Translocation of the C Terminus of a Tail-anchored Protein across the Endoplasmic Reticulum Membrane in Yeast Mutants Defective in Signal Peptide-driven Translocation*

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C-tail-anchored proteins are defined by an N-terminal cytosolic domain followed by a transmembrane anchor close to the C terminus. Their extreme C-terminal polar residues are translocated across membranes by poorly understood post-translational mechanism(s). Here we have used the yeast system to study translocation of the C terminus of a tagged form of mammalian cytochrome \( b_5 \), carrying an N-glycosylation site in its C-terminal domain \((b_5\text{-Gnglc})\). Utilization of this site was adopted as a rigorous criterion for translocation across the ER membrane of yeast wild-type and mutant cells. The C terminus of \( b_5\text{-Gnglc} \) was rapidly glycosylated in mutants where Sec61p was defective and incapable of translocating carboxypeptidase Y, a well known substrate for post-translational translocation. Likewise, inactivation of several other components of the translocon machinery had no effect on \( b_5\text{-Gnglc} \) translocation. The kinetics of translocation were faster for \( b_5\text{-Gnglc} \) than for a signal peptide-containing reporter. Depletion of the cellular ATP pool to a level that retarded Sec61p-dependent post-translational translocation still allowed translocation of \( b_5\text{-Gnglc} \). Similarly, only low ATP concentrations (below 1 \( \mu M \)), in addition to cytosolic protein(s), were required for in vitro translocation of \( b_5\text{-Gnglc} \) into mammalian microsomes. Thus, translocation of tail-anchored \( b_5\text{-Gnglc} \) proceeds by a mechanism different from that of signal peptide-driven post-translational translocation.

C-tail-anchored proteins (TA\(^2\) proteins) constitute a class of integral membrane proteins that are held in the phospholipid bilayer by a single segment of hydrophobic amino acids close to the C terminus, the entire functional N-terminal portion facing the cytosol. A large number of TA proteins have been described in animal, plant, and fungal cells, where their roles range from regulation of apoptosis to enzyme catalysis, translocation of newly synthesized polypeptides across membranes, vesicular traffic (see Refs. 1 and 2 for reviews), and control of gene expression (3). Consistent with these different functions, TA proteins are found on a variety of membranes, such as those of the endoplasmic reticulum (ER) and of the Golgi complex, the plasma membrane and the mitochondrial outer membrane. Mitochondrial targeting occurs directly from the cytosol (4), whereas TA proteins located in membranes of the exo- and endocytic pathways are first inserted into the ER and then transported to their destinations by membrane traffic (5–8).

A point that remained controversial for many years was whether TA proteins are bona fide transmembrane proteins, with the C-terminal residues exposed at the exoplasmic side of the membrane, or rather have a hairpin configuration with the hydrophobic stretch looping back to the cytosolic face of the bilayer. The difficulty in distinguishing between these alternatives was because of the small number of polar residues downstream to the hydrophobic domain, so that traditional approaches, such as protection from proteolysis or accessibility to antibodies, did not yield clear results (see Ref. 1). In the past few years, however, the use of recombinant proteins with N-glycosylation sites appended at the C terminus has allowed unequivocal demonstration that TA proteins can acquire transmembrane topology by translocating their C terminus across the ER membrane (6, 9–11). Using glycosylation as a criterion for translocation, a variety of different C-terminal sequences, deriving from cytosolic or exoplasmic portions of different proteins, have been shown to be translocated, indicating that the translocation process is not very selective with regard to the residues that are transferred to the ER lumen.

The transmembrane segment of TA proteins emerges from the ribosome only upon completion of translation; thus, translocation of the C terminus must be post-translational, as has been directly demonstrated (6, 11). However, the molecular mechanism underlying this process has not been clarified so far. Studies addressing this question have employed in vitro systems in which tight (alkaline pH-resistant) association of the protein under study to added membranes or proteoliposomes was followed (6, 12, 13). However, tight binding of TA proteins to lipid bilayers without translocation of the C terminus can occur in artificial systems, as has been demonstrated for cytochrome \( b_5 \) (14, 15).

The Sec61 translocon has been proposed as the most likely
likely without the participation of the Sec61 complex. In this study, we were able to functionally reconstitute translocation across the ER membrane in vivo and in vitro, using a yeast system. The Sec61 complex is an essential component of the ER translocon, which is responsible for the translocation of proteins across the ER membrane.

