 7) were purified by affinity chromatography on a Ni-NTA column that binds the HZZ-R4op (lanes 1 and 3) and on an amylose column that recovers the MBP-Asna1 (lane 4). Eluted fractions were characterized by SDS-PAGE and Coomassie Blue staining. Lane 2 shows a western blot with α-op antibody of proteins separated in lane 1. (**C**) Membrane insertion and glycosylation of HZZ-R4op. The affinity purified HZZ-R4op (Fig. 1B, lane 1) and MBP-Asna1/HZZ-R4op complex (Fig. 1B, lane 2) were incubated at 30°C without (lanes 1, 5, 9, 13) or with (lanes 2-4, 6-8, 10-12 and 14-16) rough microsomes (RMs). Cytosolic proteins from rabbit reticulocyte lysate (RRL) were included during incubation in the samples of lanes 1-4 and 9-12, and Mg-ATP in all the samples. The mixtures were separated into a supernatant (S) and a pellet (P) fraction. Endoglycosidase H (endoH) was added to one-half of a pellet fraction (lanes 4, 8, 12 and 16). Proteins were then separated by SDS-PAGE and MBP-Asna1, endogenous Asna1 (end) of the RRL or the RMs and HZZ-R4op and its glycosylated form (HZZ-R4opG) identified by western blotting using the indicated antibodies. (**D**) Membrane insertion of HZZ-R4op expressed in the absence of Asna1 is not restored by the addition of MBP-Asna1. Affinity purified HZZ-R4op (Fig. 1B, lane 1) was incubated with RMs and Mg-ATP (lanes 2-6) and increasing amounts of MBP-Asna1 (lanes 3-6). In lanes 7 and 8 the reaction was performed with the MBP-Asna1/HZZ-R4op complex as positive control. Proteins were then analyzed by SDS- PAGE and western blot with α-op and α-Asna1 antibodies. (**E**) Membrane insertion of HZZ-R4op from MBP-Asna1/HZZ-R4op complex does not require peripheral proteins of the RM membrane nor is it stimulated by the addition of cytosol. MBP-Asna1/HZZ-R4op was incubated with RMs (lanes 2-5) and RMs treated with puromycin and 0.5 M KCl (PKRMs) (lanes 7-10) in the presence or absence of rabbit reticulocyte lysate (RRL), salt extract derived from RMs (SE) or an *E. coli* cytosolic extract (EcL), as indicated. Mg-ATP was present in all samples. Proteins were then analyzed by SDS-PAGE and western blot with α-op antibody.

of HZZ-R4op into membranes (Fig. 2B). For the further characterization of the MBP-Asna1/HZZ-R4op complexes, we used pooled fractions 8 to 15 as they were free of DnaJ.

To see whether the amount of RMs present in the assay is limiting, we used increasing amounts/equivalents (eq) of RMs (Walter and Blobel, 1983) in the insertion assay. After the incubation, RMs were

