Distinct targeting pathways for the membrane insertion of tail-anchored (TA) proteins

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Summary

Tail-anchored (TA) proteins are characterised by a C-terminal transmembrane region that mediates post-translational insertion into the membrane of the endoplasmic reticulum (ER). We have investigated the requirements for membrane insertion of three TA proteins, RAMP4, Sec61β and cytochrome b5. We show here that newly synthesised RAMP4 and Sec61β can accumulate in a cytosolic, soluble complex with the ATPase Asna1 before insertion into ER-derived membranes. Membrane insertion of these TA proteins is stimulated by ATP, sensitive to redox conditions and blocked by alkylation of SH groups by N-ethylmaleimide (NEM). By contrast, membrane insertion of cytochrome b5 is not found to be mediated by Asna1, not stimulated by ATP and not affected by NEM or an oxidative environment. The Asna1-mediated pathway of membrane insertion of RAMP4 and Sec61β may relate to functions of these proteins in the ER stress response.

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Key words: Tail-anchored protein, RAMP4, Sec61β, Cytochrome b5, Endoplasmic reticulum, Asna-1/TRC40

Introduction

Insertion of proteins into the membrane of the endoplasmic reticulum (ER) can proceed through different types of mechanism (Rapoport et al., 1996). The best-characterised of these is the signal recognition particle (SRP)-dependent pathway in which the nascent chain ribosome complex is targeted to the ER membrane by SRP and insertion into the Sec61 translocation channel is coupled with further chain elongation (Keenan et al., 2001). Proteins inserted by this pathway usually contain an N-terminal signal or signal anchor sequence. By contrast, tail-anchored (TA) proteins represent a class of membrane proteins that lack an N-terminal signal sequence. They are anchored to the membrane by a single stretch of hydrophobic amino acid residues close to the C-terminus, exposing their larger N-terminal – and usually functional – part to the cytoplasm. Among TA proteins are components of the ER translocation site, Sec61β, Sec61γ and the ribosome-attached membrane protein 4 (RAMP4; also known as stress-associated endoplasmic reticulum protein 1, SERP1) as well as others including vesicle-associated membrane proteins (VAMPs, also known as synaptobrevins), cytochrome b5 (CYB5, hereafter referred to as b5) or members of the Bcl2 protein family (Borgese et al., 2003b; Yamaguchi et al., 1999). The latter ones are not exclusively located at the ER but are also found in the mitochondrial outer membrane (Annis et al., 2004). TA proteins studied so far can insert into membranes post-translationally but requirements for targeting to and insertion into the ER membrane are not well understood, and it seems that different pathways of membrane insertion exist (Annis et al., 2004; Borgese et al., 2003a; Borgese et al., 2003b; Janiak et al., 1994; Wattenberg and Lithgow, 2001). The integration of b5 can occur post-translationally and requires low concentrations of ATP (Anderson et al., 1983; Borgese et al., 2003b; Yabal et al., 2003). Membrane insertion of VAMP2 was found to be dependent on ATP, consistent with a role for cytosolic chaperones in maintaining the precursor in an insertion-competent state (Kim et al., 1997; Kutay et al., 1995). However, some TA proteins, namely VAMP2 and Sec61β were found to associate post-translationally with SRP, and require GTP for their membrane targeting and/or insertion (Abell et al., 2004). Although proteins are implicated in the insertion of TA proteins, there are also indications that some TA proteins insert into membranes without any assistance (Borgese et al., 2003a; Brambillasca et al., 2006; Brambillasca et al., 2005). Recently, Stefanovic and Hegde reported the identification of the 40-kDa ATPase subunit of TRC (TRC40; also known as arsenical pump-driving ATPase protein, Asna-1, and hereafter referred to as Asna1) as a component of a post-translational pathway of TA membrane protein insertion (Stefanovic and Hegde, 2007). Asna1 is homologous to bacterial ArsA and to Get3 (also known as Arr4p) of *Saccharomyces cerevisiae* (Bhattacharjee et al., 2001; Shen et al., 2003), and was found to interact with the transmembrane domain (TMD) of Sec61β, VAMP2 and a syntaxin prior to their membrane insertion. It has been suggested that Asna1, together with other proteins of the TMD recognition complex (TRC), targets TA proteins for insertion into the ER membrane (Stefanovic and Hegde, 2007). A different conclusion has been reached in another study, in which Sec61β was reported to interact with the molecular chaperones Hsc70 and Hsp40, and that these components are capable to facilitate the ATP-dependent delivery of Sec61β to the ER (Abell et al., 2007).

We have investigated the requirements for membrane insertion of three TA proteins: RAMP4, a protein of the ER translocation site that assists membrane insertion of proteins and is involved in the ER quality control and stress response (Hori et al., 2006; Schröder et al., 1999; Yamaguchi et al., 1999), Sec61β, a subunit

of the Sec61 translocation complex (Abell et al., 2007), and b5 (Borgese et al., 2003a; Brambillasca et al., 2006; Brambillasca et al., 2005; Yabal et al., 2003). On the basis of an interaction with Asna1, the requirement of ATP and the redox- or N-ethylmaleimide (NEM)-sensitivity, the membrane insertion of RAMP4 and Sec61β can clearly be distinguished from the NEM-insensitive membrane targeting and/or insertion of b5.

Results

Post-translational insertion of RAMP4

RAMP4 is a small TA membrane protein that exposes its N-terminus on the cytoplasmic and its C-terminus on the lumenal side of the ER membrane (Fig. 1A). To study membrane insertion of RAMP4 we have added an opsin tag to its C-terminus and thereby generated RAMP4op (R4op) (Fig. 1B). The opsin tag is recognised by the monoclonal anti-opsin antibody R2-15 (anti-op) and contains a N-glycosylation site. As N-glycosylation is a lumenal event this modification indicates that R4op exposes its C-terminus on the lumenal side similar to authentic RAMP4.

To investigate membrane targeting and insertion of R4op we synthesised this protein in a rabbit reticulocyte lysate (RRL) translation system. To see whether R4op can be inserted post-translationally, we added rough microsomal membranes (RMs) from canine pancreas either at the start of translation or after termination of protein synthesis following the addition of puromycin. As a control for co-translational membrane insertion we used MHC-class-II-associated invariant chain (Ii), a doubly glycosylated type II membrane protein. When Ii was synthesised in the RRL in the absence of RMs, a protein of about 26 kDa could be detected consistent with the molecular mass of unglycosylated Ii (Fig. 1D, lane 1). This protein was not present in the control reaction lacking mRNA (Fig. 1D, lane 6). When the synthesis of Ii was carried out in the presence of RMs, an additional 33-kDa protein was seen (Fig. 1D, lane 2). Removal of N-linked carbohydrates with endogylcosidase H (EndoH), led to the disappearance of the 33-kDa protein and an increase in the amount of non-glycosylated Ii (Fig. 1D, lane 3). This indicates that Ii is inserted into RMs and becomes glycosylated. When RMs were added after completion of Ii synthesis, no glycosylated Ii was detected (Fig. 1D, lanes 4 and 5), confirming that Ii can only co-translationally be inserted into RMs.