In the yeast system, the Sec61 complex is shown to be involved in the translocation of the C-terminal tail of TA proteins. This is consistent with previous studies that have shown a requirement for the Sec61 complex in the translocation of certain membrane proteins.

In summary, our results suggest that the Sec61 complex is involved in the translocation of the C-terminal tail of TA proteins. This finding is consistent with previous studies that have shown a requirement for the Sec61 complex in the translocation of certain membrane proteins.
ER Translocation of the C Terminus of Tail-anchored Proteins

ER Targeting and N-Glycosylation of b5-Nglyc in Yeast—A cDNA coding for a mammalian cytochrome b5 variant with an opsin tag and a consensus site for N-glycosylation close to the C terminus (designated b5-Nglyc; see Ref. 11 and Fig. 1A) was placed under the glucose-regulated SUC2 promoter and integrated into the genome of a yeast strain with normal Sec61p function (H1415; see Table I for yeast strains) and of the temperature-sensitive mutant sec61-3 (H1399). In the latter cells, the channel protein Sec61p of the Sec61 translocon is defective at 37°C. After induction of expression of b5-Nglyc by incubation of the cells in glucose-deficient, raffinose-containing medium for an hour at the permissive temperature (24°C), the cells were lysed and subjected to SDS-PAGE followed by Western blotting and detection of the recombinant protein with anti-opsin mAb. Parallel samples were digested with Endo H to remove expression of b5-Nglyc and lysed. One half of the samples was subjected directly to SDS-PAGE (lanes 1 and 3) and the other after Endo H digestion (lanes 2 and 4). Western blotting was performed with anti-opsin mAbs. 25- and 20-kDa forms of b5-Nglyc are indicated on the right.

ta Cruz Biotechnology). Transmission electron microscopy was performed as previously described (41).

RESULTS

ER Targeting and N-Glycosylation of b5-Nglyc—A cDNA coding for a mammalian cytochrome b5 variant with an opsin tag and a consensus site for N-glycosylation close to the C terminus (designated b5-Nglyc; see Ref. 11 and Fig. 1A) was placed under the glucose-regulated SUC2 promoter and integrated into the genome of a yeast strain with normal Sec61p function (H1415; see Table I for yeast strains) and of the temperature-sensitive mutant sec61-3 (H1399). In the latter cells, the channel protein Sec61p of the Sec61 translocon is defective at 37°C. After induction of expression of b5-Nglyc by incubation of the cells in glucose-deficient, raffinose-containing medium for an hour at the permissive temperature (24°C), the cells were lysed and subjected to SDS-PAGE followed by Western blotting and detection of the recombinant protein with anti-opsin mAb. Parallel samples were digested with Endo H to remove N-glycans prior to gel electrophoresis. In the normal (Fig. 1B, lane 1), as well as in the mutant (lane 3) cells, −25 and −20-kDa proteins were detected in the absence of Endo H treatment, whereas after digestion only the −20-kDa protein was detected (lanes 2 and 4). The parental strains lacking the recombinant gene revealed no signal (not shown). Thus, the C-terminal domain of most of the b5-Nglyc molecules had been translocated into the ER lumen and glycosylated.

ER localization was confirmed by indirect immunofluorescent staining. In cells with normal Sec61p (H1415), induced to express b5-Nglyc, staining for the recombinant protein (Fig. 2a and d) was visible in the perinuclear and peripheral regions, which correspond to the perinuclear and cortical ER, respectively (42). No staining was present in vacuoles, revealed by differential interference contrast optics (panel b). Co-staining of the sample of panel d with anti-porin antibodies resulted in a pattern typical for mitochondria (panel c), clearly different from that obtained with the anti-opsin mAbs, confirming that most of the b5-Nglyc was specifically targeted to the ER as occurs in mammalian cells (4, 11).

The yeast ER has been shown to proliferate up to 20 pairs of stacked membranes encircling the nucleus (so-called karmelae) upon overexpression for 24 h of rat cytochrome b5 (43). Therefore, we checked by electron microscopy whether the ER of our b5-Nglyc expressing cells was normal or underwent a similar proliferation. Comparison of Fig. 3, A and B shows that after 2 h of induction, within the time window of our biochemical experiments (panel B), the cortical and perinuclear components of the ER appeared indistinguishable from those of uninduced cells (panel A). After 6 h of induction, some proliferation of the ER appeared to have started (Fig. 3C), whereas after 22 h membrane stacks similar to those described by Vergères et al. (43) were clearly visible (Fig. 3D).