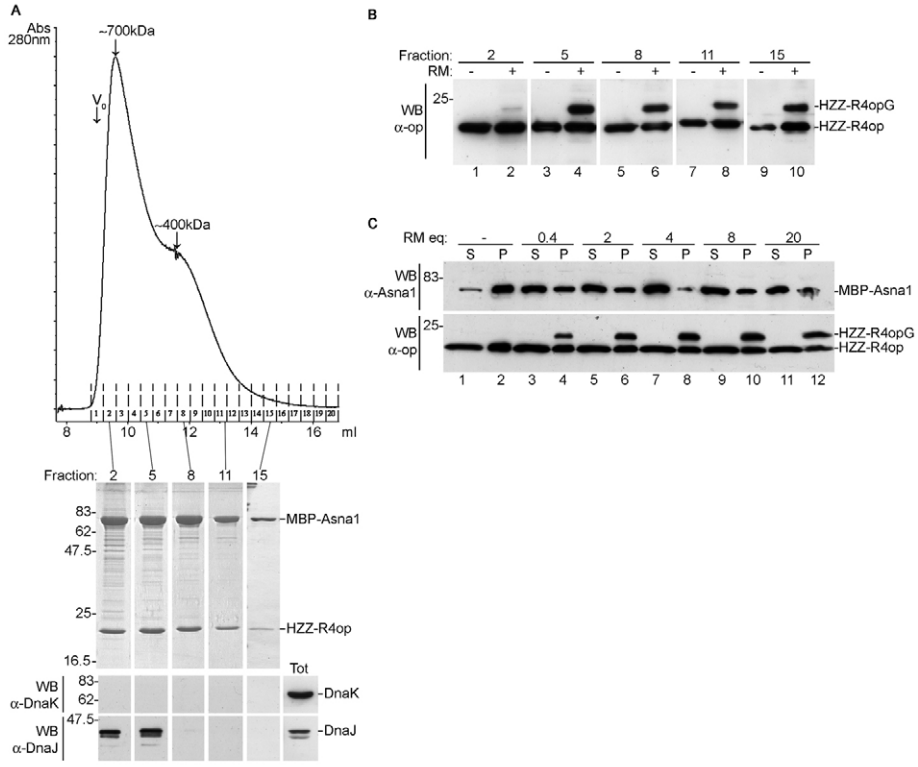

Fig. 2. Separation of MBP-Asna1/HZZ-R4op complexes by gel filtration and identification of insertion-competent complexes. (**A**) Gel filtration chromatography of the MBP-Asna1/HZZ-R4op complex on Superdex 200 column. Double affinity purified MBP-Asna1/HZZ-R4op was chromatographed on Superdex 200 and proteins in fractions under the peak and shoulder (2, 5, 8, 11, 15) characterized by SDS-PAGE and Coomassie Blue staining. Cofractionated *E. coli* chaperones DnaK and DnaJ were identified by western blotting using the respective antibodies (bottom). (**B**) Proteins from the selected fraction were tested for membrane insertion of HZZ-R4op by incubation with or without RMs. After SDS-PAGE, HZZ-R4op and its glycosylated form, HZZ-R4opG, were identified by western blot using the anti-op antibody (α-op). (**C**) Titration of the amount of RMs (equivalent) required for membrane insertion of HZZ-R4op. MBP-Asna1/HZZ-R4op complexes were incubated with no RMs (lanes 1,2) or with increasing equivalents (eq) of RMs (lanes 3-12). After incubation the samples were separated into a supernatant (S) and a pellet (P) fraction. Proteins were analyzed by SDS-PAGE and western blot as above.

pelleted by centrifugation. As can be seen in Fig. 2C, 4 eq of RMs were sufficient to give maximal membrane insertion (glycosylation) of HZZ-R4op. This analysis also shows that in the absence of RMs, the MBP-Asna1/HZZ-R4op complex is found mainly in the pellet fraction whereas, in the presence of RMs, MBP-Asna1 is found mainly in the supernatant fraction (Fig. 2C, cf. lanes1, 2 and 7, 8). This indicates that, in the absence of RMs, the MBP-Asna1/HZZ-R4op complex is prone to aggregate and that Asna1 is released from the membrane after the release of the cargo TA protein at the membrane.

Nucleotide requirements for the membrane insertion of HZZ-R4op

Asna1 is an ATPase and ATP has been shown to be required for Asna1-mediated insertion of R4op into RM membranes after synthesis of R4op in a rabbit reticulocyte lysate (Favaloro et al., 2008). To test nucleotide requirements for Asna1-mediated HZZ-R4op insertion into RM membranes we tested membrane insertion of HZZ-R4op in the absence or presence of the nucleotides indicated in Fig. 3A. We found that membrane insertion of HZZ-R4op is stimulated by ATP, ADP and the non-hydrolyzable ATP analogue AMP-PNP (lanes 3, 4 and 5), but not in the absence of added nucleotides or by AMP, GTP, GMP-PNP, GDP, GMP or CTP. To rule out contamination of AMP-PNP by ATP, we performed the reaction in the presence of Apyrase, which can hydrolyze both ATP and ADP but not AMP-PNP. In this case the insertion is stimulated only by the non-hydrolyzable AMP-PNP (lanes 13 and 14), which indicates that ATP hydrolysis is not required for membrane insertion of HZZ-R4op. Titration of the adenosine nucleotides and quantification of the membrane insertion showed that ATP stimulates the insertion more efficiently than ADP or AMP-PNP. In this case ADP was purified by HPLC to remove contaminating ATP (Fig. 3B). This indicates that, besides AMP-PNP, ADP can stimulate membrane insertion of HZZ-R4op and that the observed insertion in the presence of ADP or AMP-PNP is not due to contamination by ATP.

To see whether the kinetics of membrane insertion is affected by the type of adenosine nucleotides, we tested membrane insertion in the presence of different adenosine nucleotides and increasing amounts of RMs (Fig. 3C and supplementary material Fig. S5). When membranes are not limiting (4 and 10 eq) the insertion goes to saturation after 30 and 20 minutes, respectively, in the presence of ATP. Slower insertion kinetics were found when insertion is stimulated by ADP or AMP-PNP. By contrast, when membranes were limiting (1 eq) the linear insertion kinetics does not go to saturation within 60 minutes and in this case there is no significant difference between the added nucleotides.

Using an in vitro translation/membrane insertion system it has previously been shown that the membrane insertion of the TA protein synaptobrevin-2 requires ATP hydrolysis (Kutay et al., 1995). To see whether this is also true for RAMP4, we synthesized R4op in the reticulocyte lysate and then tested membrane insertion in the absence or presence of ATP or AMP-PNP and apyrase. As can be seen in Fig. 3D, both ATP and AMP-PNP stimulate membrane insertion of R4op (lanes 3 and 4). In the presence of apyrase, only AMP-PNP could stimulate the insertion (lane 7), which suggests that, in this system, membrane insertion of R4op does not require ATP hydrolysis.