When mRNA encoding R4op was translated in the RRL, a protein of about 8 kDa was immunoprecipitated using anti-opsin antibody (Fig. 1C, lane 1). The estimated molecular mass suggests that this is non-glycosylated R4op. When the translation of R4op mRNA was conducted in the presence of RMs, a higher molecular mass protein appeared (Fig. 1C, lane 2). Treatment with EndoH reduced the size of this larger protein and lead to an increase in the amount of non-glycosylated R4op (Fig. 1C, lane 3). When RMs were added post-translationally, glycosylated R4op was also seen (Fig. 1C, lanes 4 and 5). Taken together, these results show that R4op synthesised in vitro in the RRL can efficiently be inserted into RMs co- as well as post-translationally.

Characterisation of the cytosolic R4op complex.

The ability of newly synthesised R4op to become post-translationally inserted into RMs suggests that R4op, despite being a membrane protein, can be maintained in an insertion-competent state when exposed to the hydrophilic cytosolic environment. To see how long cytosolic R4op can be maintained in an insertion-competent state, we incubated newly synthesised R4op for different

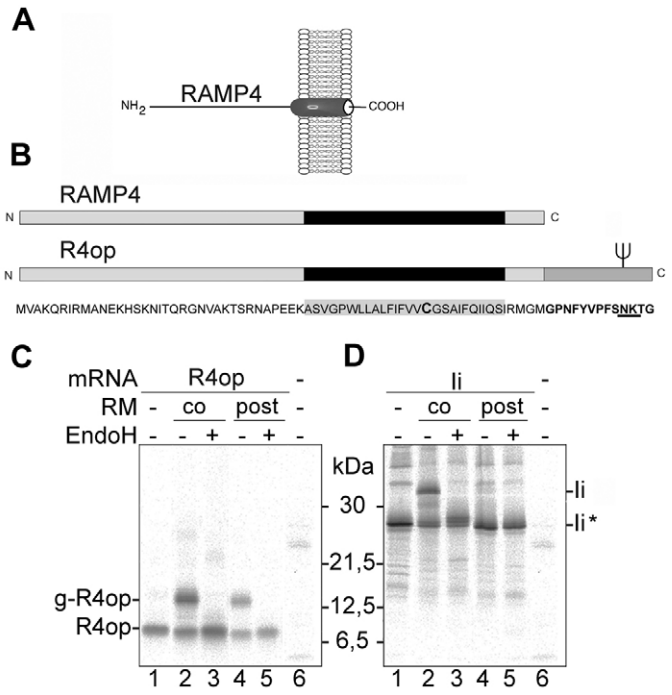

Fig. 1. Post-translational membrane insertion of R4op. (A) Topology of RAMP4 in the ER membrane. RAMP4 is a tail-anchored ER membrane protein that exposes its N-terminus on the cytosolic and the C-terminus on the lumenal side of the membrane. (B) Schematic representation of RAMP4 and RAMP4op (R4op). R4op contains at its C-terminus a bovine opsin tag comprising 13 amino acid residues (dark grey box). The tag provides an N-glycosylation site (fork). The predicted transmembrane domain (TMD) is represented as a black box. A single cysteine residue in the TMD is typed in bold. (C and D) In vitro translation and membrane insertion of R4op and the type II membrane protein Invariant chain (Ii), respectively. Proteins were synthesised in rabbit reticulocyte lysate (RRL), in the absence (lanes 1, 4, 5 and 6) or presence (lanes 2 and 3) of rough microsomes (RM co). Rough microsomes were added after completion of translation (RM post) to samples shown in lanes 4 and 5. Where indicated, samples were treated with EndoH to remove N-linked oligosaccharides. Proteins were immunoprecipitated using either anti-opsin (C) or anti-Ii (D) antibodies, were separated by SDS-PAGE and visualised by autoradiography. g-R4op: glycosylated R4op; Ii*: non-glycosylated Ii.

times before the addition of RMs. To see whether membrane insertion of R4op can proceed independently of the presence of ribosomes, we depleted ribosomes by ultracentrifugation. RMs were added at 0, 30 and 60 minutes after R4op synthesis. As shown in Fig. 2A, efficient membrane insertion and glycosylation can be detected even when the RMs were added 60 minutes after terminaton of R4op synthesis. Quantification of glycosylated and unglycosylated R4op showed that even after 1 hour incubation in the cytosol about 60% of R4op was glycosylated and, thus, membrane-inserted (see histogram). We conclude that R4op released from ribosomes can be maintained for an extended period of time in a membrane-insertion-competent state and this does not depend on the presence of ribosomes.

We next asked whether cytosolic R4op accumulates in a distinct complex. Newly synthesised R4op in RRL was separated on 10-20% sucrose gradients containing either ATP or ADP to see whether complex formation is dependent on the continued presence of these nucleotides. As shown in Fig. 2B, R4op migrates in both sucrose gradients as a distinct complex of about 60-70 kDa. In the

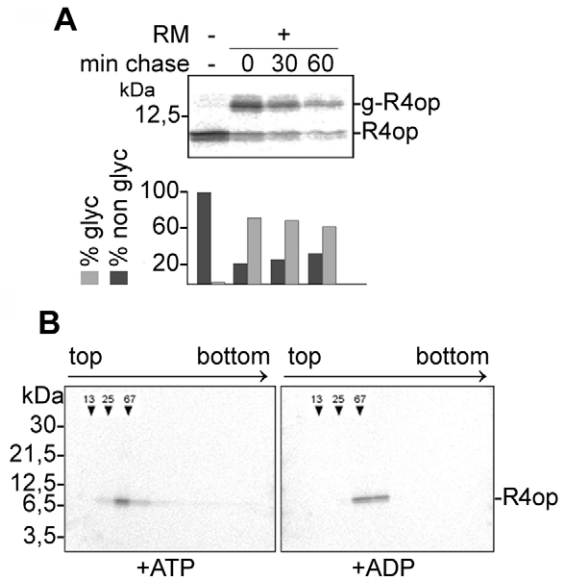

Fig. 2. Insertion competence and size of the cytosolic R4op complex. (A) Time dependence of R4op insertion into RMs. R4op was synthesised in the RRL, translation was stopped by the addition of puromycin and ribosomes were removed by sedimentation. Reactions were further incubated for the times indicated. RMs were then added and incubation continued for 30 minutes. R4op was immunoprecipitated and characterised by SDS-PAGE and autoradiography. The amounts of immunoprecipitated non-glycosylated R4op (black bars) and glycosylated g-R4op (grey bars) were quantified (see histogram). (B) Sucrose-density-gradient analysis of cytosolic R4op. R4op was synthesised in the RRL. Aliquots were loaded on top of 10-20% sucrose density gradients containing 2 mM ATP (left) or ADP (right). After centrifugation and fractionation, proteins were analyzed by SDS-PAGE and autoradiography. Black arrowheads and numbers above the gel indicate migration positions of proteins used as molecular markers and their molecular mass in kDa, respectively.

