Post-translational integration of tail-anchored proteins is facilitated by defined molecular chaperones

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Summary

Tail-anchored (TA) proteins provide an ideal model for studying post-translational integration at the endoplasmic reticulum (ER) of eukaryotes. There are multiple pathways for delivering TA proteins from the cytosol to the ER membrane yet, whereas an ATP-dependent route predominates, none of the cytosolic components involved had been identified. In this study we have directly addressed this issue and identify novel interactions between a model TA protein and the two cytosolic chaperones Hsp40 and Hsc70. To investigate their function, we have reconstituted the membrane integration of TA proteins using purified components. Remarkably, we find that a combination of Hsc70 and Hsp40 can completely substitute for the ATP-dependent factors present in cytosol. On the basis of this in vitro analysis, we conclude that this chaperone pair can efficiently facilitate the ATP-dependent integration of TA proteins.

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Introduction

The majority of proteins in higher eukaryotes are synthesised in the cytosol before being targeted to their final subcellular destination, and the presence of several distinct organelles creates a requirement for efficient and accurate targeting pathways. For the endoplasmic reticulum (ER), targeting is often co-translational and begins with the binding of the signal recognition particle (SRP) to a hydrophobic signal sequence in a nascent polypeptide chain emerging from the ribosome (Nagai et al., 2003). The resulting ribosome–nascent-chain–SRP complex is delivered to the ER membrane through interaction with the SRP receptor (Halic et al., 2006), resulting in the binding of the ribosome to the Sec61 translocon and translocation into or across the ER membrane.

Whereas the post-translational targeting of proteins to organelles, such as mitochondria, is well established, post-translational routes have also been identified for specific subsets of precursors destined for the ER. These post-translational pathways commonly depend on molecular chaperones to maintain the client polypeptide in an unfolded state, thus ensuring competency for translocation into or across the organellar membrane. In yeast, a well-defined post-translational pathway for delivering secretory proteins to the ER depends on the Hsc70-chaperone Ssa1p and the Hsp40-family co-chaperone Ydj1 (Ngosuwan et al., 2003). Hsc70 also plays an important role in protein targeting to mitochondria, with Hsc70 and Hsp90 acting cooperatively to facilitate the delivery of some precursors (Humphries et al., 2005; Young et al., 2003b). Given the pivotal role of generic chaperones such as Hsc70 in protein targeting to a variety of organelles, there must be additional factors or mechanisms that distinguish specific protein-chaperone complexes in order to ensure specificity (Reichert and Neupert, 2004; Wiedemann et al., 2004). Possible discrimination in the cytosol can be provided by factors that interact with a specific precursor-chaperone complex; thus mitochondrial targeting of some precursors is stimulated by MSF (Mihara and Omura, 1996), whereas a 14-3-3 protein binds to phosphorylated signal sequences and Hsc70 to form the guide complex implicated in chloroplast targeting (May and Soll, 2000). With the exception of a novel role for SRP (Abell et al., 2004), no specialised cytosolic factors have so far been implicated in post-translational targeting to the ER targeting.

Tail-anchored (TA) proteins are targeted to various organelles (Borgese et al., 2003), and are defined by a common C-terminal hydrophobic sequence, which functions in both targeting and membrane insertion. The location of this sequence dictates that membrane integration must occur post-translationally, because translation must terminate before the targeting sequence can emerge from the ribosomal exit tunnel (High and Abell, 2004). Thus, any direct interactions between the tail-anchor and targeting factors must also occur after translation termination. The differences in the sequences of TA proteins that are delivered to distinct subcellular organelles are often quite minor (Beilharz et al., 2003; Borgese et al., 2001) and provide no clear idea as to how specificity is achieved. Nevertheless, our previous discovery that SRP is able to target some TA proteins, such as synaptobrevin 2 (Syb2), to the ER membrane in a unique post-translational mode provides one mechanism by which specificity can be achieved (Abell et al., 2004). It was equally clear from this study that other TA proteins, including cytochrome b5 and Sec61β are targeted to

the ER in an SRP-independent manner (Abell et al., 2004; High and Abell, 2004). An ATP-dependent route for the delivery of TA proteins to the ER (Kutay et al., 1995; Yabal et al., 2003) is the prime candidate for this SRP-independent route. Whereas ATP-dependent molecular chaperones are obvious candidates for facilitating this pathway, not a single ATP-dependent factor has been identified to date (Kutay et al., 1995; Yabal et al., 2003). We therefore selected Sec61β as a model precursor to identify the cytosolic factors that mediate the ATP-dependent pathway. We show that Sec61β interacts with the molecular chaperones Hsc70 and Hsp40, and that Hsc70 binding is promoted by the presence of the TA sequence. When the integration process is reconstituted using purified components, Hsc70 stimulates membrane insertion in conjunction with Hsp40 as efficiently as complete cytosol. We conclude that Hsc70 and Hsp40 are capable of facilitating the ATP dependent delivery of TA proteins to the mammalian ER, and propose that this chaperone-mediated route is distinct from any SRP-dependent targeting.

Results

N-glycosylation of Sec61β reports membrane integration at the ER

Based on our previous study (Abell et al., 2004), we selected human Sec61β as a TA protein that can use the ATP-dependent pathway for ER integration. In order to reliably monitor its integration, we generated a version of Sec61β with a short C-terminal extension containing a site for N-glycosylation (Abell et al., 2004; Borgese et al., 2001). This polypeptide Sec61βG can only be modified if the protein is correctly inserted into the ER membrane (Fig. 1). When Sec61βG is synthesised with ER-derived microsomes (K-RM) a higher molecular-mass product is observed. This product is resistant to extraction with alkaline sodium carbonate solution and sensitive to digestion with endoglycosidase H (EndoH) (Fig. 2A, lanes 1 and 2, product 1g). We therefore conclude that it is a fully membrane integrated form of Sec61β bearing a single-N-linked glycan (Abell et al., 2004). In this case we found that 16% of the membrane-associated Sec61β was N-glycosylated. Glycosylation efficiency depends on the accessibility of the