To see whether ATP binding to Asna1 is required for membrane insertion of HZZ-R4op, we mutated the nucleotide-binding sites in MBP-Asna1 in the P-loop (MBP-Asna1^{G49R}) or in the Switch 1 and Switch 2 region (MBP-Asna1$^{D74A/D166A}$). From the structure of the bacterial homolog ArsA it is predicted that G49 binds the α-phosphate of the nucleotide and that D74 and D166 coordinate the magnesium (Zhou et al., 2000). We coexpressed these mutants and MBP-Asna1^{WT} with HZZ-R4op in *E. coli* and affinity purified the complexes. Complex formation is efficient and not affected by the

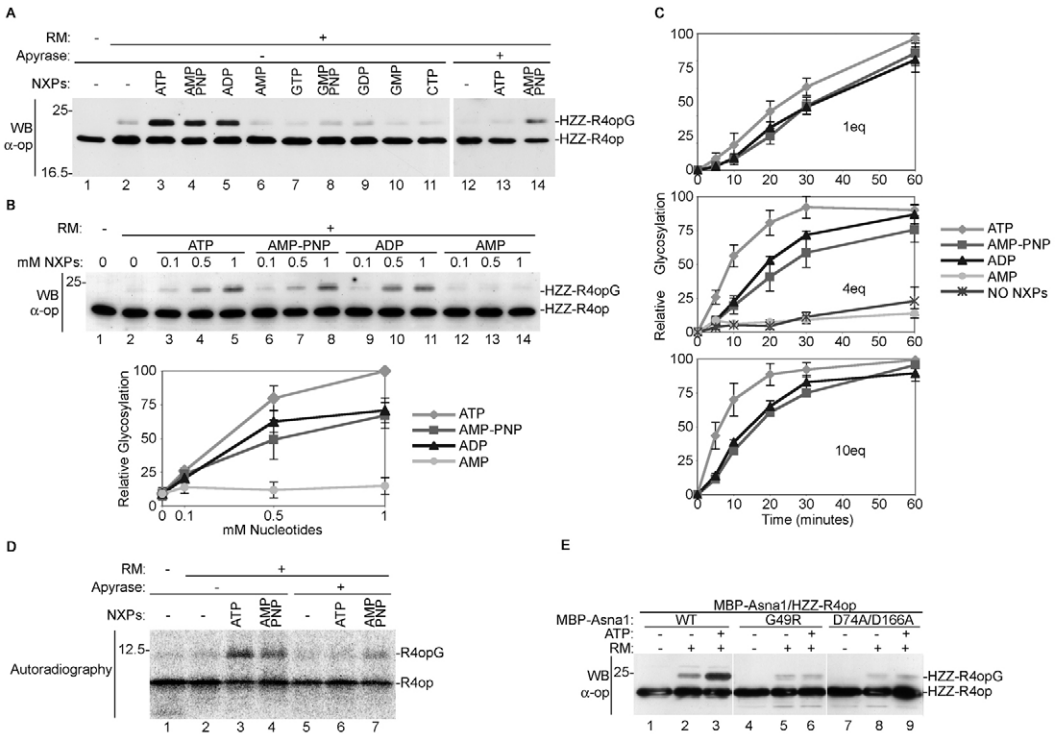

Fig. 3. Nucleotide requirement for insertion of HZZ-R4op into RM membranes. (**A**) Membrane insertion of HZZ-R4op is stimulated by ATP, AMP-PNP and ADP. MBP-Asna1/HZZ-R4op complexes from the pooled fractions 8-15 of the gel filtration (Fig. 2) were incubated with RMs in the presence of the indicated nucleotides. Apyrase was included in the insertion reaction of samples in lanes 12-14. (**B**) Titration of the amount of nucleotides required for RM membrane insertion of HZZ-R4op. MBP-Asna1/HZZ-R4op was incubated with RMs and 0.1, 0.5 and 1 mM of indicated nucleotides. The ADP used in this experiment was purified by HPLC to remove contaminant ATP. HZZ-R4op and its glycosylated form, HZZ-R4opG, were then visualized by SDS-PAGE and western blot analysis. The relative amount of HZZ-R4opG was determined by ImageJ software and normalized to the maximum value obtained. The graph represents the means±s.e.m. of three independent experiments. (**C**) Time course of insertion of HZZ-R4op into increasing amounts of RM membranes in the presence of different nucleotides. MBP-Asna1/HZZ-R4op complexes were incubated for the times indicated with 1 eq, 4 eq or 10 eq of RMs in the presence of the indicated nucleotides. In this experiment the ADP was purified by HPLC to remove contaminant ATP. After SDS-PAGE, HZZ-R4op was detected by western blot. The relative amount of HZZ-R4opG was determined by ImageJ software and normalized to the maximum value obtained. The graphs represent the means ± s.e.m. of three independent experiments. (**D**) Membrane insertion of in vitro translated R4op is stimulated by ATP and AMP-PNP. R4op was synthesised in the RRL. After the removal of small molecules by gel filtration and addition of the indicated nucleotides (lanes 3, 4 and 6, 7) and apyrase (lanes 5-7), the reactions were incubated at 30°C for 30 minutes in the presence of RMs. Labelled proteins were separated by SDS-PAGE and visualized by autoradiography. (**E**) Asna1 point mutants in the nucleotides binding site, MBP-Asna1^{G49R} and MBP-Asna1$^{D74A/D166A}$, do not stimulate membrane insertion of HZZ-R4op. WT or MBP-Asna1 mutants, G49R or D74A/D166A were coexpressed in *E. coli* with HZZ-R4op and double-affinity purified. The complexes were tested for the insertion and glycosylation of HZZ-R4op into RM membrane in the absence or presence of ATP.