Translocation of the C Terminus of de Novo Synthesized b5-Nglyc across the ER Membrane of Translocation-deficient Mutants—Next we studied whether ER translocation of newly synthesized b5-Nglyc was blocked when the Sec61 channel was inactivated for signal peptide-driven translocation at 37°C in the sec61-3 mutant (H1399). Sec61-3 cells were preincubated for 30 min in 2% raffinose to induce b5-Nglyc expression, either at the restrictive or at the permissive temperature, then labeled with [35S]met/eye for 5 min, and chased for 20 min. At both temperatures the major immunoprecipitated form of b5-Nglyc corresponded to the −25-kDa glycosylated form (Fig. 4A, lanes 1 and 3). When the incubation at the restrictive temperature was performed in the presence of TM to inhibit N-glycosylation (Fig. 4A, lane 2), only the −20-kDa polypeptide could be immunoprecipitated, confirming that the band at 25 kDa corresponds to the N-glycosylated form of b5-Nglyc. When this experiment was repeated in a sec61-41 mutant (strain H1417, Table I), which is cold-sensitive for translocation (34), similar results, showing efficient glycosylation of b5-Nglyc, were obtained both at 17 and 24°C (data not shown). When b5-Nglyc was expressed in a sbh1 strain (H1520) lacking the Sec61 translocon component Sbh1p, it again was glycosylated, as well as in a sbh1 sbh2 double deletant that also lacked Sbh2p, a component of the alternate Ssh1 translocon complex (data not shown).

The Sec63 complex (Sec62p, Sec63p, Sec71p plus Sec72p) and the luminal chaperones Kar2p/BiP and Lhs1p are required for signal peptide-driven post-translational ER translocation (19, 25, 44, 45). Therefore, we integrated our b5-Nglyc cDNA under the SUC2 promoter into the genome of yeast strains harboring temperature-sensitive mutations in the SEC63, SEC62, or KAR2 genes and of a strain lacking the LHS1 gene (37). A pulse-chase experiment carried out with a temperature-sensitive sec63-1 mutant showed that b5-Nglyc was again translocated equally efficiently at the restrictive and permissive temperatures (Fig. 4A, lanes 4–6). Similar results were obtained for the temperature-sensitive kar2Δ159 (H1424) and lhs1Δ (H1425) mutants (data not shown).

Inactivation of the Sec61 channel is lethal, whereas inactivation of proteins required for signal peptide-driven post-translational translocation only, such as Sec63p or Sec62p, can be tolerated (32). The results on the sec63-1 mutant of Fig. 4A were confirmed by using constitutive sec63-201 (H1475) and sec62-101 (H1474) mutants. Again, the mutations did not prevent translocation of the C terminus of b5-Nglyc (Fig. 4B, lanes 1–4).

Comparison of Post-translational Translocation of b5-Nglyc and Carboxypeptidase Y (CPY)—To study whether the observed glycosylation of b5-Nglyc in the translocation-deficient mutants could be explained by leakage of the mutants, we compared the fate of b5-Nglyc to that of an internal control, the vacuolar protease CPY, a protein that is post-translational translocated in a Sec61/62/63-dependent manner (32) as demonstrated before (46) and shown here for reference, in normal cells CPY is rapidly translocated into the ER, appearing after a
of the cells of mitochondria (arrows) indicate the cortical and perinuclear ER (arrowheads). In panel b, the differential interference contrast image of the cells of panel a is shown.

5 min pulse of radioactive amino acids mainly as a primary N-glycosylated 67-kDa form with a minute portion present as the cytosolic, unglycosylated, 59-kDa polypeptide (Fig. 5A, lane 7). Thereafter, the glycosylated form is transported via the Golgi, where the glycan extension causes the apparent molecular mass to increase to 69 kDa (lane 8), to the vacuole where removal of the pro-peptide results in the mature 61-kDa form (lane 9).