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Sec61β       ...GLKVGPVPVLVMSLLFIASVFMLHIWGKYTRS96
Sec61β-TM    ...GLKVGPMMEFAESADAALQGDPALQDAGDSSR96
Sec61βG      ...GLKVGPVPVLVMSLLFIASVFMLHIWGKYTRS
                GGGNKNITQA106
Sec61βOPG    ...GLKVGPVPVLVMSLLFIASVFMLHIWGKYTRS
                GPNFYVPFSNKTG109
Syb2         ...WKNLKMMIILGVICAIILIIIIVYFST116
Syb2G        ...WKNLKMMIILGVICAIILIIIIVYFSS
                SDSGGGNGGGNKNITQAPPH136
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Fig. 1. Model TA proteins. Sequences of the tail-anchor regions and C-terminal extensions of the polypeptides used in this study. Potential transmembrane (TM) domains are underlined, dots indicate hydrophilic domains extending beyond the sequence presented. Numbers in superscript show the total length of the polypeptides; –TM indicates replacement of the hydrophobic TM domain; G indicates a chimera with a C-terminal N-glycosylation site; OPG indicates a chimera with a C-terminal extension derived from bovine opsin including an N-glycosylation site; *N* indicates N-glycosylation-target residues.

recognition sequence in the ER lumen and, for TA proteins, longer extensions generally yield higher efficiencies (see also Fig. 4B below). However, a short C-terminal extension should minimise any potential impact of the tag upon ER targeting and integration (High and Abell, 2004), although the glycosylated form of Sec61β will underestimate the total population of correctly integrated polypeptides.

Sec61βG integration is post-translational and requires cytosolic factors

To confirm that Sec61βG can be integrated in a strictly post-translational fashion, and to establish a role for cytosolic factors in promoting this process, the insertion of partially purified polypeptides was investigated. Hence, nascent Sec61βG chains were prepared by translating mRNA lacking a stop codon and isolating the resulting ribosome nascent chain complexes (RNCs) by centrifugation using conditions that remove loosely bound factors, such as SRP (supplementary material Fig. S1A). The isolated nascent chains were released from the ribosome by puromycin treatment and the efficiency of membrane integration was analysed under various conditions by assessing their N-glycosylation. If no cytosol is added back to the purified chains, little authentic membrane integration is seen (Fig. 2B, lanes 1-3, product 1g). By contrast, when reticulocyte lysate is present significant integration is obtained (Fig. 2B, lane 4, product 1g). The addition of EDTA abolishes integration (Fig. 2B, lane 5), but this effect is reversed by the subsequent addition of magnesium ions (Fig. 2B, lane 6). We conclude that membrane integration is stimulated by factors present in the reticulocyte lysate used for translation, and that the process is dependent upon divalent cations consistent with a role for ATP.

Efficient membrane integration of Sec61β is dependent upon ATP

The hypothesis that Sec61β integration requires ATP is supported by the inhibition of this process upon nucleotide triphosphate depletion (NTPs) (Abell et al., 2004). To establish which nucleotide(s) promote the cytosol-dependent stimulation of Sec61β integration, small molecules were depleted from lysate by gel filtration and membrane insertion reconstituted with specific nucleotide triphosphates. Increasing the relative concentration of lysate increases membrane integration consistent with stimulation by cytosolic factors (Fig. 2C, lanes 1 and 2, product 1g), whereas gel filtration causes a fourfold reduction in membrane integration (Fig. 2C, lanes 1 and 3, product 1g). This reduction is fully reversed by adding ATP to the depleted lysate (Fig. 2C, lanes 3 and 4, product 1g), whereas GTP results in a modest recovery of integration, consistent with a proportion of Sec61β being targeted via the SRP dependent pathway (Abell et al., 2004). Since only a proportion of Sec61β chains are N-glycosylated, we compared the levels of glycosylated and non-glycosylated Sec61β that remained associated with the membrane fraction after extraction with alkaline sodium carbonate solution. The relative proportion of non-glycosylated Sec61β remaining after alkaline extraction showed a trend broadly similar to that of the N-glycosylated form (Fig. 2C, quantification of 0g and 1g products). However, because we can only be certain that the N-glycosylated chains are fully membrane integrated, we focused on these glycosylated chains for the remainder of the study.
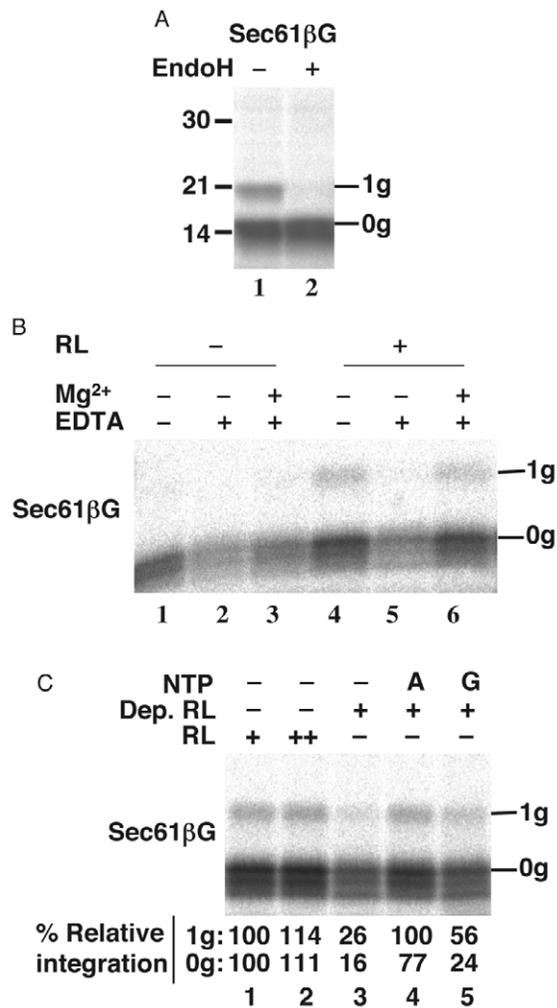
Fig. 2. N-glycosylation indicates ATP-dependent post-translational integration. (A) mRNA encoding the full-length Sec61βG polypeptide but lacking a stop codon to terminate protein synthesis (see Fig. 1) was translated for 20 minutes, and nascent-chain release synchronised by the addition of puromycin. Incubation was continued in the presence of microsomes for 30 minutes and one sample was treated with EndoH. Glycosylated and non-glycosylated Sec61β are indicated (1g and 0g respectively). Quantification showed that, in the absence of EndoH treatment, 16% of the membrane-associated chains remaining after extraction with alkaline sodium carbonate solution were N-glycosylated. Molecular mass is indicated on the left (in kDa). (B) Sec61βG was released from isolated RNCs by puromycin treatment in the presence or absence of reticulocyte lysate (RL), then treated with or without 10 mM EDTA, followed by treatment with or without 10 mM Mg(OAc)$_2$ as shown. Samples were finally incubated with microsomes for 30 minutes and membrane-associated material was isolated by extraction with alkaline sodium carbonate solution. Of the membrane-associated products recovered, 8% were N-glycosylated for the control sample (lane 4). Lower molecular weight forms of non-glycosylated Sec61β were more prevalent after RNC preparation (lanes 1-6, product 0g and below), most likely as a result of ribosome stacking (Ismail et al., 2006). We confirmed that EDTA treatment does not prevent N-glycosylation per se (data not shown), hence, a lack of glycosylated Sec61β reflects a lack of integration. (C) Sec61βG was released from isolated RNCs by puromycin treatment in the presence of buffer, reticulocyte lysate (RL) or lysate depleted of small molecules by gel filtration (Dep. RL), with additional ATP (A) or GTP (G) as shown. In one case, a double quantity of normal lysate was added (++). Samples were incubated with microsomes for 30 minutes and the membrane fraction was recovered after extraction with alkaline sodium carbonate solution as for B. The resulting material corresponding to non-glycosylated polypeptides (0g) and glycosylated polypeptides (1g) was quantified and standardised to the sample incubated with reticulocyte lysate (lane 1, relative integration=100). In this case, 6% of the membrane-associated products recovered were N-glycosylated for the control sample (lane 1).