mutations (supplementary material Fig. S2, lanes 3, 6 and 9) but HZZ-R4op membrane insertion mediated by mutant MBP-Asna1 is not stimulated by ATP (Fig. 3E, cf. lanes 3, 6 and 9). To determine whether nucleotides were still bound to the complexes after the purification, we determined the nucleotide contents in the affinity-purified complexes by HPLC analyses (data not shown). We found that 13% of the MBP-Asna1^{WT}/HZZ-R4op complexes are associated with ADP and 3% with ATP, whereas 5% of the complexes with MBP-Asna1 mutants contain ADP and 2% ATP. The basal level of membrane insertion observed in the absence of nucleotides is probably mediated by the nucleotides still bound to purified MBP-Asna1/HZZ-R4op complexes (Fig. 3E cf. lanes 2, 5 and 8). Taken together, our data show that ATP or ADP binding to Asna1 but not ATP hydrolysis is required for HZZ-R4op membrane insertion.

Requirements for membrane insertion of Sec61β and Cytb5

Previous studies have shown that requirements for membrane insertion of RAMP4 and Sec61β are quite different from those for Cytb5 (Favaloro et al., 2008). Membrane insertion of RAMP4 and Sec61β was found to depend on high concentrations of ATP while only low concentrations of ATP were required for membrane insertion of Cytb5 (Favaloro et al., 2008). Furthermore Cytb5 has been shown previously to insert into ER membranes unassisted and even inserts into liposomes (Brambillasca et al., 2006; Colombo et al., 2009; Kim et al., 1997). However, Cytb5 has also been found to interact with Asna1 (Colombo et al., 2009; Stefanovic and Hegde, 2007). To see whether Cytb5 binds to Asna1 after coexpression in *E. coli* and to compare requirements for membrane insertion with those of R4op and S61β, we expressed Sec61β (HZZ-S61βop) and Cytb5 (HZZ-Cb5op) either alone or together with MBP-Asna1 in *E. coli*. Soluble fractions of the respective *E. coli* lysates were affinity purified and proteins characterized by SDS-PAGE and western blotting (supplementary material Fig. S2, lanes 10-45 and 19-24). When HZZ-S61βop or HZZ-Cb5op were expressed in the absence of MBP-Asna1, only a small amount of soluble HZZ-S61βop was obtained while a substantially higher amount of HZZ-Cb5op was found in the

soluble fraction (supplementary material Fig. S2, lanes 20 and 23). This difference is probably due to the lower hydrophobicity of the TMD of Cb5. Both proteins were found to co-purify with several *E. coli* proteins, among them the chaperone DnaK (supplementary material Fig. S2, lanes 19-24). When coexpressed with MBP-Asna1, both HZZ-S61βop and HZZ-Cb5op co-purified with MBP-Asna1 (supplementary material Fig. S2, lanes 10-15). Membrane insertion of HZZ-S61βop was found only when it was coexpressed with MBP-Asna1 and this insertion was ATP dependent (Fig. 4A, lanes 1-3 and 7-9). By contrast, HZZ-Cb5op inserted into membranes even when not in complex with MBP-Asna1 and when ATP was absent (Fig. 4A, lanes 4-6 and 10-12). The kinetics of membrane insertion of the two TA proteins under the different conditions was very similar, showing half maximal insertion after about 15 to 20 minutes (supplementary material Fig. S6).

As HZZ-Cb5op can be released from MBP-Asna1 and inserts into RM membrane in the absence of ATP, we asked whether the MBP-Asna1 mutants that are unable to bind ATP, can support HZZ-Cb5op membrane insertion. We coexpressed and copurified HZZ-Cb5op with MBP-Asna1^{G49R} and MBP-Asna1$^{D74A/D166A}$ (data not shown) and tested membrane insertion of HZZ-Cb5op. Both mutants supported efficient membrane insertion of the cargo HZZ-Cb5op protein independently of the presence of ATP (Fig. 4B). This shows that release of a TA protein from Asna1 and its membrane insertion is not always dependent on ATP.

For Get3, the yeast homolog of Asna1, receptor proteins in the ER membrane, Get1 and Get2, have been found to be required for membrane insertion of a subset of TA proteins (Schuldiner et al., 2008). An involvement of receptor proteins has also been suggested for the ER membrane insertion of Sec61β, but not for Cytb5 (Kim et al., 1999; Kim et al., 1997; Kutay et al., 1995). To test for the need of receptor proteins, we released cytosolically exposed domains of membrane proteins by treating RMs with trypsin, puromycin and high salt (T-PKRMs). The efficiency of this treatment was monitored by the cleavage of selected ER membrane proteins (supplementary material Fig. S3B). When we used these membranes in the insertion assay, we found no insertion of HZZ-R4op and HZZ-S61βop, but still efficient membrane insertion of HZZ-Cb5 (Fig. 4C). This suggests that membrane proteins required for membrane insertion of HZZ-R4op and HZZ-S61βop are not needed for membrane insertion of HZZ-Cb5op.