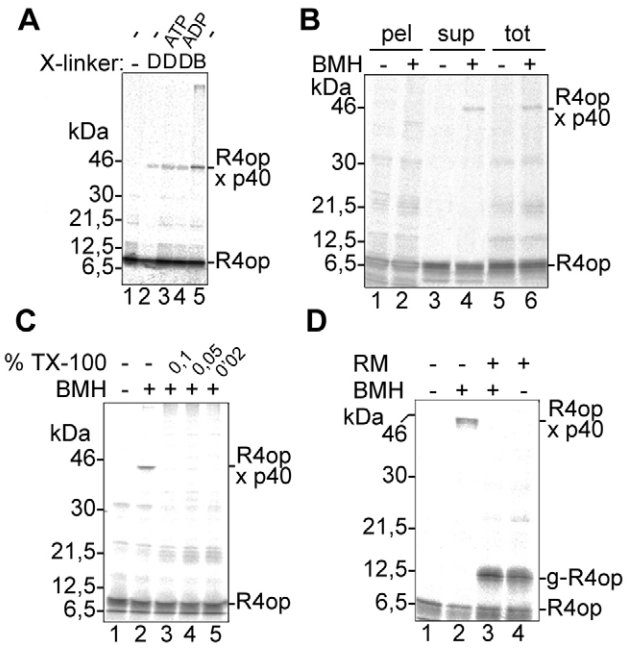

Fig. 3. Crosslinking of R4op. (A) Crosslinking of cytosolic R4op in the presence or absence of nucleotides. After termination of R4op translation in RRL, nucleotides were removed and either no nucleotides or 2 mM ATP or ADP added. After the crosslinking by either DSS (D) or BMH (B), R4op was immunoprecipitated and characterised by SDS-PAGE and autoradiography. (B) Crosslinking of R4op in the presence or absence of ribosomes. After translation, ribosomes were removed by ultracentrifugation. A total fraction (total), the resuspended pellet (pellet) and the supernatants (Sn) were crosslinked by BMH or left uncrossliked (–). (C) Crosslinking of R4op in the presence of Triton X-100. After synthesis of R4op in the RRL, the samples were adjusted to the indicated amount of Triton X-100 and then BMH was added. (D) Crosslinking of R4op in the absence or presence of RMs. After synthesis of R4op, BMH was added either immediately or after the additional incubation with RMs. g-R4op, glycosylated R4op. R4op × p40, crosslinked product of R4op and a 40-kDa protein.

presence of ATP, R4op migrated with a slightly lower molecular mass than in the presence of ADP. These data suggest that newly synthesised R4op can be maintained in a distinct soluble complex in the absence of RMs. Judged by the migration distance within the gradient we deduce that cytosolic RAMP4 is associated with a cytosolic partner (or partners) of about 50 kDa.

Probing the molecular environment of newly synthesised R4op by chemical crosslinking

To directly investigate the molecular surrounding of cytosolic R4op in RRL, we applied chemical crosslinking using a crosslinker specific for NH_2-groups (DSS) or for SH groups (BMH). Amino acids with NH_2 groups are found in the hydrophilic parts of R4op, whereas a single cysteine residue is found close to the middle of the transmembrane domain (TMD) of R4op (Fig. 1B). R4op was synthesised in the RRL and crosslinkers were added in the absence of nucleotides and small molecules or in the presence of ATP or ADP. As shown in Fig. 3A, both DSS (lanes 2-4) and BMH (lane 5) induce formation of a crosslinked product of about 46 kDa that is not seen in the absence of crosslinker (compare lane 1 with lanes 2-5). Consistently, we found that the amount of crosslinked product was highest when BMH was used. As the 46 kDa crosslinked product was obtained after immunoprecipitation with the anti-opsin antibody it must be an adduct of R4op. Taking into the account the molecular mass of R4op, we estimate the size of its interacting partner to be about 40 kDa and, accordingly, named it p40. Presence of ATP (lane 3) or ADP (lane 4) did not affect the efficiency of R4op crosslinking to p40. Crosslinking between R4op and p40 does not depend on the presence of the opsin tag because the same crosslinking partner is also seen when RAMP4 is used in the reaction instead of R4op (supplementary material Fig. S1).

To determine whether p40 is a ribosomal protein, we sedimented ribosomes from the R4op translation mixture prior to crosslinking. As shown in Fig. 3B, the crosslinked product between R4op and p40 was detected exclusively in the supernatant fraction (compare lanes 4 and 2), suggesting that p40 is not a ribosomal protein. The efficiency of sedimentation was confirmed by western blot analysis of aliquots of the supernatant and pellet fractions using an antibody against the ribosomal protein L23a (supplementary material Fig. S2).

Crosslinking of R4op to p40 through the single cysteine residue present within the TMD domain of R4op (Fig. 3A, lane 5) suggests that hydrophobic interactions have a role in the interaction between R4op and p40. To test this possibility we added different amounts of the detergent Triton X-100 before adding BMH. Very low concentrations of Triton X-100 (0.02% v/v) were sufficient to completely abolish crosslinking between R4op and p40 (Fig. 3C). By contrast, ionic interactions do not break the association between these two proteins because crosslinking was observed even when

high ionic strength buffer (1M KOAc) was used in the crosslinking reaction (supplementary material Fig. S3).

When RMs are added to newly synthesised R4op in the RRL, R4op is inserted into the membranes and becomes glycosylated. To see whether R4op is released from p40 when RMs are added, we crosslinked before and after the addition of RMs. In the absence of RMs, newly synthesised R4op was found crosslinked to p40 (Fig. 3D, lane 2). When RMs were present in the reaction, no crosslinking to R4op was seen anymore. Instead glycosylated R4op appeared (Fig. 3D, lane 3). This indicates that, upon RM addition, R4op is released from p40 followed by its insertion into RMs and its glycosylation.

Requirements for membrane insertion of R4op

ER membrane insertion of some TA proteins can depend on SRP and the SRP receptor (Abell et al., 2004). To test whether the SRP system is involved in the insertion of R4op we treated RMs with a high-salt solution containing puromycin (PKRM) and low amounts (1 or 2 μg/ml) of trypsin (PKRM-T1 or PKRM-T2, respectively). Under these conditions the cytoplasmically exposed part of the SRP receptor is liberated from the membrane rendering RMs incompetent for the SRP-dependent translocation (Meyer and Dobberstein, 1980). Recombinant soluble SRP-receptor (SR) can then be added to restore the SRP-dependent translocation (Fulga et al., 2001). We tested such trypsin-treated membranes for their ability to allow insertion of the type II membrane protein invariant chain Ii or of R4op. Membrane insertion was again determined by glycosylation of these proteins and a shift to higher molecular forms. Fig. 4 shows that Ii is inserted into PKRM and mock-treated PKRM (lanes 1-4), but not into the trypsin-treated membranes (PKRM-T1 or PKRM-T2; lanes 5 and 7, respectively). When the purified SRP-receptor was added, membrane insertion of Ii was re-established (lanes 6 and 8). This confirms that the trypsin treatment was effective and that the soluble SRP-receptor can restore membrane insertion of Ii. When membrane insertion of R4op was tested similarly, the trypsin-treated membranes were found to still promote membrane insertion (glycosylation) of RAMP4 (Fig. 4, lanes 13 and 15). This demonstrates that membrane insertion of R4op does not depend on the SRP system.