Identification of cytosolic factors associated with Sec61β by crosslinking

To identify candidates for the ATP-dependent cytosolic factors that stimulate the membrane integration of Sec61β, we used a crosslinking approach. Nascent Sec61β chains were released from the ribosome by puromycin treatment in the presence of reticulocyte lysate. The reaction mixture was then depleted of ATP to stabilise transient interactions with components such as ATPases. Treatment with the bifunctional crosslinking reagents SMCC and BMH generated several discrete adducts, and suggested that Sec61β associates with several proteins of between 10 kDa and 90 kDa see (Fig. 3A, lanes 1-4). Using immunoprecipitation, we confirmed the identity of a 60 kDa interacting partner as the SRP54 subunit (Fig. 3B, lane 2) (Abell et al., 2004), whereas a 35 kDa partner was identified as a new adduct with Hsp40 (Fig. 3B, lane 3). Likewise a ~70 kDa interacting partner was identified as Hsc70 (Fig. 3C, lane 5). When a version of Sec61β with its tail-anchor replaced by a hydrophilic stretch of residues (Fig. 1) was analysed in the same assay, we found that Hsc70 crosslinking was clearly promoted by the presence of the tail-anchor (Fig. 3C, lanes 5 and 10). Taken together, these results show that newly synthesised Sec61β associates with defined molecular chaperones present in mammalian cytosol, and that Hsc70 binding is promoted by the presence of a tail-anchor domain.

Purified chaperones can facilitate Sec61β integration

To determine the functional role of the molecular chaperones that we had shown to be associated with Sec61β chains we reconstituted membrane insertion in the presence of different combinations of defined components in place of complete lysate (Fig. 2B,C). This approach relied on the prior depletion of stimulatory chaperones by the isolation of ribosome–nascent-chain complexes from the translation reaction by centrifugation, and we first established the efficiency of this process. This analysis showed that – in contrast to SRP, which was efficiently removed by the purification procedure – a residual amount of Hsc70 was co-purified with the RNC complexes (supplementary material Fig. S1A,B). Thus, the background level of 30% relative membrane integration (Fig. 4A, lanes 2 and 12), obtained in the absence of any exogenously added factors, may reflect the activity of such residual chaperones that remain associated with the ribosome–nascent-chain complexes during the purification process.

When the purified Sec61βG chains are supplemented with purified chaperones prior to puromycin-mediated release from the ribosome, membrane integration shows some apparent stimulation by Hsc70 alone (Fig. 4A, lanes 2 and 4). More strikingly, a combination of Hsp40 and Hsc70 together results in a level of integration equivalent to that seen with complete
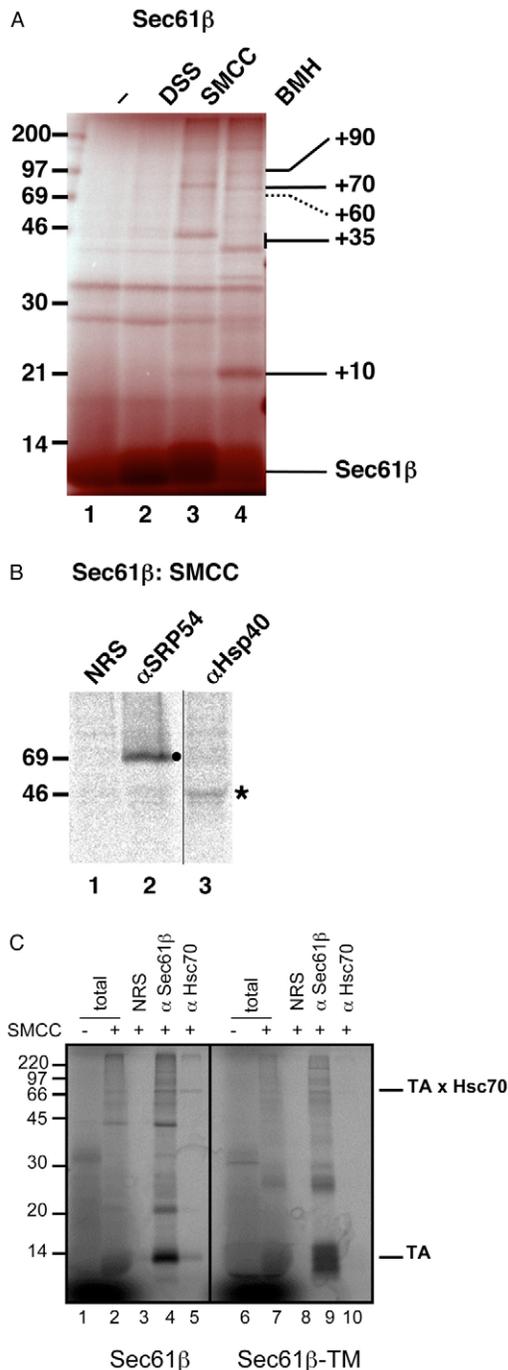
Fig. 3. Sec61β associates with cytosolic chaperones. (A) Sec61β was synthesised as in described for Fig. 2 and polypeptide chains were released from the ribosome by puromycin treatment. Samples were treated with apyrase to deplete nucleotide triphosphates, crosslinking reagents were added as shown and the resulting products resolved by SDS-PAGE. The location of Sec61β chains and the approximate molecular mass of major adducts are indicated (in kDa). (B) Products of SMCC cross-linking were subjected to immunoprecipitation with antisera recognising specific cytosolic components or a non-related serum (NRS). Adducts with SRP54 (filled circle) and Hsp40 (star) are shown. (C) Sec61β (lanes 1 to 5) or a version without the hydrophobic TM region, Sec61β-TM (lanes 6 to 10), were synthesised as for A) and total products analysed either before (lanes 1 and 6) or after (lanes 2 and 7) SMCC mediated cross-linking. Adducts were identified by immunoprecipitation carried out in the absence of prior SDS denaturation and using antisera specific for either Sec61β (lanes 4 and 9) or Hsc70 (lanes 5 and 10). A non-related serum was used as a control (lanes 3 and 8), adducts with Hsc70 are identified (filled square).