In the constitutive sec62–101 and sec62–201 mutants, it has previously been demonstrated that CPY accumulates in the cytosolic form (32). Here, we have compared the fates of β7-Nglyc and CPY in the conditional translocation-defective mutants, sec61–3 and sec63–1. After preincubation and pulse-labeling for 5 min at the restrictive temperature, one half of each lysate was exposed to anti-opsin mAbs to immunoprecipitate β7-Nglyc (Fig. 5A), while CPY was immunoprecipitated from the other half (Fig. 5B). In sec63–1 (lane 2) and sec61–3 (lane 3) cells, which were induced for β7-Nglyc expression, CPY occurred mostly as the untranslocated 59-kDa form, as in a sec63–1 strain lacking the recombinant gene (lane 1). Similar results were obtained for these same strains under conditions where β7-Nglyc expression was not induced (lanes 4–6). In contrast, β7-Nglyc was glycosylated in sec63–1 (Fig. 5B, lane 2) and sec61–3 (lane 3) cells. No signal was obtained in the sec63–1 strain lacking the recombinant gene (lane 1) or in any of the three strains when induction in low glucose was omitted (lanes 4–6). We conclude that under conditions in which prepro-CPY remained in the cytosol because of a non-functional translocon channel or Sec63p, the C terminus of β7-Nglyc was rapidly translocated into the ER.

Kinetics of Translocation of β7-Nglyc and HSPΔ-β-Lactamase—We have previously shown that the β-lactamase portion of a fusion protein, Hisp150Δ-β-lactamase, folds in the cytosol to a native-like conformation and is then translocated into the ER. Penetration across the Sec61 translocon requires unfolding, and this apparently takes time because the translocation rate is slow, occurring with a half time of about 5 min (38, 47)

**Table I**

**Yeast strains constructed for this study**

| Strain       | Genotype                                      |
|--------------|-----------------------------------------------|
| H823         | MATa sec23-1 leu2-3,112 ura3-52 URA3::Hsp150Δ-β-lactamase URA3::loxP-kanMX-loxP |
| H996         | MATa sec18-1 trp1-289 ura3-52 LEU2::Hsp150Δ-β-lactamase |
| H1415        | MATa can-100 leu2-3,112 his3-11,15 trp1-1 ade2-1 URA3::β7-Nglyc |
| H1417        | MATa sec61::His3 sec61-1 can-1-100 leu2-3,112 his3-11,15 trp1-1 ade2-1 URA3::β7-Nglyc |
| H1424        | MATa bar2-159 ura3-52 leu2-3,112 LEU2::β7-Nglyc |
| H1425        | MATa sec23-1 leu2-3,112 ura3-52 URA3::loxP-kanMX-loxP URA3::Hsp150Δ-β-lactamase LEU2::β7-Nglyc |
| H1474        | MATa sec62-101 ura399 leu2-1 trp99 ade2-101ochre LEU2::β7-Nglyc |
| H1475        | MATa sec63-201 ura399 leu2-1 trp99 ade2-101ochre LEU2::β7-Nglyc |
| H1520        | MATa leu2-3,112 ura3-52 ssh1::URA3 LEU2::β7-Nglyc |
| H1521        | MATa leu2-3,112 ura3-52 ssh1::URA3 ssh2::G418 LEU2::β7-Nglyc |
| H1641        | MATa sec18-1 trp1-289 ura3-52 LEU2::Hsp150Δ-β-lactamase URA3::β7-Nglyc |

**Fig. 2. Immunofluorescent staining of β7-Nglyc-expressing cells.** Control cells (H1415) were induced in 2% raffinose for 3 h at 24 °C and stained with anti-opsin mAbs (a) to visualize the recombinant protein or doubly immunostained with anti-opsin mAbs and antiporin antibodies to compare the distribution of β7-Nglyc with that of mitochondria (c and d). Panels c and d show the same field of cells viewed with the rhodamine filter for the anti-porin antibodies and under the fluorescein isothiocyanate filter for the anti-opsin antibodies, respectively. Arrows indicate the cortical and perinuclear ER (a and d) and mitochondria (c). In panel b, the differential interference contrast image of the cells of panel a is shown.