Discussion

Previous studies employing a cell free system, cross-linking and a dominant negative mutant have revealed that the mammalian ATPase Asna1/TRC40 is involved in ER membrane insertion of TA proteins (Favaloro et al., 2008; Stefanovic and Hegde, 2007). This conclusion is also supported by studies in yeast in which the homologue of Asna1, Get3, or its receptor were deleted (Schuldiner et al., 2008).

Here we directly demonstrate with purified Asna1/TA protein complexes and RMs, that Asna1 is an essential targeting factor for some TA proteins (RAMP4 and Sec61β) and for their ATP/ADP-dependent insertion into the RM membranes; however, not for all TA proteins. Asna1 is not essential for the membrane insertion of Cytb5, although it can associate with Cytb5 and release the cargo protein into the membrane in a nucleotide-independent manner. As the Asna1/Cytb5 complex has been assembled in a heterologous *E. coli* cytosol, it remains open whether complex formation also occurs in a eukaryotic cytosol.

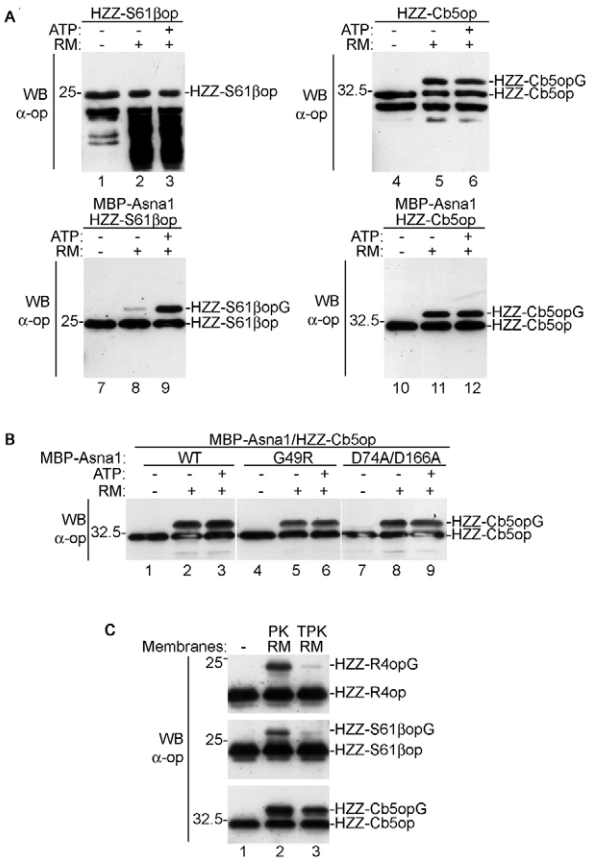

Fig. 4. RM membrane insertion of tagged Sec61β (HZZ-S61βop) and cytocrome b5 (HZZ-Cb5op). (**A**) Affinity purified complexes of recombinant MBP-Asna1/HZZ-S61βop, HZZ-S61βop alone, MBP-Asna1/HZZ-Cb5op and HZZ-Cb5op alone were incubated with or without RMs in the absence or presence of ATP as indicated. Membrane insertion was monitored after western blot analysis by glycosylation (G) of HZZ-S61βop and HZZ-Cb5op. (**B**) WT Asna1 and point mutants in the nucleotide-binding site, MBP-Asna1^{G49R} and MBP-Asna1$^{D74A/D166A}$, support membrane insertion of HZZ-Cb5op. WT, MBP-Asna1 mutants, G49R and D74A/D166A were coexpressed in *E. coli* with HZZ-Cb5op and double-affinity-purified complexes tested for the insertion of HZZ-Cb5op into RM membranes by western blotting. Membrane inserted and glycosylated HZZ-Cb5opG is indicated. (**C**) Trypsin-sensitive proteins of RMs are required for membrane insertion of HZZ-R4op and HZZ-S61βop but not of HZZ-Cb5op. Affinity purified complexes of recombinant MBP-Asna1/HZZ-R4op, MBP-Asna1/HZZ-S61βop, and MBP-Asna1/HZZ-Cb5op were incubated with RMs (lane 2) or with trypsin-treated RMs (lane 3) in the presence of ATP. Membrane insertion was monitored by western blot analysis.

Expression and characterization of recombinant Asna1/TA protein complexes

Recombinant Asna1, when coexpressed in *E. coli* with tagged forms of RAMP4, Sec61β or Cytb5, associates with these proteins into soluble complexes and maintains these proteins competent for post-translational insertion into RM membranes (Fig. 1). The Asna1-RAMP4 complexes are heterogeneous in size, showing two major size classes after size-exclusion chromatography. Complexes of ~700 kDa also contain the *E. coli* chaperone DnaJ, whih suggests that at least some of these protein complexes are not yet completely folded or assembled. Asna1/TA protein complexes of 400 kDa and

smaller do not contain the *E. coli* chaperones DnaK or DnaJ and efficiently promote the membrane insertion of the TA protein RAMP4 (Fig. 2). This indicates that these *E. coli* chaperones do not contribute to membrane insertion of the TA proteins.