glycosylation. In order to quantitatively and rigorously analyse the chaperone-dependent stimulation of TA protein integration, we constructed a version of Sec61 designed to be more efficiently glycosylated. We replaced the original glycosylation tag with a short section from the N-terminus of opsin that had proven well-suited to this purpose in previous studies of cytochrome b5 (Borgese et al., 2001). We found that the single site for N-glycosylation in the resulting chimera Sec61βOPG (see Fig. 1) is used very efficiently during a simple post-translational integration assay performed after puromycin release (37% of chains are N-glycosylated, see supplementary material Fig. S3A). Base-level integration of the RNC-purified Sec61βOPG chains was clearly detectable (Fig. 4B, lane 7), consistent with the co-purification of cytosolic chaperones including Hsc70 (supplementary material Fig. S1B). However, puromycin release in the presence of reticulocyte lysate, resulted in a threefold increase in membrane integration compared with control samples receiving buffer alone (Fig. 4B, lanes 6 and 7). When various combinations of purified chaperones are analysed using this second Sec61β derivative, we find that a combination of Hsp40 and Hsc70 consistently stimulates membrane integration with a high degree of statistical significance (Fig. 4B, lanes 3, 5 and 7, $P<0.01$). As previously, any stimulation by individual chaperones appears modest and the effect of combining Hsp40-Hsc70 with Hsp90 is, if anything, inhibitory. Taken together, these data suggest that Hsp40 and Hsc70 can play a major role in facilitating the ATP-dependent post-translational integration of Sec61β at the ER membrane.

To further define the significance of the Hsc70-Hsp40-mediated stimulation of TA protein membrane integration, we examined the effect of the nucleotide-exchange factor BAG1. Hsc70 activity is stimulated by BAG1 in combination with Hsp40 (Hohfeld and Jentsch, 1997) and we therefore investigated the effect of CBAG, an active fragment of BAG1 (Sondermann et al., 2001), upon the Hsc70-Hsp40-mediated membrane integration. We find that CBAG levels have a clear impact upon Hsc70-Hsp40-mediated membrane integration of Sec61βG (Fig. 4C), with maximal CBAG-dependent stimulation obtained at an estimated ratio of 1:8 (CBAG:Hsc70). This reflects a typical physiological ratio for these components (Terada and Mori, 2000), and further

lysate (Fig. 4A, lanes 5 and 12), but only when ATP is also included in the reaction consistent with an authentic chaperone-mediated event (Fig. 4A, lanes 5 and 9). Any effect of Hsp90 is rather modest (Fig. 4A, lanes 2 and 6), and we find no indication of synergy between Hsc70-Hsp40 and Hsp90 (Fig. 4A, lanes 5 and 7). Furthermore, the purified mammalian Hsp60 complex TRiC/CCT has no effect on membrane integration (Fig. 4A, lanes 2 and 11), consistent with Hsc70-Hsp40 exerting a specific effect.

As with many reconstituted processes, we find that the purification of nascent Sec61βG chains as ribosome-bound nascent polypeptides lead to a reduction in the efficiency of N-