**Fig. 3. Ultrastructure of the ER in cells induced or not induced to express β7-Nglyc.** Yeast cells (H1415) were fixed and processed for transmission electron microscopy before induction (A) or after 2 (B), 6 (C), or 22 h (D) of induction of β7-Nglyc expression with 2% raffinose at 30 °C. Panel D shows two different images illustrating formation of karmellae (arrowheads). ER, endoplasmic reticulum. N, nucleus. V, vacuole. Bar, 0.5 m.
Because the catalytic N-terminal portions of TA proteins also fold in the cytosol, we compared the translocation kinetics of Hsp150Δ-β-lactamase and b$_{5}$-Nglyc. Both proteins were expressed in a sec18–1 mutant where translocated proteins are prevented from escaping the pre-Golgi compartment at 37 °C, allowing facile comparison of the cytosolic and ER forms. After induction of b$_{5}$-Nglyc expression at the restrictive temperature and pulse-labeling with [35S]met/cys, immunoprecipitation of half of the cell lysates with β-lactamase antisem showed that most of the Hsp150Δ-β-lactamase was in its non-glycosylated cytosolic form of 66 kDa and some in the primary glycosylated ER form of 110 kDa (Fig. 6A, lane 1). After a 10-min chase, most of the protein was in the ER form (lane 2). In contrast, and in agreement with the results of Fig. 5, immunoprecipitation of the other half of the lysates with anti-opsin mAb showed that almost all of the b$_{5}$-Nglyc was already in the ER form at the end of the pulse (Fig. 6A, lanes 3 and 4). The rapid rate of glycosylation of b$_{5}$-Nglyc in comparison to Hsp150Δ-β-lactamase suggests that its translocation is not limited by a folding/unfolding process.

Translocation of Hsp150Δ-β-lactamase is slowed under high cell density conditions, apparently because of glucose limitation that results in reduction of the ATP pool (38). To compare the energy requirements of b$_{5}$-Nglyc and Hsp150Δ-β-lactamase, the above pulse-chase experiment was repeated in high cell density conditions. After the pulse, only the cytosolic form of Hsp150Δ-β-lactamase could be detected (Fig. 6B, lane 1), and after a 10-min chase most of the protein still was in the cytosolic form (lane 2). In contrast, growth under high cell density conditions had no effect on the glycosylation of b$_{5}$-Nglyc (lanes 3 and 4). Thus, the TA protein had lower energy requirements for translocation than signal peptide-containing Hsp150Δ-β-lactamase.

**ATP and Cytosolic Protein Requirement for b$_{5}$-Nglyc Translocation into Mammalian Microsomes**—We further investigated the energy requirements for post-translational translocation of b$_{5}$-Nglyc in the mammalian reticulocyte lysate cell-free system supplemented with dog pancreas microsomes (DPMs). As shown in Fig. 7A, lane 1, when in vitro-translated b$_{5}$-Nglyc was incubated with DPMs about half of the radioactively labeled protein was glycosylated, as indicated by the presence of the ~25-kDa band, in agreement with our previous results (4, 11). However, if the sample was exposed to apyrase to hydrolyze ATP before incubation with membranes, post-translational glycosylation did not occur (Fig. 7A, lane 2). To
more precisely define the ATP requirement, we used the hexokinase/glucose trap as an alternative procedure for energy depletion. Samples were preincubated for 10 min with 30 mM glucose and varying concentrations of hexokinase to obtain different degrees of ATP depletion. ATP levels obtained after the preincubation were monitored directly with the luciferin-luciferase assay and are reported below the lanes of Fig. 7B. As shown in panel B, lanes 1–4, if hexokinase/glucose treatment was carried out before translation, production of b$_2$-N glyc was already reduced with the lowest concentration of hexokinase (0.4 μM/ml) and was completely abolished at higher concentrations. In contrast, when the energy-depleting treatment was carried out after translation but before addition of DPMs, glycosylation occurred at all concentrations of hexokinase (Fig. 7B, lanes 5–8). Significant glycosylation, albeit with somewhat reduced efficiency, also occurred with the highest amount of enzyme (25 μM/ml, lane 8), resulting in an ATP concentration as low as 0.2 μM at the start of the post-translational incubation with DPMs. This ATP concentration was totally non-permissive for protein synthesis (Fig. 7B, lanes 1–4) and was about 4 orders of magnitude lower than that of intracellular ATP under physiological conditions.

