Biogenesis of tail-anchored proteins

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Abstract
A group of integral membrane proteins, known as C-tail anchored, is defined by the presence of a cytosolic N-terminal domain that is anchored to the phospholipid bilayer by a single segment of hydrophobic amino acids close to the C-terminus. The mode of insertion into membranes of these proteins, many of which play key roles in fundamental intracellular processes, is obligatorily post-translational, is highly specific and may be subject to regulatory processes that modulate the protein’s function. Recent work has demonstrated that tail-anchored proteins translocate their C-terminals across the endoplasmic reticulum membrane by a mechanism different from that used for Sec61-dependent post-translational signal-peptide-driven translocation. Here we summarize recent results on the insertion of tail-anchored proteins and discuss possible mechanisms that could be involved.

Introduction
A group of integral membrane proteins, known as TA (C-tail anchored), is defined by the presence of a cytosolic N-terminal domain that is anchored to the phospholipid bilayer by a single segment of hydrophobic amino acids close to the C-terminus (Figure 1). TA proteins carry out their functions at the cytosolic face of essentially all membranes, and many of them are key players in fundamental cell biological phenomena. For example, processes as important as targeted vesicle fusion and regulation of apoptosis involve TA proteins, the SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors; reviewed in [1]) and Bcl-2 family members (reviewed in [2]) respectively. Other examples are offered by adaptor proteins, tethering proteins, components of the translocation machinery of the ER (endoplasmic reticulum), of the MOM (mitochondrial outer membrane) and of peroxisomes, as well as numerous enzymes and viral proteins (reviewed in [3]). For all these different proteins, localization on a precise intracellular membrane or set of membranes is clearly a prerequisite for their function. Thus understanding the specific targeting, membrane insertion and intracellular trafficking of these proteins has wide implications for cell biology.

TA proteins do not carry an N-terminal signal peptide, and the membrane-anchoring segment is so close to the C-terminus that it emerges from the ribosome only upon termination of translation. Therefore, the Signal Recognition Particle is not involved in the targeting of these proteins, and their insertion into membranes occurs post-translationally. Once released from the ribosome, they are specifically targeted to a limited subset of membranes (the ER and MOM, and, in plants, presumably the chloroplast envelope) and translocate their C-terminus across the bilayer. From the ER, they may traffic further to downstream compartments of the secretory pathway. The targeting and intracellular transport pathways of TA proteins are summarized in Figure 2, and have recently been reviewed [3]. In this article, we will focus on one important aspect of TA protein biogenesis: the mechanism of translocation of the C-terminus across the ER membrane.

TA proteins translocate their C-terminus across the ER membrane by a mechanism distinct from the one used in signal-peptide-driven translocation
The Sec61 machinery is the only protein-translocation system characterized in the ER so far (Figure 3A). It is composed of the heterotrimeric Sec61 complex, consisting of the channel protein Sec61p together with two other proteins (Sbh1p and Sss1p in Saccharomyces cerevisiae, and Sec61β and Sec61γ in mammalian cells). The Sec61 complex operates as the channel in co- and post-translational translocation of signal-peptide-bearing, soluble and membrane-anchored polypeptides (reviewed in [4]). For post-translational translocation in yeast, the Sec61 complex interacts with the accessory proteins Sec62/63p and Sec71/72p, and requires co-operation with the luminal ER chaperone BiP (Ig heavy-chain-binding protein)/Kar2p. An alternative yeast channel, composed of Shl1p, Sbh2p and Sss1p, functions in signal-peptide-driven co-translational translocation [5,6].

Key words: endoplasmic reticulum, membrane protein insertion, protein targeting, protein translocation, Sec61 translocan, transmembrane domain.

Abbreviations used: MOM, mitochondrial outer membrane; ER, endoplasmic reticulum; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; TA, C-tail-anchored, TM2, transmembrane domain; N, N-ethylmaleimide-sensitive factor attachment protein receptor; C-terminal, C-terminal; BiP, Ig heavy-chain-binding protein.

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Figure 1 | Examples of TA proteins
Shown are cytochrome \(b_5\) (pink, with the haem in red) on the ER membrane, a syntaxin (Sso1p; in blue) on the plasma membrane, and Bcl-xL (yellow) on the MOM. In all cases, the active domain is exposed to the cytosol and the C-terminus is on the exoplasmic side of the membrane. The structures of the cytosolic domains of the three proteins are from the Brookhaven Protein DataBank; the C-terminal tails, whose structures have not been resolved, are shown as transmembrane \(\alpha\)-helices.