Fig. 4. Specific chaperones stimulate the membrane integration of TA proteins. (A) Sec61βG was released from isolated RNCs by puromycin treatment for 5 minutes in the presence of ATP (except for –ATP) and various molecular chaperones or reticulocyte lysate (RL) as shown. Samples were incubated with ER-derived microsomes (K-RM) for 30 minutes, and membrane-associated material was isolated as before. N-glycosylated material was quantified after extraction with alkaline sodium carbonate solution and standardised relative to the sample incubated with reticulocyte lysate (set to 100). Of the membrane associated products recovered, 10% were N-glycosylated for the control sample (lane 12). (B) Sec61βOPG was used to analyse the role of molecular chaperones as described in A. In this case, the membrane-associated material was analysed directly after the isolation of the membrane fraction through a high-salt sucrose cushion because a comparison with subsequent alkaline extraction revealed that the two procedures give similar results with this precursor (supplementary material Fig. S3B). Combinations of chaperones were added together with ATP and integration efficiency was analysed on the basis of N-glycosylation efficiency in four independent experiments. One such experiment is presented together with the average level of stimulation for the different treatments and the ±s.e.m. For the experiment shown, 42% of the membrane-associated products recovered were N-glycosylated when the sample was incubated with reticulocyte lysate (lane 6). **$P<0.01$ for these chaperone combinations causing a stimulation of membrane integration when compared to the control (lane 7). (C) Sec61βG was treated as described for A, except that varying concentrations of CBAG, a C-terminal fragment of Bag1, were included. N-glycosylation was used to measure membrane integration, and the values were standardised relative to those obtained with Hsp40 and Hsc70 alone.
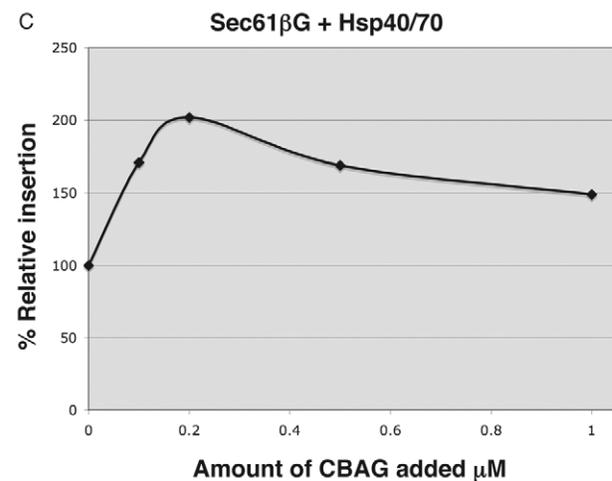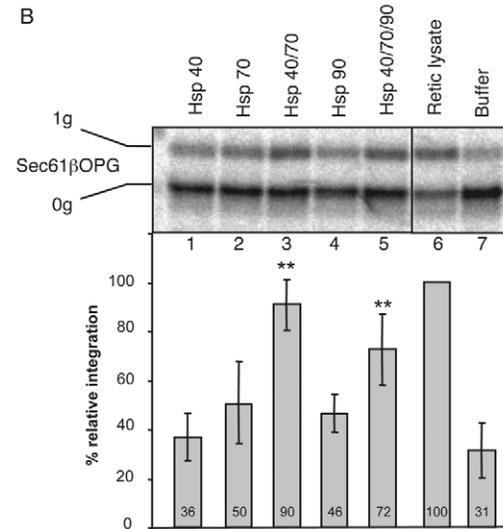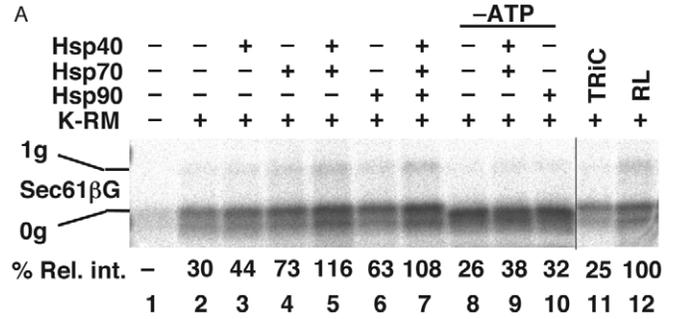
supports the proposal that the activity of the Hsc70-Hsp40 combination in our assay reflects an authentic biological chaperone function (see Takayama and Reed, 2001).

Different factors act at distinct stages during biosynthesis

We have previously suggested that the role of SRP during TA protein integration is largely restricted to a short period immediately after biosynthesis, whereas the factors responsible for the alternative, ATP-dependent route acted over a much longer period (Abell et al., 2004). Having now identified Hsc70-Hsp40 as one of the major cytosolic factors responsible for the ATP-dependent route in our in vitro system, we carried out a time-course analysis of Sec61βG integration in the presence of SRP, Hsc70-Hsp40 or complete lysate. This was achieved by releasing the nascent Sec61βG chains from the ribosome in the presence of the different factors for 5 minutes, followed by the addition of ER-derived microsomes and monitoring of membrane integration over a 30-minute period. The SRP-dependent integration of Sec61βG was complete within 5 minutes (Fig. 5A, ▲), consistent with our previous proposal that there is a very short window of opportunity during which SRP can target TA proteins (Abell et al., 2004). By contrast, the rate of integration achieved by Hsc70-Hsp40 was significantly slower, but the effect was sustained across the whole of the 30 minutes and ultimately surpassed the level supported by SRP (Fig. 5A, ■). The effect of complete cytosol, in the form of reticulocyte lysate, was quite distinct; hence, we observed a delay in integration of 5 minutes (Fig. 5A, ◇). However, after this delay the slopes of the curve describing membrane integration are entirely consistent with the

hypothesis that, even in complete lysate, any role played by SRP is restricted to a short period after the nascent TA protein is released from the ribosome. Hence, after 10 minutes it appears that any SRP-mediated membrane integration is largely complete, and subsequent integration is presumably driven largely by Hsc70-Hsp40 (Fig. 5A, ◇ and ■).

Interplay between the SRP and chaperone pathways for TA protein integration

The time-course analysis outlined above, together with our previous work (Abell et al., 2004), suggest that the stimulation of membrane integration by SRP and Hsc70-Hsp40 are

complementary. Furthermore, whereas SRP can have a very high affinity for its substrates (Flanagan et al., 2003), it is estimated to be at least 100-fold less abundant than Hsc70 in typical mammalian cytosols (Frydman et al., 1994; Siegel and Walter, 1988), raising the possibility that its availability for the post-translational route may be limited (Abell et al., 2004). To address these issues more directly, we compared the integration of Sec61βG using nucleotide-depleted reticulocyte lysate supplemented with combinations of ATP, GTP and purified SRP. Previous studies have shown that reticulocyte lysate typically contains 5 nM endogenous SRP, and that its in vitro

Fig. 5. Chaperone-mediated pathways operate in parallel with SRP-mediated targeting. (A) Sec61βG was released from isolated RNCs by puromycin treatment in the presence of reticulocyte lysate (◇), purified Hsp40 and Hsc70 with ATP (■) or purified SRP with GTP (▲). Samples were incubated with membranes for 0 to 30 minutes, membrane-associated material resistant to extraction with alkaline sodium carbonate solution was recovered as described above, and relative integration efficiency measured by N-glycosylation was compared with the value obtained with reticulocyte lysate after 30 minutes. (B) Sec61βG or Syb2G were isolated as RNCs and the polypeptides released from the ribosomes by puromycin treatment in the presence of reticulocyte lysate, or depleted reticulocyte lysate supplemented with SRP, ATP and GTP, as indicated. Samples were then incubated with membranes for up to 30 minutes and relative integration was measured by N-glycosylation as compared to the level obtained with reticulocyte lysate after 30 minutes.

effects can be artificially accentuated simply by increasing its concentration (Wolin and Walter, 1989). Hence, ~12 nM purified canine SRP was added to the reaction to significantly increase the estimated SRP concentration and to establish whether this influenced TA protein integration (Wolin and Walter, 1989).

In the case of Sec61βG, the addition of ATP resulted in a clear stimulation of membrane integration over the appropriate control (Fig. 5B, lanes 1 and 2) consistent with the use of ATP-dependent chaperones, as described above. The addition of GTP alone resulted in a more modest stimulation (Fig. 5B, lanes 1 and 6), whereas the inclusion of additional SRP enhanced this effect somewhat, consistent with SRP being limiting in this reconstituted in vitro system (Fig. 5B, lanes 4, 6 and 7). However, the effects of ATP, GTP and SRP were not additive (Fig. 5B, lanes 2, 4 and 5), suggesting that for Sec61βG there is most probably some redundancy in this in vitro system, with a fraction of chains using either the SRP-GTP-dependent, or the Hsc70-Hsp40-ATP-dependent, pathway (see Fig. 6 and Discussion).