Immunoaffinity purification of the R4op-p40 complex and identification of p40 as Asna1

To isolate and identify p40, the interaction partner of newly synthesised R4op, we pursued an immunoaffinity purification strategy using a large volume of RRL in which R4op was synthesised in the absence of membranes. As a control, another in vitro translation reaction was used, to which buffer was added instead of R4op mRNA. Both reaction mixtures were batch-adsorbed to CNBr-sepharose beads coupled to the anti-opsin antibody. After binding, the beads were extensively washed with a high-salt buffer, a treatment that does not interfere with the R4op-p40 association (supplementary material Fig. S3). p40 was then released from R4op using a buffer containing Triton X-100, and remaining proteins were finally eluted from the affinity matrix using an acidic glycine buffer.

As shown in Fig. 5A, lane 1, a prominent band of a protein with an apparent molecular mass of ~40 kDa was detected after silver staining of a gel containing the sample eluted with Triton X-100. No such protein was found in the sample obtained from the control reaction that lacked newly synthesised R4op (lane 3). The 40-kDa protein band was cut out from the gel, eluted and analysed by ESI-QUAD-TOF mass spectrometry. Fourteen of the detected peptides correspond to sequences of human Asna1, an arsenite-stimulated ATPase (protein coverage of 41%). To confirm the identity of p40, we immunoprecipitated crosslinked R4op-p40 (Asna1) using anti-Asna1 antibody (Fig. 5B, lane 6). This antibody immunoprecipitated exclusively the 46-kDa crosslinked product, but not non-crosslinked R4op. In the reaction without BMH, anti-Asna1 did not precipitate any radiolabelled protein (Fig. 5B, lane 5). The antibody directed against the opsin tag immunoprecipitated both, non-crosslinked and crosslinked R4op (Fig. 5B, lanes 3 and 4, respectively). We conclude that the 40 kDa cytosolic interacting partner of R4op that was synthesised in the RRL in the absence of RMs is the ATPase Asna1.

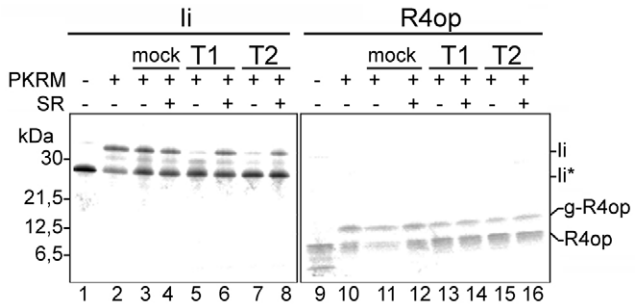

Fig. 4. Membrane requirements for R4op and Ii insertion into PKRM. R4op and Ii were synthesised in the RRL. Membranes washed in high-salt buffer supplemented with puromycin (PKRM), mock-treated (PKRM mock) or treated with 1 or 2 μg/ml trypsin (PKRM-T 1 and PKRM-T 2, respectively) were present during the synthesis of Ii. R4op was incubated with these membranes post-translationally. Where indicated, 100 nM of soluble SRP receptor (SR) was added. Proteins were analysed by SDS-PAGE and autoradiography. Ii*, nonglycosylated Ii; g-R4op, glycosylated R4op.

Does Asna1 also interact with other membrane proteins?

To see whether Asna1 interacts also with other membrane proteins we tested the C-terminally tagged TA proteins Sec61βop (S61βop), cytochrome b5op (b5op) and Ii. S61βop and b5op, similar to R4op, are efficiently post-translationally inserted into ER membranes, whereas Ii is not (supplementary material Fig. S4). To see whether these proteins associate with Asna1, we used again the crosslinkers BMH and DSS. As b5op does not contain a cystein residue (Fig. 6A), we used in this case only DSS. After translation in RRL and following crosslinking, we immunoprecipitated the crosslinked complexes using an anti-opsin, anti-Asna1 or a non-related anti-Myc antibody. As shown Fig. 6B-E, efficient crosslinking to Asna1 and Asna1 in higher molecular mass complexes (◆) was only found for R4op and S61βop, but not for b5op (lanes 3 and 4) and Ii (lanes 5 and 6).

Nucleotide- and redox-state dependence of TA-protein insertion into the ER membrane

Previous studies by several groups have shown that membrane insertion of TA proteins can depend on the presence of ATP or GTP, or even proceed in the absence of nucleotides. On the basis of these observations, different pathways for the membrane insertion of TA proteins have been suggested (Abell et al., 2007; Borgese et al., 2003b; Brambillasca et al., 2006). To determine the requirements for nucleotides, we synthesised R4op, S61βop and b5op in the RRL, and released nucleotides from proteins by chelating Mg^{2+} with

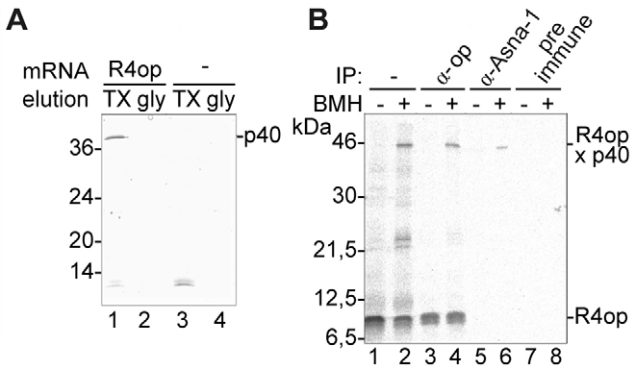

Fig. 5. Identification of p40. (A) Immunoaffinity purification of p40 associated with R4op. Large-volume RRL translation reactions were incubated with R4op mRNA (lanes 1 and 2) or without (lanes 3 and 4). R4op-containing complexes were affinity-purified using anti-opsin antibody beads and proteins released from R4op by elution with 0.1% Triton X-100 (TX) (lanes 1 and 3). Remaining bound proteins were eluted from the column by using an acidic glycine buffer (gly) (lanes 2 and 4). Eluted proteins were separated by SDS-PAGE and silver stained. The protein band of about 40 kDa was cut out, proteins were eluted and peptide sequences determined by mass spectroscopy. Peptide sequences identified p40 as Asna1. (B) Immunoprecipitation of R4op × p40 crosslinked product. R4op was synthesised in RRL and aliquots of the reaction were either crosslinked with BMH (+) or incubated with DMSO solvent alone (–). Aliquots of both reactions were either directly applied to the gel (lanes 1 and 2) or immunoprecipitated by anti-opsin antibody (lanes 3 and 4), an anti-Asna1 antibody (lanes 5 and 6) or a pre-immune serum (lanes 7 and 8) and characterised by SDS-PAGE and autoradiography.