When Asna1/TA protein complexes are incubated in the insertion assay in the absence of RMs, the proteins aggregate and are found in the pellet after centrifugation (Fig. 2C). In addition, the yeast homolog of Asna1, Get3, forms aggregates when expressed in the absence of the membrane receptor Get1/Get2 (Schuldiner et al., 2008).

Asna1 alone is sufficient for membrane insertion of TA proteins

We show that Asna1 can associate with TA proteins in the absence of other eukaryotic proteins. This does not exclude the possibility that there are other factors in eukaryotic cells that mediate the efficient assembly of Asna1 with newly synthesized TA proteins. Large size complexes containing Asna1/TRC40 and TA proteins have previously been found in eukaryotic cell-free translation systems and additional cytosolic proteins could be associated with Asna1/TA protein complexes in the eukaryotic cytosol (Stefanovic and Hegde, 2007). Additional proteins associated with Get3, named Get4 and Get5, have been identified in yeast and it has been suggested that they associate with ribosomes and promote the delivery of TA proteins to Get3 (Fleischer et al., 2006; Jonikas et al., 2009). Our data show that, once an Asna1/TA protein complex is formed, the TA protein can insert into ER membranes in the absence of additional soluble eukaryotic proteins. At this step, additional proteins may exist to modulate delivery of TA proteins to the membrane.

Nucleotide requirement for RM-membrane insertion of TA proteins

Asna1 is an ATPase and previous data strongly suggest that Asna1-mediated TA protein insertion is ATP-dependent. However the molecular details of the ATPase cycle in this process are not known. Using non-hydrolyzable ATP, ADP and Asna1 mutants defective in nucleotide binding, we show that ATP or ADP binding but not hydrolysis of ATP is required for membrane insertion of RAMP4 or Sec61β (Fig. 3). However, in the presence of ATP, when the membranes are not limiting, the rate of insertion is higher than in presence of ADP or AMP-PNP. This suggests that the hydrolysis of ATP might accelerate the release of the TA protein from Asna1.

A similar role of the ATP hydrolysis has been observed for the release of the substrate from the chaperone Hsp90. Binding of ATP to Hsp90 causes only a slow release of substrate. Conversion of Hsp90 to the ADP state through hydrolysis of the bound ATP leads to fast release of substrate (Young and Hartl, 2000). By contrast, the membrane insertion of Cytb5 is not dependent on the presence of ATP (Fig. 4A). Moreover, nucleotide binding mutants of Asna1 do not negatively affect Cytb5 membrane insertion (Fig. 4B). This suggests that there are at least two mechanisms by which TA proteins are released from Asna1: an ATP/ADP dependent and an ATP/ADP independent one. As RAMP4 and Sec61β have a more hydrophobic TMD compared with Cytb5 (Rabu et al., 2008), it is conceivable that the interaction between Asna1 and a more hydrophobic TMD is so tight that the conformational change induced by ATP/ADP binding, is required to release the TA protein. By contrast, the TMD of Cytb5 is less hydrophobic and thus may be bound more weakly to Asna1 and does not need nucleotide induced conformational changes of Asna1 to be released and insert into RM membranes.

Recently, several crystal structures of the yeast homolog of Asna1, Get3, have been determined (Bozkurt et al., 2009; Hu et al., 2009; Mateja et al., 2009; Suloway et al., 2009; Yamagata et al., 2009). Get3 forms symmetric homodimers which show, in the nucleotide-free state, an open dimer architecture in which hydrophobic patches are shielded (Hu et al., 2009; Mateja et al., 2009; Suloway et al., 2009; Yamagata et al., 2009). In the presence of AMPPNP-Mg^{2+}, ADP-AlF_4^- or ADP-Mg^{2+}, the Get3 dimer is in a closed conformation and exposes a putative binding site for cargo TA proteins (Bozkurt et al., 2009; Hu et al., 2009; Mateja et al., 2009). Using recombinant Get3/RAMP4 complexes we have shown that Get3-mediated membrane insertion of RAMP4 also occurs in the presence of ATP-Mg^{2+}, AMPPNP-Mg^{2+} or ADP-Mg^{2+} but not in the absence of these nucleotides (Bozkurt et al., 2009). This suggests that Asna1 and Get3 mediate membrane insertion of RAMP4 in the nucleotide-bound, closed conformation. Release of nucleotides may then be linked to the liberation of Asna1 and Get3 from their receptors.