Syb2G, a glycosylated form of Syb2 was specifically included for comparison in this analysis, because we have previously found its integration to be strongly SRP dependent (Abell et al., 2004). In this case, the ATP-mediated integration was comparatively modest, whereas the addition of SRP and GTP was almost three times more effective (Fig. 5B, combinations 1, 2 and 4). Hence, these data are consistent with a major in vitro role for SRP (Wolin and Walter, 1989) and support our previous hypothesis that the membrane integration of Syb2 is particularly SRP dependent (Abell et al., 2004). Nevertheless, the effects of ATP, GTP and SRP on membrane integration appear to be additive (Fig. 5B, combinations 2, 4 and 5), suggesting that a distinct fraction of Syb2 polypeptide chains can use the alternative, chaperone-mediated, ATP-dependent route. We conclude that the ATP- and SRP-dependent pathways for TA protein biogenesis most likely operate in parallel, and that different precursors exploit these distinct routes to varying degrees.

Discussion

The ATP-dependent stimulation of TA protein integration at the ER has been well documented but is still poorly understood (Kim et al., 1997; Kutay et al., 1995; Yabal et al., 2003). In this study, we have for the first time identified the Hsc70 chaperone system as a cytosolic factor that can facilitate this ATP-dependent pathway. Using Sec61β as a model TA precursor, we confirmed that its membrane integration is stimulated by one or more factors, present in reticulocyte lysate, that require ATP. We used a bifunctional crosslinking approach to identify candidate cytosolic factors, and confirmed our previous finding that Sec61β is a potential substrate for SRP (Abell et al., 2004). However, we now identified new interactions of Sec61β with Hsc70 (the cytosolic form of the highly conserved DnaK/Hsp70 family) and Hsp40, the cytosolic DnaJ-related co-chaperone of Hsc70. Most compelling, we show that Hsc70 functions in combination with the stimulatory co-chaperone Hsp40 to promote the ATP-dependent membrane integration of Sec61β with full efficiency when analysed in vitro. Thus, the actions of this chaperone complex conform to the established biochemical mechanisms that underlie Hsc70 functions (Mayer and Bukau, 2005; Young et al., 2004).
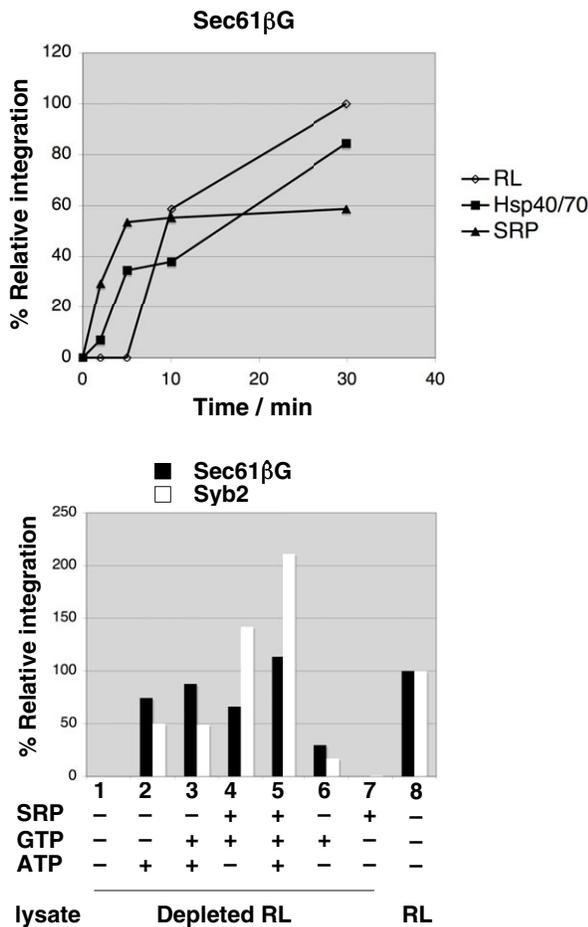
Early studies had implicated chaperones of the Hsp70-family in the post-translational translocation of the *S. cerevisiae* secretory protein pre-pro-α-factor into yeast microsomes (Chirico et al., 1988), and the post-translational integration of the M13 phage coat protein into canine pancreatic microsomes (Zimmermann et al., 1988). However, these studies left open the possibility that any role for Hsp70s was restricted to very specialised precursors and/or accentuated by the use of a heterologous model substrate. Our present study suggests that the Hsc70-mediated pathway is also used by TA proteins, a large class of proteins with a range of important cellular functions. For TA proteins, such as Sec61β and Syb2, although the Hsc70-Hsp40-mediated pathway appears to complement the recently described SRP-dependent post-translational pathway (Abell et al., 2004), it is mechanistically distinct.

We considered the possibility that the interactions of Sec61β with Hsc70 and its co-chaperone Hsp40 simply reflect the well-established binding of cytosolic molecular chaperones to many nascent polypeptides, both during and shortly after their synthesis (Frydman et al., 1994; Young et al., 2004). However, when a version of Sec61β that lacked its TA sequence was analysed, the binding of Hsc70 to the polypeptide was almost completely abolished. This strongly suggests that the hydrophobic TA sequence acts to continuously recruit Hsc70 onto the newly synthesised polypeptide chain, and that Hsc70 binding represents more than a transient interaction occurring during the folding of the soluble region of the newly made protein. A precedent for such a function exists in chloroplast targeting, where the binding of Hsp70 to the ferredoxin-NADP$^+$ reductase precursor has been shown to require an intact transit peptide (Rial et al., 2000). Such data support a model that in which various chaperones may play a key role during the translocation of precursor proteins into and across the membranes of a number of subcellular organelles (Young et al., 2003a).