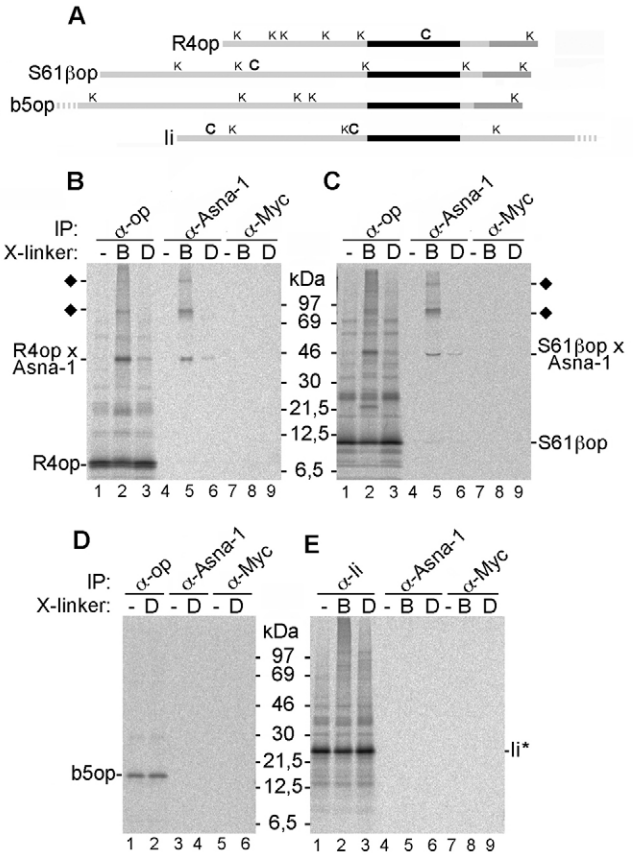

Fig. 6. Cytosolic R4op (RAMP4op) and S61βop (Sec61βop) but not b5op (cytochrome-b5op) or Ii (invariant chain) can be crosslinked to Asna1. (A) Outline of the sequences around the TM (black) of R4op, S61βop, b5op and Ii. The sequences are aligned by the relative position of their TM domains. Lysine (K) and cysteine (C) residues that can function in crosslinking with DSS and BMH, respectively, are indicated. (B-E) Crosslinking of the TA proteins and Ii in the RRL: R4op (A), S61βop (B), b5op (C) and Ii (D) were in vitro synthesised in the RRL and small molecules were removed by gel filtration and either DMSO (–) or the crosslinker BMH (B) or DSS (D) were added. The TA proteins were immunoprecipitated with anti-opsin (α-op), anti-Asna1 (α-Asna-1) or the unrelated anti-Myc (α-Myc) antibodies and characterised by SDS-PAGE and autoradiography. R4op × Asna-1 and S61βop × Asna-1, R4op and S61βop, respectively, crosslinked to Asna1. ◆, yet-unidentified complexes of higher molecular mass.

EDTA. We then removed small molecules from the lysates by gel filtration and supplied $Mg(OAc)_2$ to all further reactions.

The homologue of Asna1 in *Saccharomyces cerevisiae*, Get3 may be a redox-sensitive protein because Cu^{2+}-mediated redox stress changes the conformation of the protein, as detected in non-reducing SDS-PAGE gels (Metz et al., 2006). Furthermore five of the eight cysteine residues are conserved from yeast to human. We therefore tested whether the redox state of the cytosol influences the nucleotide requirement for the membrane targeting and/or insertion of the TA proteins. We depleted lysates of small molecules and first added H_2O_2 (to 2 mM) (Fig. 7A, lanes 3-6) or DTT (to 2 mM) (Fig. 7A, lanes 7-10), and then added either no nucleotides or ATP (A), GTP (G) or CTP (C) to lysates depleted of small molecules (Fig. 7A). After the addition of RMs the lysates were incubated and the labeled TA proteins analysed by SDS PAGE and autoradiography. The percentage of glycosylated TA proteins was determined densitometrically. Most efficient membrane insertion (glycosylation) of R4op and S61βop is seen in the presence of ATP under all redox conditions tested (Fig. 7A; and supplementary material Fig. S5, 0.25-8 mM H_2O_2 or DTT) although glycosylation is slightly less efficient under oxidising conditions. Under reducing conditions a lower level of membrane insertion is seen in the presence of GTP or CTP, or when nucleotides were removed (Fig. 7A). Membrane insertion of b5op, by contrast, was not affected by the removal of nucleotides or the addition of nucleotides, but is slightly more efficient under reducing (DTT) than oxidising (H_2O_2) conditions (Fig. 7A).

As membrane insertion of b5 is known to require low concentrations of ATP (Yabal et al., 2003) and gel filtration might not have removed all nucleotides, we used in addition apyrase to deplete nucleotides. As shown in Fig. 7B all three TA proteins were not glycosylated to a further extent, neither under oxidising nor reducing conditions (lanes 5 and 8). Complete depletion of nucleotides led to a reduction in the amounts of S61βop and to the accumulation of smaller-molecular-mass forms of these proteins, which suggests proteolytic processing. Efficient membrane insertion of R4op and S61βop required the addition of ATP, whereas efficient membrane insertion of b5op was not increased by the addition of ATP (lanes 3, 4 and 6, 7).

To see how the redox conditions affect the interaction (crosslinking) between R4op and Asna1, and the release of Asna1 upon addition of RMs, we crosslinked proteins after the membrane-insertion reaction with BMH. R4op-Asna1 complexes were either immunoprecipitated using anti-opsin antibody (Fig. 7C) or anti-Asna1 antibody (supplementary material Fig. S6). We find that release of R4op from Asna1 and also membrane insertion (glycosylation) require the presence of RMs and ATP (Fig. 7C, lanes 13 and 15), which suggests that the ATP-dependent pathway is

mediated by Asna1. The release, however, is less efficient under oxidising conditions (Fig. 7C, lanes 5 and 7). R4op is not released from Asna1 in the absence of ATP (lanes 5 and 13). A low amount of glycosylated R4op is also seen when ATP was not added.

To test whether free sulfhydryl (SH) groups on cytosolic proteins are essential for membrane insertion of the three TA proteins, we treated the translation reaction with NEM, eliminated excess NEM by gel filtration, added H_2O_2 or DTT or ATP to the lysates where indicated, and incubated them with RMs (Fig. 7D). We find that NEM-treated cytosol prevents membrane insertion (glycosylation) of R4op and S61βop, but not of b5op (Fig. 7D).