Since the membrane-anchoring segment of TA proteins resembles the signal anchor of type II membrane proteins, the difference being only in its position within the polypeptide, it seemed plausible that the post-translational integration of these proteins could be mediated by the Sec61 translocon as well. This possibility has been investigated by a number of groups, using \textit{in vitro} assays, in which insertion of the TA proteins cytochrome \(b_5\) and synaptobrevin I was monitored by assessing carbonate-resistant binding to microsomal membranes. The conclusion from these investigations has been that the two proteins may use different insertion mechanisms [7,8], but that the classical Sec61-based translocation machinery of the ER is not involved in either case. Indeed, synaptobrevin insertion occurred both into mammalian microsomes depleted of Sec61 [7] and into microsomes from yeast strains defective in the Sec61 machinery [9], and cytochrome \(b_5\) binding occurred also in protease-treated microsomes [8].

Recently, a different experimental approach, based on chemical cross-linking, has been used to investigate the possible involvement of the known ER translocons in TA protein insertion in living yeast cells by applying a rigorous assay for translocation, based on the \textit{in vivo} utilization of an N-glycosylation consensus sequence engineered to the C-terminus of mammalian cytochrome \(b_5\) [11]. A large number of mutant yeast strains were utilized in this study, including conditional and constitutive mutants or knockout strains defective in the translocon protein Sec61p (sec61-3 and sec61-41), accessory protein Sec62p (sec62-101), the co-chaperone Sec63p (sec63-1 and sec63-202), or the ER luminal chaperones Bip/Kar2 (kar2-159) and Lhs1p (\(\Delta\)lhs1). We also assayed cytochrome \(b_5\) translocation in a mutant lacking a component of the alternate yeast translocon, Sbh2p (\(\Delta\)sbh2). None of these mutations or deletions had detectable effects on the translocation of the tail of the \(b_5\) construct (called \(b_5\)-Nglyc, with an N-glycosylation site in the luminal C-terminal fragment), which was rapidly glycosylated in all the strains at permissive and restrictive temperatures, under conditions in which signal-peptide-driven post-translational translocation was abolished.

The energy requirements for insertion of cytochrome \(b_5\) were also different from those of signal-peptide-driven translocation. In yeast cells, glycosylation of \(b_5\)-Nglyc occurred rapidly after partial ATP depletion, which was possible to achieve \textit{in vivo} by limiting the availability of the

Figure 2 | Intracellular trafficking of TA proteins
After release from the ribosome, a TA protein, depicted here as a black mouse, is specifically targeted either to the ER or to the MOM (red arrows). Not shown is the targeting pathway to the plastid surface in plants, which appears to be similar to the one for the MOM. The backward movement of the mouse is meant to indicate the importance of features in the tail for this targeting process. From the ER, TA proteins undergo further sorting: they may remain as ER residents (blue arrow), travel down the secretory pathway (cyan arrows) or be transported to peroxisomes (green arrow).

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**Possible modes of translocation of TA proteins (B and C) in comparison with signal-peptide-driven post-translational translocation (A)**

(A) The heterotrimeric Sec61 translocon with its accessory Sec62/63p complex, required together with Sec71/72 proteins (not shown) and luminal BiP/Kar2p for post-translational signal-peptide-driven translocation. The white box represents the signal peptide and the ellipsoids are chaperones. The known functions of the Sec61 translocon are not involved in TA protein translocation [11], but Sec61 could be involved via a novel mechanism. (B) Insertion of the short luminal portion and the TMD of TA proteins directly across the lipid bilayer in the absence of a protein channel, either spontaneously or in an enzyme-assisted manner. The C-terminus might flip (red arrow) from a hairpin conformation to the stable, transmembrane topology. (C) Insertion of the luminal and TMD portions into the ER membrane through a novel, hitherto undetected protein channel. As is the case for the Sec61p translocon, this novel translocation channel would have to open laterally (horizontal red arrows) to allow contact of the TMD with the lipid bilayer.

Taken together, the results of Yabal et al. [11] clearly demonstrate that TA protein insertion is mechanistically different from signal-peptide-driven post-translational translocation. In the following section, we discuss possible mechanisms that we envisage for this process.

### Possible mechanisms for TA protein insertion

We consider here four possibilities (see Figure 3). Discovering which one is operating in cells is the challenge for future research in this area.