In order to directly address the issue of function, we purified the nascent Sec61β chains away from cytosolic factors in the reticulocyte lysate and reconstituted membrane integration using purified components. Decisively, a combination of Hsc70 and Hsp40 could efficiently substitute for complete lysate. The amount of Hsc70 used in these experiments (1.7 µM) reflects estimates of typical Hsc70 concentrations found in reticulocyte lysate (Frydman et al., 1994; Zimmermann et al., 1988). Furthermore, the Hsc70-Hsp40-mediated stimulation of membrane integration was ATP dependent and modulated by the Hsc70 co-chaperone BAG1, which acts as a nucleotide-exchange factor and functions to modulate substrate binding and release. These features confirm that Hsc70 and Hsp40 behave as authentic molecular chaperones within the context of our in vitro system (Mayer and Bukau, 2005; Young et al., 2004). By contrast, we found that the mammalian Hsp60 chaperone TRiC-CCT
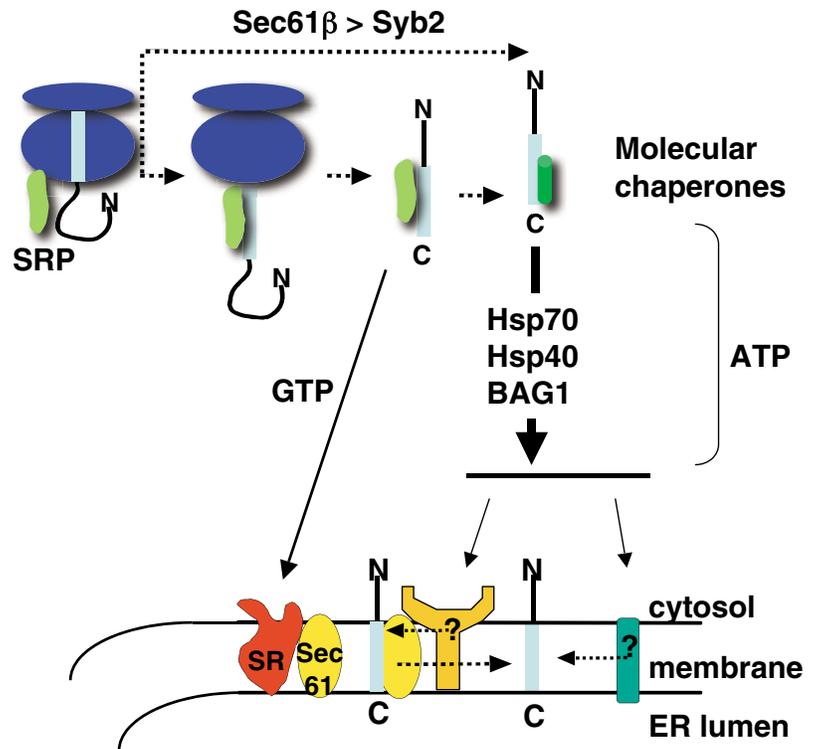

Fig. 6. A combination of SRP and molecular chaperones mediate TA protein integration. The binding of SRP to TA proteins occurs at an early stage of biosynthesis, shortly after the nascent chain is released from the ribosome. If the nascent TA protein is a poor substrate for SRP, it can use the chaperone-mediated pathway. The Hsc70-Hsp40 combination mediates the major ATP-dependent route and their activity can be modulated by co-chaperones such as BAG1. The identity of the membrane component(s) to which the molecular chaperones deliver their substrates remains unclear (High and Abell, 2004).

provides no stimulation of Sec61β integration in vitro. On this basis, we conclude that the Hsc70-Hsp40 present in the reticulocyte lysate can facilitate TA protein integration into the ER membrane (Fig. 6). Although a role for Hsp90 has been established in assisting mitochondrial import (Young et al., 2003b), a previous study of M13 phage coat protein integration found no evidence for a role in post-translational integration at the ER (Wiech et al., 1993). We find that purified Hsp90 can mediate very little, if any, stimulation of Sec61βG integration, and found no synergistic effect with the actions of Hsc70-Hsp40. Thus, any role for Hsp90s during TA protein integration remains unclear.

By comparing the kinetics of Sec61β integration in the presence of different components, we found experimental evidence to support our earlier hypothesis that the SRP-mediated pathway for TA protein biogenesis operates primarily during a short period after the release of the nascent chain from the ribosome (Abell et al., 2004). By contrast, we show that the Hsc70-Hsp40-mediated route remains active throughout the course of the experiment. When complete lysate is used to better reflect the physiological integration process for Sec61β, we find that the process appears to reflect a combination of the SRP and Hsc70-Hsp40-mediated pathways. In *S. cerevisiae*, the operation of two parallel ER-

targeting pathways is well-established, with the route taken being determined by the properties of at the signal sequence of a precursor, and a number of precursors being shown to exploit both targeting routes (Ng et al., 1996). We propose that a comparable system may operate for TA proteins and that, in this case, a combination of SRP-dependent and Hsc70-Hsp40-dependent routes are used for their delivery to the ER membrane (Fig. 6). Presumably, the relative importance of each route is also determined by the properties of the TA protein, for example the length and/or hydrophobicity of the TA sequence, although this has yet to be studied in any detail.

Our conclusion that the Hsc70 chaperone system is the primary mediator for the post-translational integration of TA proteins at the ER raises the question as to the mechanism of this function. One possibility is that Hsc70 simply maintains newly made TA proteins in an 'integration-competent' form by keeping the transmembrane domains soluble, thereby inhibiting protein aggregation. A similar mechanism has been suggested for yeast pre-pro-α-factor, where Hsc70 (Ssa1p) prevented aggregation of the precursor before translocation (Ngosuwan et al., 2003). Alternatively, or in addition to any such role, there may also be ER-specific receptors or co-chaperones that recognise the Hsc70-Hsp40-bound TA proteins. For organelles such as mitochondria and chloroplasts, there is good evidence that precursor/chaperone complexes can bind to specific receptors on the cytosolic face of the membrane (Qbadou et al., 2006; Soll and Schleiff, 2004; Young et al., 2003a). However, the identities of any membrane components that mediate the actual integration of TA proteins at the ER are poorly defined and controversial (Fig. 6). Current models range from those that suggest the process maybe entirely lipid dependent (Brambillasca et al., 2006) move through those that conclude novel integration sites may be used (Steel et al., 2002; Yabal et al., 2003), and include the possibility that the well-defined Sec61 translocon may mediate integration (Abell et al., 2003). The in vivo role of Hsc70-Hsp40 chaperones during TA protein biogenesis and the identity of any ER specific receptors for these components are a key questions for future studies.