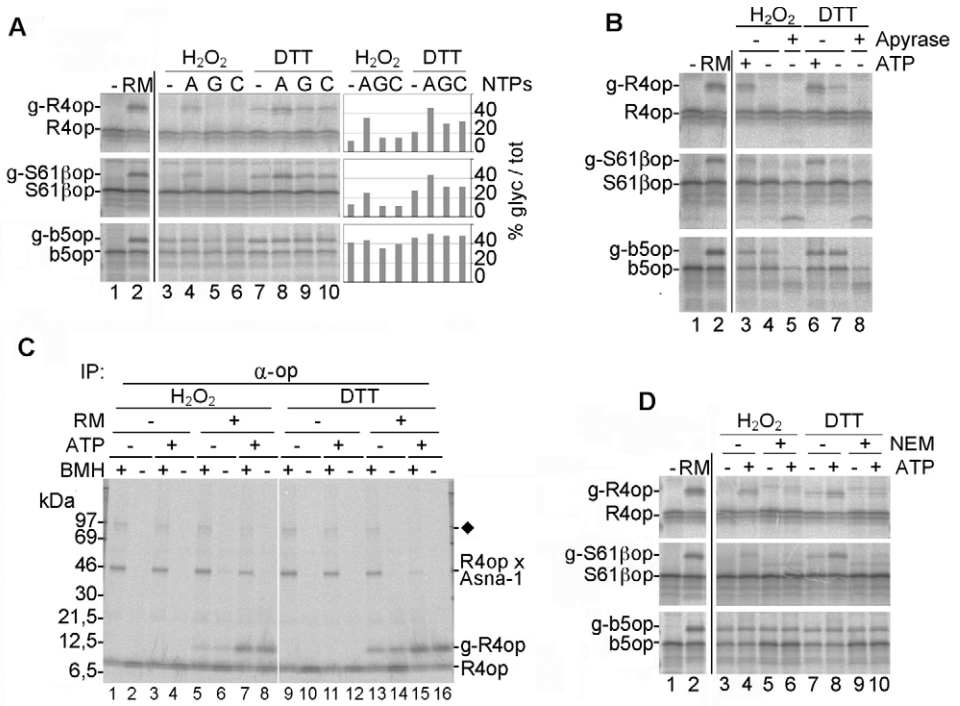

Fig. 7. Comparison of the requirements for membrane insertion of R4op, S61βop and b5op. (A) Nucleotide- and redox-state dependence of the post-translational membrane insertion. After the synthesis of the TA proteins in the RRL (lane 1) RMs were added either directly (lane 2) or after treatments as indicated (lanes 3-10). To test nucleotide tri-phosphate (NTP) and redox conditions required for membrane insertion of these TA proteins, small molecules were removed from the lysates by gel filtration (lanes 3-10) and addition of 2 mM H_2O_2, 2 mM DTT, 3 mM ATP (A), GTP (G) or CTP (C) as indicated. After incubation, proteins were separated by SDS-PAGE and visualised by autoradiography. Glycosylated TA protein (glyc) was quantified in percent (right panel). (B) Nucleotide depletion by apyrase and membrane insertion of R4op, S61βop and b5op. The three TA proteins were synthesised in the RRL (lane 1) and RMs added either directly (lane 2) or after removal of small molecules by gel filtration and addition of H_2O_2, DTT, apyrase or ATP as indicated (lanes 3-8). (C) Release of R4op from Asna1 and membrane insertion. After synthesis of R4op in the RRL, small molecules were removed by gel filtration and lysates were adjusted to either 2 mM H_2O_2 (lanes 1-8) or 2 mM DTT (lanes 9-16) and 3 mM ATP and then incubated with RMs as indicated. After the membrane insertion small molecules were removed by gel filtration and BMH crosslinking induced where indicated. Proteins were immunoprecipitated using anti-opsin antibodies (α-op), separated by SDS-PAGE and visualised by autoradiography. (D) Free sulfhydryl (SH) groups on cytosolic proteins are required for the membrane insertion of R4op and S61βop but not of b5op. After synthesis of the three TA proteins in the RRL (lane 1) RMs were added (lane 2) and the insertion reaction was incubated for 30 minutes at 30°C. To test whether free SH-groups are needed for membrane insertion, lysates were adjusted to 5 mM NEM where indicated. After incubation, small molecules were removed by gel filtration and the lysates adjusted to 2 mM H_2O_2 (lanes 3-6), 2 mM DTT (lanes 7-10) and 3 mM ATP as indicated and incubated with RMs. Proteins were separated by SDS-PAGE and visualised by autoradiography.

Discussion

We have investigated requirements for the membrane insertion of opsin-tagged RAMP4, Sec61 and cytochrome b5 (R4op, S61βop and b5op, respectively). We show here that these three TA proteins can insert post-translationally into membranes of the ER in a cell free system. In the absence of membranes newly synthesised R4op and S61βop but not b5op can be crosslinked to Asna1, an arsenite-stimulated ATPase that promotes various membrane related functions (Auld et al., 2006; Kao et al., 2007; Shen et al., 2003; Tseng et al., 2007). We identified Asna1 after immunoisolation of the soluble R4op complex by mass spectrometry and by immunoprecipitation with an anti-Asna1 antibody. As the release of R4op from Asna1 requires the presence of RMs and ATP we propose that Asna1 is a functional intermediate in the membrane insertion of R4op and, probably, also of S61βop. Asna1-mediated targeting and/or membrane insertion of R4op and S61βop is sensitive to oxidising conditions and is blocked when free SH groups are modified by NEM. By contrast, membrane insertion of b5op is not blocked by NEM and low levels of nucleotides are sufficient for membrane insertion of this TA protein (Borgese et al., 2003a; Yabal et al., 2003).

While the initial version of this manuscript was prepared Stefanovic and Hegde reported the finding that Asna1 interacts with transmembrane domains (TMDs) of several TA proteins, such as Sec61β, and VAMP2 as well as the two members of the syntaxin family Nsyn1 and Stx1 (Stefanovic and Hegde, 2007). Our findings add RAMP4 to this group of TA proteins that interact with Asna1 in a cytosolic complex before membrane insertion. Using sucrose-gradient centrifugation we find a size of 60-70 kDa for the soluble R4op complex. However, we identified distinct higher-molecular-mass complexes by crosslinking with R4op as well as with S61βop (Fig. 6B,C); these might represent crosslinks to dimers of Asna1 (Kurdi-Haidar et al., 1998a). A size of 100-500 kDa is found by Stefanovic and Hegde for the soluble Sec61β complex, named TMD recognition complex (TRC) (Stefanovic and Hegde, 2007). This complex is proposed to contain, apart from Asna1, additional proteins that function in the membrane targeting of TA proteins (Stefanovic and Hegde, 2007).

Several lines of evidence show that Asna1 interacts with the TMD of TA proteins. We show that Asna1 can be crosslinked to a single SH group (Cys) in the transmembrane region of R4op. Binding to a hydrophobic region can also be deduced from the fact that a mild detergent but not a high-salt solution can break the interaction of Asna1 with R4op. Stefanovic and Hegde show directly that the TMD of Sec61β is required for an interaction with Asna1 (Stefanovic and Hegde, 2007). Deletion of the TMD of Sec61β prevents interaction with Asna1. However, the interaction of Asna1 with TA

Table 1. Requirements for membrane insertion of R4op and S61bop, and b5op

	DTT				H_2O_2			
	+ATP	low NTP	–NTP	NEM+ATP	+ATP	lowNTP	–NTP	NEM+ATP
RAMP4 and Sec61β	+++	+	–	–	++	–	–	–
Cytochrome-b5	+++	+++	–	+++	++	++	–	++

Summarised results for the requirements for membrane insertion of R4op and S61bop, and b5op.
Evaluation of glycosylation (membrane insertion): +++, most efficient; ++, less efficient; +, drastically reduced; –, not detected.

proteins is probably not restricted to the TMD because crosslinking to the single cysteine in the N-terminal hydrophilic region of S61βop is also observed (Fig. 6A). Asna1 does not seem to interact with b5op or Ii because no crosslinking of Asna1 to these proteins could be observed (Fig. 6D,E). Consistent with this conclusion is also the fact that membrane insertion of b5op is not stimulated by additional ATP (Fig. 7B). The hydrophobic signal anchor domain of nascent Ii is known to interact with SRP (High et al., 1993). Our data show that Asna1 is not in proximity to any hydrophobic TMD of newly synthesised membrane proteins that accumulate in the cytosol.