It has been reported that binding of another TA protein, VAMP-1B, to mitochondrial outer membranes can occur without ATP if cytosolic chaperones are absent (48). We tested a possible role of chaperones in our system by comparing the glycosylation of b$_2$-N glyc obtained in an undiluted translated sample (Fig. 7C, lane 1) with that obtained after dilution of the sample in lysate (Fig. 7C, lanes 2 and 3) or in buffer (lanes 4 and 5). The concentrations of ions, nucleotides, and components of the energy-regenerating system (creatine phosphate and creatine kinase) were held constant, and all samples received the same amount of DPMs. As shown in Fig. 7C, both with a 1:5 and a 1:10 dilution of the lysate in buffer (lanes 4 and 5), glycosylation of b$_2$-N glyc was severely impaired, indicating that its delivery to ER membranes in a translocation-competent form requires at least one cytosolic protein.

**DISCUSSION**

In recent years it has become clear that TA proteins can translocate their C-terminal residues across the ER membrane. However, the mechanism of membrane penetration has remained unsolved. The transmembrane domains (TMD) of TA proteins resemble classic signal-anchor sequences of type II monotopic proteins, with the difference that they emerge from the ribosome only upon termination of translation and thus must interact with the target membrane post-translationally. Because the Sec61 translocon can operate in both co- and post-translational modes (19), it seemed plausible that it could be involved in translocation of TA protein tails as well, as proposed by Tyedmers et al. (16) and Wattenberg and Isenmann (17). This hypothesis was supported by the observations that (i) TA proteins localized to compartments of the secretory pathway must first insert into the ER (5–8) and (ii) glycosylation sites at the C terminus of TA proteins are good substrates for oligosaccharyltransferase (6, 9–11), an enzyme complex that is closely associated with the translocon (49). The finding that another translocon-associated enzyme, signal peptidase, can cleave a modified TA protein further strengthened this idea (18). The translocon hypothesis was, however, not supported by data obtained from in vitro binding assays that showed that carbonate-resistant binding of synaptobrevin to mammalian or yeast microsomes can occur in the absence of Sec61p function (6, 13). In these in vitro experiments, however, no evidence for translocation of the C terminus of synaptobrevin was presented.

In the present study we have employed a rigorous assay for translocation, based on utilization of an N-glycosylation consensus sequence engineered to the C terminus of mammalian cytochrome b$_6$ (b$_6$-N glyc; see scheme in Fig. 1). We have taken advantage of the yeast system to assess in ultrastructurally normal living cells a possible role of the Sec61 translocation machinery in the translocation of the C terminus of b$_6$-N glyc. The large number of mutant yeast strains utilized here included conditional and constitutive mutants or knock-out strains defective in the translocon protein Sec61p (sec61p [sec61–1] or accessory proteins Sbh1p [sbh1] and Sec62p [sec62–101], in Sbh2p [sbh2], a component of an alternate yeast translocon), in the ER lumenal chaperones Bip/Kar2p (kar2–159) and Lha1p (lha1), and in the co-chaperone Sec63p (sec63–1 and sec63–202). None of these mutations or deletions had detectable effects on the translocation of the tail of b$_6$-N glyc, which was rapidly glycosylated in all the strains at permissive and restrictive temperatures, under conditions in which translocation of CPY was abolished. CPY harbors a signal peptide driving post-translational translocation (32) and was used as an internal control to monitor eventual leakage of the mutants.
The most likely interpretation of these results is that translocation of TA protein tails occurs by a mechanism not involving the Sec61 (or the alternate Shs1p) translocon machinery. However, another possibility is that the tails are inserted into the ER membrane via the Sec61 pore but using a novel function of Sec61p that is not compromised by the sec61Δ-3 and sec61Δ-41 mutations. Indeed, Sec61 appears to be a multifunctional protein. So far, three activities have been assigned to it: the two types of signal peptide-dependent ER entry and dislocation by which short-lived or misfolded proteins are translocated from the ER lumen back to the cytosol for ubiquitinylation and proteasomal degradation (20, 50). The proteins to be dislocated differ markedly from those entering the ER lumen because they have lost the signal peptide and undergone co- and post-translational modifications and folding. Accordingly, distinct TMDs of Sec61p appear to operate in ER entry and dislocation. Deletion analysis showed that TMD2 has specific functions in post-translational translocation, whereas TMD3 is needed for efficient dislocation (51). It is interesting to note that the sec61Δ-41 mutation (V134I), which did not affect ER insertion of b5-Nglyc, resides in TMD3. It blocks entry at low temperature and dislocation at all temperatures (34). Thus, should the Sec61 complex have a role in TA protein translocation, it must be independent from the known entry and dislocation functions.