Materials and Methods

Materials
Anti-SRP54 was a gift from B. Dobberstein (ZMBH, Heidelberg, Germany), whereas anti-Hsp40 and anti-Hsp70 antibodies were from Stressgen. Canine SRP was prepared using established protocols (Walter and Blobel, 1983b), but omitting low concentrations of the detergent Nikkol in any buffers. Canine pancreatic microsomes (Walter and Blobel, 1983a) were depleted of endogenous SRP (supplementary material Fig. S1A) by washing in high-salt buffer (Walter and Blobel, 1983b). Hsc70 was purified from bovine brain by chromatography on DEAE-cellulose, ATP-agarose and hydroxyapatite (supplementary material Fig. S2). Recombinant human Hsp40 and Hsp90 were obtained from Stressgen. The C-terminal domain of human Bag-1M [C-BAG, residues 151-264) in the vector pPROEXHTa (Invitrogen)] was expressed in BL21(DE3) *E. coli* and purified by Ni-Sepharose and Mono Q chromatography (supplementary material Fig. S2) as previously described (Sondermann et al., 2001).

Transcription
cDNAs encoding human Sec61β and rat synaptobrevin 2 were cloned in to pSPUTK (Abell et al., 2004) and transcription templates incorporating a C-terminal glycosylation tag or replacing the hydrophobic tail-anchor region were prepared by PCR using appropriate reverse primers (see supplementary material Table S1). Sec61βOPG was created in pCDNA5 (Invitrogen) by mutagenesis and the transcription template obtained by PCR from the resulting construct (supplementary

material Table S1). In all cases, the mRNAs lacked a stop codon causing the resulting polypeptides to remain associated with the ribosome after synthesis (see Fig. 1A for protein sequences). Transcripts were synthesised using SP6 or T7 RNA polymerase, according to manufacturer's instructions (New England Biolabs or Promega, respectively).

Translation and membrane insertion
Proteins were synthesised using rabbit reticulocyte lysate with incubations at 30°C in the presence of [^{35}S]-methionine, according to manufacturer's instructions (Promega). Puromycin was used at 1 mM with subsequent incubation at 30°C for 5 minutes to elicit efficient release of the stalled peptidyl-tRNAs from the ribosome (Abell et al., 2004). SRP-depleted microsomes (K-RM) were added to a final concentration of 1.5-2.0 OD_{280} per ml, and were analysed for TA protein insertion on the basis of relative N-glycosylation efficiency following recovery by centrifugation through 100 μl HSC (500 mM sucrose, 500 mM KOAc, 5 mM $Mg(OAc)_2$, 50 mM Hepes-KOH pH 7.9) at 100,000 *g* for 10 minutes or 132,000 *g* for 5 minutes. Where indicated, the resulting membrane pellet was resuspended in 100 μl of cold 0.1 M Na_2CO_3, incubated on ice for 10 minutes and recovered by centrifugation at 132,000 *g* for 5 minutes to confirm membrane integration. De-glycosylation was performed with endoglycosidase H (EndoH) according to manufacturer's instructions (New England Biolabs).

Nucleotide depletion
Reticulocyte lysate was depleted of nucleotides by loading 70 μl onto a Biospin 6 column (Bio-Rad) equilibrated with LSC buffer (100 mM sucrose, 100 mM KOAc, 5 mM $Mg(OAc)_2$, 50 mM Hepes-KOH pH 7.9, 1 mM DTT), following manufacturer's instructions, repeating the process once. A parallel depletion using a translation of Syb2 showed a 49% recovery rate and a double volume of depleted lysate was used for comparative experiments with non-depleted lysate.

Crosslinking and immunoprecipitation
Following puromycin treatment, translation products were treated with 1 μg of apyrase per 40 μl volume for 5 minutes at 30°C, then incubated on ice for 5 minutes followed by incubation at 30°C for 5 minutes with either 1 mM disuccinimidyl suberate (DSS; Pierce), 1 mM succinimidyl *trans*-4-(maleimidylmethyl) cyclohexane-1-carboxylate (SMCC; Pierce) or bismaleimidohexane (BMH; Pierce) diluted from a 20 mM stock in DMSO. Crosslinking was stopped with 50 mM glycine (DSS), 10 mM 2-mercaptoethanol (BMH) or both (SMCC). Samples were denatured with SDS unless otherwise stated; specific adducts were recovered by immunoprecipitation (Abell et al., 2003).

Reconstitution of ER integration
Ribosome–nascent-chain complexes (RNCs) were generated by translating transcripts lacking a stop codon for 7 minutes. Reactions of 200 μl were supplemented with 2.5 mM cycloheximide and 500 mM KOAc, and the final 240 μl sample was layered over 400 μl HSCC (HSC with 2.5 mM cycloheximide and 1 mM DTT), followed by centrifugation at 213,000 *g* for 20 minutes. The pellet was resuspended in 50 μl HSCC with reduced sucrose (100 mM), layered onto 150 μl HSCC, and centrifuged at 213,000 *g* for 20 minutes. The pellet was finally resuspended in 40 μl LSC. Membrane-insertion reactions comprised 2 μl of isolated RNCs made up to a final volume of 10 μl by LSC and various additions. Hsp40 was added at 3 μM, Hsc70 was added at 1.7 μM, Hsp90 was added at 1.3 μM, TRiC (gift from Judith Frydman, James Clark Center, Stanford University, CA) was added at 0.6 μM, SRP was added at ~12.5 nM, prespun reticulocyte lysate was added at 20% v/v, and depleted lysate was added at 40% v/v. ATP or GTP was added at 1 mM. Following the addition of all cytosolic targeting factors and treatments, puromycin was added at 1 mM and the sample incubated for 5 minutes at 30°C. Membrane insertion was achieved by incubation with K-RMs (final concentration of 1.5-2.0 OD_{280} per ml) at 30°C.

Gel electrophoresis
Samples were heated to 70°C for 10 minutes in SDS-PAGE sample buffer and then resolved on 16% polyacrylamide Tris-glycine gels under denaturing conditions. Gels were fixed, dried and then exposed to phosphorimage plates, which were read using a Fuji BAS-3000 phosphorimager. Radiolabelled products separated by SDS-PAGE were quantified using Aida software.

Note added in proof

While this work was under review, the Asna-1 protein was independently identified as a cytosolic ATPase that can promote the membrane insertion of TA proteins (Stefanovic and Hegde, 2007). We therefore conclude that the ATP-dependent integration of TA proteins at the ER is most probably complex and multifaceted.

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