Different targeting pathways for the membrane insertion of R4op and S61βop, and b5op

As RAMP4 and Sec61β are ER-stress-induced proteins (Hori et al., 2006) and one of the functions of Asna1/Get3 is related to cellular stress (Metz et al., 2006; Shen et al., 2003), we investigated how cytosolic redox conditions and nucleotides affect membrane insertion of these proteins. Our results are schematically summarised in Table 1. We find that the cytosolic requirements for membrane insertion of R4op and S61βop are very similar: free SH groups in a cytosolic protein are required for membrane insertion of these proteins because NEM-treated cytosol does not support membrane insertion, neither under oxidising nor reducing conditions. Efficient membrane insertion of R4op and S61βop requires ATP and is not supported by GTP, CTP or non-hydrolysable nucleotides (AMPPNP, GMPPNP) (Fig. 7A and data not shown). The reduction of nucleotides by gel filtration revealed a striking difference between the membrane insertion of R4op and S61βop on the one hand and b5op on the other hand: whereas low levels of nucleotides fully support the membrane insertion of b5op, they support only a basal level of insertion of R4op and S61βop. Additional ATP is required for the release of R4op from Asna1, and for the efficient membrane insertion of R4op and S61βop. In contrast to R4op and S61βop, membrane insertion of b5op is not NEM-sensitive and is not affected by oxidative cytosolic conditions. The free SH group(s) that are affected by NEM might be present in the substrate protein, in Asna1 or in another targeting factor. Both, R4op and S61βop contain a cysteine residue in the TMD and the N-terminal domain, respectively, whereas b5op does not contain a cysteine.

Different pathways for the membrane insertion of TA proteins have been proposed previously. They were defined by the requirement for different cytosolic factors and ATP, GTP or the absence of nucleotides (Abell et al., 2003; Abell et al., 2004; Borgese et al., 2003a; Kim et al., 1997; Kutay et al., 1995; Kutay et al., 1993; Rabu and High, 2007; Steel et al., 2002). Sec61β was found to interact with SRP and the molecular chaperones Hsc70 and Hsp40, and the presence of Hsc70 stimulates membrane insertion in conjunction with Hsp40 (Abell et al., 2007). It is well-conceivable that these chaperones, in addition to Asna1, mediate membrane insertion of TA proteins such as RAMP4 and Sec61β. The higher-molecular-mass complexes seen in the R4op and S61βop crosslinks might represent Asna1 dimers (Kurdi-Haidar et al., 1998a) and/or complexes with such chaperones (Fig. 6B,C) (Rabu and High, 2007). Another possibility is that the membrane insertion of R4op and S61βop (which requires a low concentration of nucleotides) is mediated by the HSP70-HSP40 chaperone system and by SRP (Abell et al., 2003; Abell et al., 2004; Abell et al., 2007).

The ATPase Asna1 is thought to function in membrane-associated processes

Mammalian Asna1 is a 37 -kDa cytoplasmic ATPase with a single ATP-binding cassette (Kurdi-Haidar et al., 1998b). Asna1 is homologous to bacterial ArsA and to Get3 of *Saccharomyces cerevisiae* (Bhattacharjee et al., 2001; Shen et al., 2003). Yeast Get3 ATPase has been implicated in multiple membrane-dependent pathways including metal ion homeostasis and heat tolerance (Metz et al., 2006; Shen et al., 2003). In *C. elegans* and in mammals Asna1 is mainly expressed in cells with high secretory activity, in particular in β-islet cells (Kurdi-Haidar et al., 1996; Kurdi-Haidar et al., 1998c). The data presented here and previously by Stefanovic and Hegde suggest that membrane insertion of a subset of TA proteins is mediated by Asna1 (Stefanovic and Hegde, 2007). Our data furthermore suggest, that the Asna1 pathway may not be the only pathway by which TA proteins like R4op and S61βop can be inserted into the ER membrane. In the presence of low nucleotides, when Asna1 is not released from R4op, a proportion of R4op is found inserted into membranes (Fig. 7A,C). This pool is rather sensitive to oxidative conditions (Fig. 7A). Such conditions may arise when for instance metal ions accumulate in the cytosol. Such an assumption is also supported by considering functions observed for the yeast homologue of Get3, which has been implicated in many membrane-dependent pathways including ion homeostasis, ER-associated degradation and regulation of membrane transport processes (Auld et al., 2006; Metz et al., 2006; Schuldiner et al., 2005; Shen et al., 2003). Get3 and Asna1 might contribute to these functions by mediating efficient membrane insertion of a certain subset of TA proteins (Auld et al., 2006; Metz et al., 2006; Schuldiner et al., 2005). Clearly, more work is required to elucidate the role of Asna1 in membrane insertion of RAMP4 and other TA proteins.

Materials and Methods

Cloning procedures and plasmids

Constructs used in this study were made by standard methods (Maniatis et al., 1982) and verified by sequencing. Vector pGem4Ii used for the synthesis of invariant chain was described previously (High et al., 1993). A plasmid containing mouse RAMP4 cDNA (MNCb-2442) was obtained from the National Institute of Infectious Diseases, Division of Genetic Resources, Tokyo, Japan. An *Eco*RI-*Pst*I fragment was ligated into the pGEM4Z plasmid (Promega) to give pGEM4Z-MNCb-RAMP4. A tag containing the N-terminal 13 amino acid residues of bovine opsin was added to the C-terminus of mouse RAMP4 encoded in the plasmid pGEM4Z-RAMP4/3′UTR (mouse RAMP4 with authentic 3′ UTR) using PCR-based mutagenesis ExSite kit from Stratagene and the following oligonucleotides: GG_F1 (5′-GGCCCAAAC-TTCTACGTGCCTTTCTCCAACAAGACGGGCTGAAGTGACTGACCTTGA-3′) pGG_R1 (5′-CATGCCCATCCTGATACTTTGAATAATCTGGAAAATTGCAGA-GCCACAGACAA-3′). Plasmids encoding the human cytochrome b5 (b5) and Sec61β

cDNAs comprising the C-terminal opsin tag, pcDNA5-Cb5OPG and pcDNA5-Sec61βOPG (Abell et al., 2007) were a kind gift from Stephen High.

In vitro transcription and translation, membrane insertion and denaturing immunoprecipitation

mRNA was synthesised from the SP6 or T7 promotor using linearised plasmid DNA and standard methods as described previously (Schröder et al., 1999). Proteins were synthesised in the rabbit reticulocytes lysate (RRL) according to the manufacturer's instructions (Promega RRL kit for in vitro translation) and by using L-[^{35}S]methionine (7.5 µCi per 10 µl reaction). 150 ng of in-vitro-synthesised mRNA was used per 10 µl reaction.