Different membrane requirements for the insertion of TA proteins

The three tested TA proteins efficiently assemble into soluble complexes with Asna1 in *E. coli* but only RAMP4 and Sec61β require a trypsin-sensitive membrane protein for their release from Asna1 and RM membrane insertion. Trypsin-treated PKRMs support membrane insertion of Cytb5 but not of RAMP4 or Sec61β. We can envision different possibilities to explain the release of Cytb5 from Asna1 and its insertion into T-PKRMs: as cytoplasmic domains of membrane proteins are cleaved off, it might be the contact of Asna1/Cytb5 complexes with the lipid bilayer that induces the release of Cytb5 from Asna1 and allows its insertion into the membrane. This possibility is consistent with the finding that Cytb5 is inserted into liposomes even when in complex with eukaryotic cytosolic factors (Brambillasca et al., 2006; Colombo et al., 2009). Alternatively, it can be argued that there is a receptor complex, of which a trypsin-sensitive component is essential for the ATP/ADP-dependent insertion of TA proteins such as RAMP4 and Sec61β, but not for the nucleotide-independent insertion of TA proteins such as Cytb5. A receptor complex consisting of two membrane proteins, Get1 and Get2, has been identified in yeast and shown to mediate Get3 and ATP-dependent membrane insertion of TA proteins (Schuldiner et al., 2008).

In conclusion, our approach allowed us to characterize at the molecular level one stage in the Asna1-mediated pathway of TA protein insertion into ER membranes and revealed two different modes of release of TA proteins from Asna1. As our work has been performed with a purified Asna1/TA protein complex it does not necessarily recapitulate the physiological situation. Like in the yeast system, there may be additional factors which assist loading of cargo TA proteins onto Asna1 or modulate membrane insertion under certain physiological conditions (Jonikas et al., 2009; Schuldiner et al., 2008). To further characterize Asna1-mediated membrane insertion in the cellular context, cell-based assays are required.

Materials and Methods

Plasmid constructs

Constructs used in this study were made by standard methods (Maniatis et al., 1982) and verified by sequencing. The coding sequence for human Asna1was amplified by PCR from pCDNA3-hAsna1 (a kind gift of Blanche Schwappach, Faculty of Life Sciences, University of Manchester, UK). The coding sequences of the TA proteins were amplified from plasmids previously described (Favaloro et al., 2008). Asna1 and the TA proteins were cloned as fusion proteins in pT5*L*/T7 vector, derived from pQE80 (Qiagen) and pET3d (Novagen). MBP-Asna1 contains an N-terminal MBP

tag followed by a TEV-protease recognition site and the Asna1 coding sequence; HZZ-R4op, HZZ-Cb5op and HZZ-S61βop contain an N-terminal 10His and two Z tags (the IgG binding domain of Protein A) followed by a TEV-protease recognition site. *MBP-Asna1* was cloned behind the T5/*Lac* promoter and *HZZ-TAproteins* behind the T7 promoter. The final plasmid, pT5*L*/T7-MBP-Asna1/HZZ-TAproteins allows for independent induction of MBP-Asna1 by IPTG and HZZ-TAprotein by arabinose in the BL21-AI *E. coli* strain (Invitrogen). For single expression of MBP-Asna1, the coding sequence was cloned in pQE80. Point mutants of Asna1 were generated by site directed mutagenesis using the following primers: G49R, 5′-CAAGGGTGG-TGTGCGCAAGACCACCTG-3′ and 5′-CAGGTGGTCTTGCGCACACCACC-CTTG-3′; D74A, 5′-TCATCTCCACAGCCCCAGCACACAAC-3′ and 5′-GTT-GTGTGCTGGGGCTGTGGAGATGA-3′; D166A, 5′-GTGGTATTTGCCACG-GCACCCAC-3′ and 5′-GTGGGTGCCGTGGCAAATACCAC-3′.

Antibodies

Anti-opsin (anti-op) and anti-SRP54 antibodies were described previously (Favaloro et al., 2008; Scoulica et al., 1987). Anti-calnexin, anti-PDI and anti-Hsc70 were from Stressgen. Anti-Hsp40 was from Neomarkers. Anti-DnaJ and anti-DnaK were a kind gift of Bernd Bukau (ZMBH, Heidelberg). Anti-TRAM was previously described (Gorlich et al., 1992). Anti-SRα, anti-SRβ, anti-Sec61α C-term and anti-Sec61α 1° loop were raised in rabbits against the synthetic peptides KKFEDSEKAKKPVR, ADIQDLEKWLAKIA, KEQSEVGSMGALLF and IIEVGDTPKDRALFNGAQKL, respectively. Anti-Asna1 was raised against four OVA-conjugated synthetic peptides (EAEEFEDAPDVEPL; FDQKFSKVPTKVKGYD; VSEQFKDPEQTT; LEPYK-PPSAQ) in rabbits (Peptide Speciality Laboratories, Heidelberg). Secondary antibodies were purchased from Sigma-Aldrich.

ER-derived membranes

Rough microsomes (RMs) and membranes treated with puromycin/high-salt buffer (PKRMs) were prepared as described by Walter and Blobel (Walter and Blobel, 1983) and resuspended at 50 OD_{280} per ml in RM buffer (50 mM HEPES-KOH pH 7.6, 50 mM KOAc, 2 mM $Mg(OAc)_2$, 2 mM DTT, 250 mM sucrose). 1 µl of this supension is defined as 1 eq (Walter and Blobel, 1983). To prepare trypsin-treated PKRMs (T-PKRMs), 20 µg/ml of trypsin (Type XI, Sigma-Aldrich) was added to 1 ml of PKRMs (Meyer and Dobberstein, 1980). This mixture was incubated for 60 minutes on ice. The reaction was stopped by addition of RM buffer containing 2 mM PMSF and 10 µg/ml aprotinin. Membranes were pelleted by centrifugation and resuspended in RM buffer.

