Minireview

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Stitching proteins into membranes, not sew simple

Abstract: Most integral membrane proteins located within the endomembrane system of eukaryotic cells are first assembled co-translationally into the endoplasmic reticulum (ER) before being sorted and trafficked to other organelles. The assembly of membrane proteins is mediated by the ER translocon, which allows passage of lumenal domains through and lateral integration of transmembrane (TM) domains into the ER membrane. It may be convenient to imagine multi-TM domain containing membrane proteins being assembled by inserting their first TM domain in the correct orientation, with subsequent TM domains inserting with alternating orientations. However a simple threading model of assembly, with sequential insertion of one TM domain into the membrane after another, does not universally stand up to scrutiny. In this article we review some of the literature illustrating the complexities of membrane protein assembly. We also present our own thoughts on aspects that we feel are poorly understood. In short we hope to convince the readers that threading of membrane proteins into membranes is 'not sew simple' and a topic that requires further investigation.

Keywords: co-translational; hydrophobic; integration; translocation; translocon; transmembrane.

Introduction

The majority of proteins that are targeted to the endoplasmic reticulum (ER) have an N-terminal hydrophobic stretch

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of amino acid residues that is recognised by signal recognition particle (SRP) upon emergence from the ribosome. Binding of SRP halts translation and the ribosome/nascent chain/SRP complex is targeted to the ER in a GTP-dependent manner where it interacts with the SRP receptor (Rapoport, 2008). Once at the ER membrane the ribosome is thought to engage with a protein conducting channel, the ER translocon (Johnson and van Waes, 1999), with translation resuming once SRP dissociates from the complex following GTP hydrolysis. This co-ordinated process ensures that proteins to be translocated across, or integrated into, the ER membrane are synthesised (apart from their N-termini, Figure 1) in the vicinity of the translocon.

The ER translocon forms a channel through which newly translated polypeptide chains can travel through into the ER lumen. It must also allow transmembrane (TM) domains of membrane proteins to move laterally into the lipid bilayer. For the purpose of this review we consider the core of a single ER translocon to be a heterotrimeric complex of Sec61α, β and γ. The Sec61α subunit has 10 TM domains forming a proposed 'channel' through and 'gate' into the membrane. Sec61γ contributes a single TM domain situated at the 'hinge' of Sec61α with Sec61β also contributing a single TM domain to the complex. A plausible model of how a single ER translocon, rather than a higher order assembly, may function mechanistically (reviewed in Martinez-Gil et al., 2011b) has been proposed based on the structures of homologous translocons from *Methanococcus jannaschii* in a closed conformation without a translocating nascent chain (Van den Berg et al., 2004) and *Pyrococcus furiosus* in an 'open conformation' because of a pseudo substrate being present in the translocon (Egea and Stroud, 2010). Recent cryo-EM studies on translocons actively engaged with nascent chains are broadly supportive of the 'single translocon' model (Gogala et al., 2014; Park et al., 2014).

TM domains of integral membrane proteins are in general hydrophobic α-helices. It is well accepted that thermodynamically favourable partitioning of TM domains from the translocon, via a lateral gate, into a more hydrophobic (lipidic) environment is important in
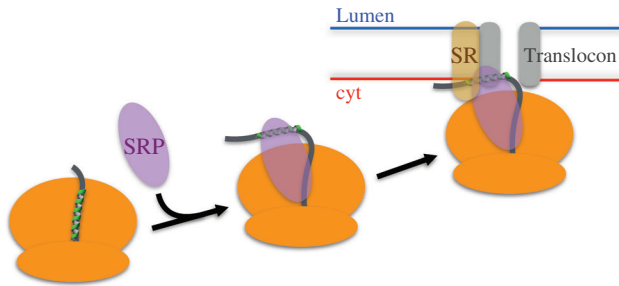
**Figure 1** Targeting of secreted and membrane proteins to the ER translocon.
A ribosome translating the mRNA of a membrane or secreted protein is targeted to the membrane through the SRP (purple). SRP recognises the emerging hydrophobic signal (green helix), binds to the ribosome and cause arrest of nascent chain elongation. The ribosome/nascent chain/SRP complex binds to membrane-bound SRP receptor (SR, brown), which associates dynamically with the translocon (grey).

TM domain membrane integration (Hessa et al., 2005, 2007). However, how hydrophobic stretches of amino acids get positioned in the translocon and where they fold (i.e., adopt a helical conformation) prior to partitioning into the membrane, is less well understood. Furthermore, hydrophobic partitioning does not account for how marginally hydrophobic TM domains become membrane integrated.

We will highlight some of the possible complexities of membrane integration when considering different mechanisms by which the first two TM domains of membrane proteins might achieve their final topology in the ER membrane.