Rough microsomes (RMs) and membranes washed in puromycin/high-salt buffer (PKRM) were prepared as described by Walter and Blobel 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] (Walter and Blobel, 1983). To prepare trypsin-treated PKRM, the amount of trypsin indicated in the figure legend was added to 1 ml of PKRM (Meyer and Dobberstein, 1980). This mixture was incubated for 60 minutes on ice. The reaction was stopped by addition of RM buffer containing 1 mM PMSF, 10 µg/ml aprotinin and 0.75 M KOAc. Membranes were pelleted by centrifugation and resuspended in 1 ml of RM buffer. Mock-treated membranes were prepared in the same way, except water was used instead of trypsin.

Translation reactions were incubated for 30 minutes at 30°C and stopped by addition of puromycin to the final concentration of 2 mM. In reactions where proteins were inserted co-translationally, RMs from canine pancreas were present during translation. For post-translational insertion RMs were added after termination of translation and incubation continued for 30 minutes at 30°C. When insertion into trypsin-treated microsomal membranes was reconstituted, soluble recombinant SRP receptor SRhisα/βΔN was added to the final concentration of 100 nM (Fulga et al., 2001).

To test the redox state and nucleotide requirements for the membrane insertion of TA proteins, we synthesised the TA proteins in the RRL and then chelated Mg^{2+} by adding EDTA to 5 mM. To test the effect of NEM on membrane insertion of the TA proteins, NEM (Sigma-Aldrich, Steinheim, Germany) freshly dissolved in water, was added to the lysates after translation to a final concentration of 5 mM. Where indicated in figure legends, small molecules were removed by gel filtration using prepacked G-25 MicroSpin columns (GE Healthcare) that were equilibrated in 50 mM HEPES-KOH pH 7.6 and 80 mM KOAc. Reactions were then adjusted to 10 mM $Mg(OAc)_2$, 2 mM H_2O_2 or DTT and 3 mM of the nucleotide indicated in the figures. In some experiments apyrase was added to a final concentration of 0.4 U/µl and the samples incubated for 30 minutes at 30°C.

At the end of the RM insertion assays, proteins were precipitated with ammonium sulfate and one-fifth of the starting reaction was prepared for SDS PAGE. Where indicated antigens were immunoprecipitated under denaturing conditions using specific antibodies and proteinA-coupled sepharose beads (Amersham Pharmacia) as previously described (High et al., 1993; Schröder et al., 1999). Proteins were then separated by SDS-PAGE on 15% gels and radiolabelled molecules were visualised by autoradiography.

Chemical crosslinking

Crosslinkers were purchased from Pierce, dissolved in DMSO and stored at –20°C. Before crosslinking, small molecules were removed by gel filtration using Microspin columns pre-packed with Sephadex G-25 (GE Healthcare) that were equilibrated in 20 mM HEPES-KOH pH 7.6, 80 mM KOAc, 0.5 mM $Mg(OAc)_2$. If not specified otherwise, crosslinker were used at a final concentration of 250 µM. Crosslinking was conducted for 15 minutes at room temperature. The reaction was quenched by the addition of glycine and DTT to the final concentration of 10 mM each and incubation on ice for further 5 minutes. Samples were then processed for denaturing immunoprecipitation and analysed by a 15% or 6-15% gradient SDS gel and using autoradiography. As a control, one sample was incubated with DMSO lacking the crosslinker.

Sucrose-density-gradient analysis

To analyse R4op-containing cytosolic complexes, R4op was synthesised in vitro in 20 µl RRL reaction. After termination of protein synthesis by puromycin, aggregates were removed by centrifugation for 10 minutes at 13,000 rpm in a tabletop centrifuge. Supernatants and molecular mass marker proteins were loaded onto linear 10-20% sucrose gradients containing 50 mM HEPES-KOH pH 7.5, 80 mM KOAc, 1 mM Mg(OAc)2, 1 mM DTT and either 2 mM ATP or 2 mM ADP. Gradients were centrifuged in a SW60 rotor at 4°C for 15 hours at 45,000 rpm. Fractions were collected from the bottom using an ISCO density gradient fractionator. Fraction 12 contains the resuspended pellet. Proteins in the fractions were precipitated by TCA resuspended in 1% SDS, 10 mM Tris-HCl pH 7.5, 80 mM KOAc, 1 mM Mg(OAc)2 and R4op was immunoprecipitated by the anti-opsin antibody. Proteins were then characterised by SDS-PAGE and autoradiography. Marker proteins, run on parallel gradients were lysozyme (13 kDa), chymotrypsin A (25 kDa), albumin (67 kDa).

Affinity-purification of R4op and associated proteins

To purify R4op-containing complexes, we coupled the monoclonal anti-opsin R2-15 antibody to CNBr-Sepharose beads (Amersham Pharmacia). The beads were washed with buffer I (0.5 M KOAc, 10 mM Tris-HCl pH 7.6) and equilibrated in ice-cold buffer II (250 mM KOAc, 10 mM Tris-HCl pH 7.6).

R4op or no protein were synthesised in each 5 ml of rabbit reticulocyte lysates obtained from Green Hectares (Wisconsin). After the synthesis aggregates were removed by centrifugation in a Sorvall SS34 rotor for 15 minutes at 10,000 rpm. The resulting supernatants were added to 200 µl anti-opsin-antibody-coupled beads. The mixture was incubated with shaking for 3 hours at room temperature. Beads were then washed twice with 4 ml of buffer W1 (1 M KOAc, 10 mM Tris-Cl pH 7.6), transferred to fresh tubes and washed eight times with 4 ml of W2 buffer (500 mM KOAc, 10 mM Tris-Cl pH 7.6). Proteins were then eluted by incubating the beads with 150 µl of buffer E1 (0.1% Triton X-100, 500 mM KOAc, 10 mM Tris-HCl pH 7.6) for 10 minutes at room temperature. After centrifugation, proteins in the eluate were precipitated with 20% TCA, 80% acetone and resuspended in 30 µl sample buffer for SDS-PAGE. Beads were washed once with 4 ml of buffer II, and then incubated in 150 µl of 100 mM glycine (pH 2.5) for 10 minutes. After centrifugation proteins in the glycine eluate were precipitated by TCA. Proteins were separated on a 15% SDS gel and visualised by standard silver staining except that the gel was developed in a solution of 0.01% formaldehyde (v/v) and 2% sodium carbonate (w/v) followed by washing with 1% acetic acid. To determine the identity of the ~40 kDa protein, the band was cut from the gel and analysed by ESI-QUAD-TOF mass spectrometry.

Antibodies

Rabbit antibodies against invariant chain (anti-Ii), ribosomal protein L23 (anti-L23) and RAMP4 (anti-RAMP4) have been described previously (High et al., 1993; Lipp and Dobberstein, 1986; Pool et al., 2002). Hybridoma cell line secreting mouse monoclonal anti-opsin antibody (R2-15) was generated by Paul Hargrave (Adamus et al., 1991). Antibodies against Asna1 were raised against a maltose-binding protein Asna1-fusion protein in guinea pig (Peptide Speciality Laboratories, Heidelberg). Secondary antibodies used for western blotting were purchased from Sigma-Aldrich, Steinheim Germany.

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