Fusion protein expression and purification

Tagged Asna1 and TA proteins were coexpressed in the BL21-AI *E. coli* strain (Invitrogen). The bacterial culture was grown in 2YT medium at 30°C under shaking at 220 rpm. First, the expression of MBP-Asna1 was induced with 0.05 mM IPTG at 0.5 OD_{600} for 1 hour, and then the HZZ-TA protein was induced with 0.5% arabinose for 4 hours. Cells were then harvested and resuspended in ice-cold HS buffer (50 mM HEPES, 500 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, 1 mM PMSF, pH 7.0) containing 40 mM imidazole and 10 µg/ml DNase I, lysed using Avestin Emulsiflex-C5 and aggregates pelleted by centrifugation for 30 minutes at 200,000 ***g***. The supernatant was filtered and loaded onto a HisTrap-HP column (GE Healthcare), washed with HS buffer containing 40 mM imidazole and proteins were eluted with HS buffer containing 500 mM imidazole. Eluted samples were loaded onto an MBPTrap-HP column (GE Healthcare) and the column washed with HS buffer, LS-ATP buffer (50 mM HEPES, 150 mM NaCl, 10 mM $MgCl_2$, 10 mM ATP, 10% glycerol, 1 mM PMSF, pH 7.0) and HS-EDTA buffer (50 mM HEPES, 500 mM NaCl, 50 mM EDTA, 10% glycerol, 1 mM PMSF, pH 7.0). The column was finally washed with HS buffer containing 10 mM arginine and 1 mM DTT (HSAD) and proteins eluted with HSAD buffer containing 20 mM maltose. An ÄKTAdesign system, Superdex 200 10/300 GL column (GE Healthcare) and buffer HSAD were used for gel filtration. TEV cleavage was performed overnight at 4°C.

Single expression of MBP-Asna1 was performed in the BL21-AI *E. coli* strain at 30°C and was induced with 0.05 mM IPTG at 0.5 OD_{600} for 4 hours. Harvested cells were resuspended in ice-cold HSAD buffer containing 10 µg/ml DNase I, lysed and centrifuged as before. The supernatant was filtered and loaded onto an MBPTrap-HP column, washed with HSAD buffer and MBP-Asna1 eluted with HSAD buffer containing 20 mM maltose.

In vitro reconstitution of post-translational membrane insertion

Purified MBP-Asna1/HZZ-TA protein complexes were incubated in a 10 µl reaction at 200 nM final concentration (or 32.5 nM when the glycosylated TA protein was quantified; Fig. 3B,C; supplementary material Figs S5 and S7) with 4 eq of RMs, 1 mM ATP or other nucleotides (unless otherwise indicated), 1 mM $Mg(OAc)_2$, 40 mM HEPES pH 7.6 and 80 mM KOAc. Where indicated, Apyrase (grade III, Sigma-Aldrich) was added to a final concentration of 0.4 U/µl. The membrane insertion reaction was carried out for 30 minutes at 30°C and stopped by adding SDS-PAGE sample buffer. In some experiments, N-linked oligosaccharides were removed by EndoH treatment, according to the manufacturer's instructions (New England Biolabs). Proteins were then separated by SDS-PAGE on 12.5% tris/glycine gel and characterized by western blot analysis with anti-opsin antibodies.

In vitro transcription/translation and post-translational membrane insertion of R4op in rabbit reticulocytes lysate (RRL)

Messenger RNA was synthesized from the SP6 promoter using linearized plasmid DNA and standard methods, as described previously (Favaloro et al., 2008; Schröder et al., 1999). Proteins were synthesized in the rabbit reticulocytes lysate (RRL) according to the manufacturer's instructions (Promega RRL kit for in vitro translation) using L-[^{35}S]methionine (7.5 µCi per 10 µl reaction). 200 ng of in vitro synthesized mRNA was used per 10 µl reaction. Translation reactions were incubated for 30 minutes at 30°C and stopped by addition of puromycin to the final concentration of 2 mM.

To test nucleotide requirements for membrane insertion of R4op, we chelated Mg^{2+} by adding EDTA to 5 mM and removed small molecules by gel filtration using prepacked G-25 MicroSpin columns (GE Healthcare) that were equilibrated in 50 mM Hepes-KOH pH 7.6, 80 mM KOAc, 10 mM $Mg(OAc)_2$ and 2 mM DTT. Where indicated, 1 mM of nucleotides and/or 30 mU/µl of Apyrase were added. Finally RMs were added and the mixture incubated for 30 minutes at 30°C. At the end of the incubation, proteins were precipitated with ammonium sulfate and one-quarter of the starting reaction was prepared for SDS-PAGE. Proteins were separated by SDS-PAGE on 16.5% Tris/tricine gel and radiolabelled molecules were visualized by autoradiography.

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