# Integration of the first TM domain into the ER membrane: deciding how to make the first stitch

Integration of the first TM segment of a membrane protein into the ER membrane in the correct orientation is considered important in defining the overall topology of an integral membrane protein. The first TM domain of an integral membrane protein can either be inserted with its N-terminus in the cytoplasm and C-terminus in the ER lumen as a signal anchor (SAII), or with its C-terminus in the cytoplasm and N-terminus in the ER lumen (Figure 2). In the latter case the N-terminus of the mature protein may be directed into the lumen of the ER by a cleavable signal sequence (SS, not discussed in this review) or a reverse signal anchor (SAI).
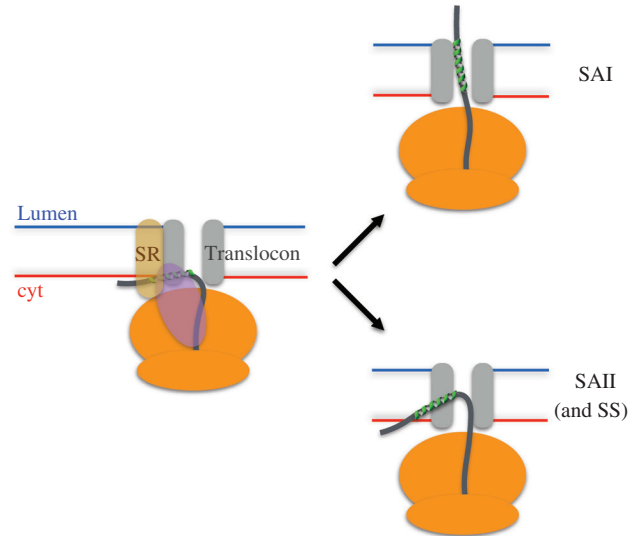
Figure 2 Integration of the first TM segment of membrane proteins. SRP dissociation from the SR and the ribosome transfers the hydrophobic signal to the translocon and elongation of the nascent polypeptide resumes. The insertion of the first transmembrane domain may be a simple head-first process (SAI), or may require a hairpin rearrangement (SAII and SS).

Before discussing how these first (N-terminal) topogenic sequences may engage with the ER translocon, the pre-integration folding of nascent chains containing TM domains will be briefly addressed. The first hydrophobic stretch [signal sequence (SS) or signal anchor (SA), green helices in Figures 1–3] of amino acids to emerge from the ribosome is bound by the SRP54 subunit of SRP (Zopf et al., 1990). It is likely that these SS or SA are in an α-helical conformation when bound to SRP54 as shown in the crystal structure of *Sulfolobus solfataricus* SRP54 (Ffh) with a signal sequence positioned in its peptide binding groove (Janda et al., 2010). There is no reason to suppose that mammalian SRP54 recognises hydrophobic signals any differently than their archaeal or bacterial equivalents. It is possible, indeed likely, that the helical conformation of SS and SA are adopted in the ribosome before recognition by SRP as it has been shown that the ribosomal exit tunnel can accommodate nascent chains folded into α-helices (Mingarro et al., 2000; Woolhead et al., 2004; Lu and Deutsch, 2005; Bhushan et al., 2010). Thus, when considering how the most N-terminal topogenic sequences interact with the translocon, it is necessary to explain how fully formed hydrophobic helices re-locate from a cytoplasmic location upon SRP release into a 'membrane-spanning' orientation (Figure 2).

It is known that positively charged amino acid residues flanking the hydrophobic core of the first topogenic
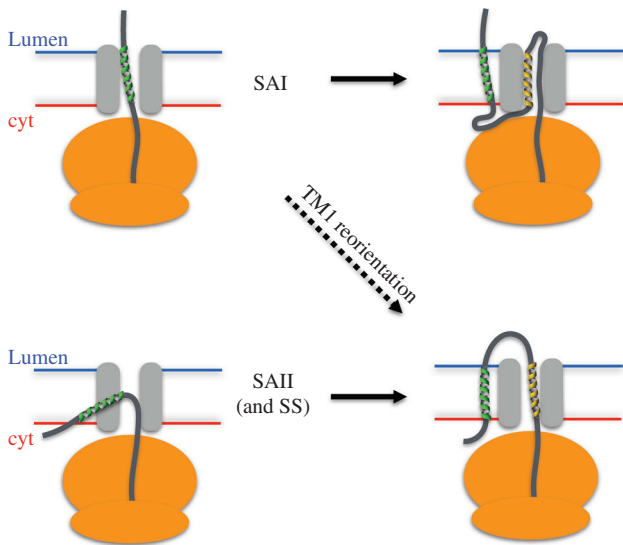
Figure 3 Routes of possible integration of the first two TM segments of membrane proteins.

A second hydrophobic region (yellow helix) downstream of an SAI sequence would require TM2 flipping (top), while downstream of an SAII sequence TM2 should be able to insert in a head-first basis (bottom). Initial insertion of TM1 segments with SAI orientation can be reversed (dotted arrow). Other possibilities are discussed further in the text.

sequence (SS or SA) are major, but not the only, determinants of orientation in the membrane (Higy et al., 2004). The charge distribution of residues flanking SA sequences favours positively charged amino acids on the cytoplasmic side of the membrane (von Heijne and Gavel, 1988; Hartmann et al., 1989). They could possibly assert their topogenic effects by electrostatic interactions using negatively charged molecules (amino acids in the translocon or lipid head groups) on the cytoplasmic side of the ER membrane as 'anchorage' points (Junne et al., 2007; Bogdanov et al., 2008). However, in reality, lipids are likely to play a much more complex and dynamic role in exerting their topological effects [reviewed in Bogdanov et al. (2014)].