#### A novel function of the Sec61 translocon?

Considering the number of components of the Sec61 machinery and the different mutations analysed in the study of Yabal et al. [11], the most likely interpretation of the results is that the classical translocon is not involved in TA protein insertion. Nonetheless, it must be remembered that Sec61p is a multifunctional protein, and that the mutations analysed may not necessarily affect all of its functions. In addition to co- and post-translational translocation, the Sec61 translocon is also involved in dislocation of short-lived or misfolded proteins from the ER lumen back to the cytosol for ubiquitinylation followed by proteasomal degradation [14,15]. The proteins to be dislocated differ markedly from those entering the ER lumen because they have lost the signal peptide and have undergone co- and post-translational modifications, as well as folding attempts. Accordingly, distinct TMDs (transmembrane domains) 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 [16]. Interestingly, the cold-sensitive sec61-41 mutation, which did not affect glycosylation of b5-N glyc [11], resides in TMD3, and blocks entry into the ER at low temperature and dislocation at all temperatures [17]. Thus should the Sec61 complex have a role in TA protein translocation, it would involve a novel function, independent from the known entry and dislocation mechanisms.

#### Lipids-only hypothesis

After release from ribosomes, TA proteins select their target membrane (MOM or ER) with a high degree of accuracy. The specificity of the targeting process does suggest that proteins are involved; however, differences in the lipid composition of the MOM and the ER membrane [18] could also be important. Whether or not targeting is assisted by dedicated proteins, the subsequent translocation of the C-terminus plus the TMD of TA proteins could occur spontaneously across the lipid bilayer (Figure 3B). The luminal portions of the known ER TA proteins are generally quite short (<10 amino acids). Although they often carry a net negative or positive charge, it is not inconceivable that they could penetrate spontaneously across the membrane. This idea is attractive from the evolutionary point of view. TA proteins might be a class of very primitive transmembrane proteins that were
able to integrate into membranes early in evolution, before the development of the modern translocation machinery. Subsequently, one or some of these TA proteins could have formed a primordial translocation pore that permitted the insertion of membrane proteins with larger exoplasmic domains. Consistent with this idea, the current translocation machines of ER, MOM and peroxisomes all contain some TA protein subunits.

**Could TA protein insertion be related to phospholipid flip-flop?**

A particular feature of the ER membrane is its capacity to support rapid, bidirectional transport of phospholipids across the bilayer (reviewed in [19]). This capacity is required in the context of the phospholipid biosynthetic activity of the ER membrane. The phospholipid-synthesizing enzymes have their active sites on the cytoplasmic face of the membrane; thus newly synthesized lipid molecules must be able to translocate to the opposite side of the bilayer. There is general agreement that phospholipid movement between the monolayers in the ER is bi-directional and ATP-independent. Thus the activity responsible for this movement qualifies as a ‘flippase’ [19]. A number of studies have implicated proteins, without specificity for the headgroup of phospholipids, as ‘flippase’ [19]. An extreme possibility is that many different transmembrane proteins in the ER, with different primary functions, also incidentally accelerate phospholipid flip-flop.

So, how could TA protein insertion be related to phospholipid flip-flop? First, if dedicated flippases exist, given their lack of specificity for the polar head group, it is feasible that they could translocate the C-terminal polar residues of TA proteins as well. Secondly, if transbilayer polypeptide helices facilitate phospholipid flip-flop, the reverse could also be true: phospholipid flip-flop could facilitate helix insertion. One might even conceive that TA protein insertion and phospholipid flip-flop are coupled processes; however, such a mechanism could not account for transport of phospholipids in the opposite direction, from the luminal to the cytosolic face of the bilayer, unless some TA proteins alternate between a hairpin and a transmembrane conformation.

**A novel proteinaceous translocation channel for TA proteins?**

Finally, the translocation field probably still reserves many surprises. TA proteins could be inserted by a hitherto undetected proteinaceous channel (Figure 3C). Indeed, novel translocation channels have been discovered rather recently in bacterial, chloroplast and mitochondrial membranes. Of these, the OXA system, conserved in mitochondria and prokaryotic cells, appears to be specialized in the integration of membrane proteins [21–23]. Interestingly, bacterial membrane proteins that exhibit no dependence on the Sec transloacase and that were previously thought to insert into the membrane without the aid of other proteins were later shown to depend on the OXA system for insertion [23]. Could a similar system be present in the ER membrane of eukaryotic cells and be responsible for the integration of TA and also of other membrane proteins?

**Conclusions and perspectives**

Although TA proteins have attracted increasing interest over the past few years, the molecular mechanism of their integration into membranes is still a mystery. Recent work has clearly shown that they translocate their C-terminal by a mechanism distinct from that of signal-peptide-driven translocation. This negative result sets the stage for future research aimed at precisely defining the molecular components involved in their integration. With the use of appropriate assays that rigorously test for translocation of the C-terminus, it should be possible to carry out both genetic screens in yeast and in vitro assays with reconstituted membranes. It is hoped that these approaches will soon bring clear answers to the question of TA protein integration.

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