In addition to investigating whether translocon components are required for TA protein insertion, in the present study we have also analyzed the energy requirements of the process. On this question, previous studies with in vitro binding assays have yielded diverging results. Whereas there is general agreement that synaptobrevin (VAMP) requires ATP for membrane association (6, 12), other TA proteins seem to have either lower energy requirements (e.g. bcl-2 and Nyv1p) or none at all (cytochrome b5) (12, 13). Moreover, in the case of the mitochondrial outer membrane isoform of synaptobrevin, VAMP-1B, the need for energy was found to be related to chaperone function. In the absence of cytosolic proteins, VAMP-1B could bind tightly to added mitochondria in the absence of ATP (48).

We first examined b5-Nglyc translocation in yeast cells grown at high density, a condition that results in partial energy depletion (38, 47), and found that its glycosylation was not affected, whereas translocation of Escherichia coli β-lactamase, expressed in S. cerevisiae as a fusion protein with a post-translationally operating signal peptide, was drastically slowed. We have previously demonstrated that the β-lactamase fusion protein folds to a native-like protease-resistant and catalytically active conformation in the cytosol, is then unfolded, and is thereafter translocated (38, 47). Its slow and energy-sensitive rate of translocation may be because of the time required for the folding/unfolding process. The catalytic domain of b5 also folds in the cytosol, but the rapid rate of glycosylation observed here suggests that the conformation of the N-terminal domain does not influence the translocation of the C-terminal region.

To better define the energy requirements for translocation of the tail of b5-Nglyc, we turned to a mammalian in vitro translocation-translocation system. N-glycosylation in cell-free systems is based on the utilization of preassembled oligosaccharidol dolichol molecules and thus does not in itself require energy. Therefore, this post-translational modification could be used to report on translocation of the tail of b5-Nglyc, also under conditions of energy depletion. Complete depletion of ATP with apyrase resulted in failure of translocation of b5-Nglyc into dog pancreas microsomes. This is in agreement with what has been reported for a synaptobrevin variant carrying an N-glycosylation consensus close to the C terminus (6) but contrasts with the widely believed notion that cytochrome b5 does not require energy for membrane insertion (12) and illustrates how in vitro binding data must be interpreted with caution. When ATP depletion was achieved in a more controlled fashion using a hexokinase/glucose trap, we observed that translocation of b5-Nglyc occurred even at extremely low concentrations of ATP, in agreement with the results we obtained in vitro in yeast. We also found that dilution of the lysate severely impaired glycosylation of b5-Nglyc, demonstrating for the first time the involvement of cytosolic proteins in b5 membrane insertion. Taken together, the in vitro experiments suggest that cytosolic chaperones for may be needed for delivery of b5 and other TA proteins to the ER in a translocation-competent form and that the ATP requirement may be related to chaperone function. However, we cannot exclude that ATP also has a direct function in translocation.

In conclusion, our results based on in vitro glycosylation of the C terminus of a cytochrome b5 construct in yeast mutants, combined with in vitro experiments in the mammalian system, demonstrate that post-translational translocation of a TA protein can occur by a mechanism distinct from that of signal peptide-dependent translocation, characterized by fast kinetics, low energy requirements, and the participation of at least one cytosolic protein. The obvious question now is what molecular functions are involved in this alternative translocation process, whether only lipids, protein(s), or both. TA proteins are targeted specifically from the cytosol to ER or mitochondrial outer membranes, the specificity depending on residues adjacent to the TMD as well as on features of the TMD itself (4, 52–54). This specificity argues in favor of proteins playing a role in the targeting process. Consistently, a proteinaceous receptor has been implicated in the binding of synaptobrevin and Nyv1p to microsomal membranes (6, 12, 13). However, once a TA protein is delivered to its target membrane, it is conceivable that its translocation could occur directly across the lipid bilayer without the aid of proteins. A combined effort, involving yeast genetics and biochemistry, will hopefully lead to the elucidation of the molecular details of this novel translocation process.

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