SAI (N-lumen C-cyt) insertion

The insertion of SAI sequences may be conveniently considered as a head-first insertion of the N-terminus of the protein, driven by the hydrophobic domain engaging with the translocon without having to disrupt its helical conformation (Figure 2, top). In a single spanning membrane protein, SAI integration would give rise to a protein with a Type III (or Type I for membrane proteins with cleavable SS) membrane topology with their N-termini translocated (Goder and Spiess, 2001). It is relatively straightforward to

imagine that head-first (needle like) insertion of an unbroken hydrophobic helix may drive short N-termini through the translocon but is less clear how longer N-termini can be pushed through by the same mechanism (analogous to threading a very blunt needle). In order for long N-termini to be translocated it seems that there is a requirement for them not to be tightly folded (Kida et al., 2005). In fact, it would seem that rapidly folding hydrophilic domains N-terminal to the hydrophobic core may inhibit SAI insertion and promote integration in the opposite orientation (Denzer et al., 1995). This is likely a result of folded domains not being compatible with transfer through the translocon pore into the ER lumen for steric reasons. The inability to translocate tightly folded structures has been elegantly shown in experiments where folding of an N-terminal domain consisting of dihydrofolate reductase (DHFR, approximately 200 amino acids) could be controlled by the addition of methotrexate (MTX). In the absence of MTX, 'loosely folded' DHFR could be efficiently transported into the ER lumen, driven by a hydrophobic domain; while in the presence of MTX, 'tightly folded' DHFR was not (Kida et al., 2005). These observations suggest that positioning of the hydrophobic domain in the translocon provides a 'power stroke' sufficient for unfolding and initiating translocation of loosely folded but not tightly folded DHFR N-terminal domains.

In our opinion, it is difficult to comprehend how a head-first insertion could facilitate translocation of long N-terminal domains, even unfolded ones. There is possibly an initial translocation of a hairpin loop of a few amino acids adjacent to the hydrophobic domain followed by a ratcheting of the remainder of the N-terminus through the translocon pore. This, however, cannot strictly be considered as head-first insertion because the extreme N-terminal amino acids would not be the first to enter the lumen of the ER. Proteins on the lumenal side of the ER membrane such as BiP may function as a molecular ratchet to prevent 'backwards translocation' (Matlack et al., 1999). This brings us back to the question as to how else, upon release from SRP, may a 'presumed' fully folded helix adopt its initial TM orientation. 'Flipping in' from parallel to perpendicular to the membrane (see Figure 2), of a 30 Å long hydrophobic domain (the thickness of the hydrophobic core of a 'typical' membrane) as an unbroken helix seems likely to be sterically hindered given the dimensions of the translocon, approximately 20 Å in diameter at its widest point on the cytoplasmic side (Clemons et al., 2004). Flexibility of the translocon and 'sliding in' of the hydrophobic helix facilitated by the lateral gate and/or involvement of other proteins in addition to the core translocon subunits could overcome this

issue. A 'single helix hairpin' insertion with a whip-like power stroke would involve disrupting a pre-existing helix followed by its reformation once positioned in the translocon is another potential mechanism.

SAII (N-cyt, C-lumen) insertion

In a single spanning membrane protein, SAII integration would give rise to a protein with a Type II membrane topology (N-terminal cytosolic/C-terminal lumenal disposition). Thus, SAII sequences translocate their C-terminal flanking residues into the ER lumen. This is often depicted as the N-terminus of an SAII being held on the cytoplasmic side of the membrane and a hairpin structure inserting into the translocon (Figure 2, bottom). As translation proceeds the SAII straightens, orients perpendicular to the translocon and partitions into the lateral gate. Translocation of the newly synthesising chain of amino acids is then pushed through the central translocon pore into the lumen of the ER. In contrast to translocation of long N-terminal domains preceding an SAI sequence, translocation of ER lumenal domains following an SAII sequence will probably not normally require unfolding of this region as they are newly synthesised and presumably have not had opportunity to form tightly folded structures inside the ribosome. Uni-directional translocation is probably powered by elongation of the polypeptide chain with there being no obvious requirement for a ratchet mechanism in this case. While being convenient as a concept, this 'classical' model of SAII insertion (as with SAI above) requires that the presumed helical nature of the SAII bound to SRP is disrupted or at least exhibits some plasticity (to allow hairpin formation) during the insertion process. An alternative variation on the classical model is that the pre-formed TM helix, somehow positions itself correctly in the translocon without helix disruption. As mentioned previously, we would envisage that the lateral gate would have to have a key role in positioning a 30 Å long SAII helix because of the geometry of the translocon. Cross-linking experiments have indicated that the SAII of aquaporin-4 (Sadlish et al., 2005) and of a viral movement protein (Sauri et al., 2007) progresses in the translocon in an 'ordered and sequential' fashion. Despite such studies we feel that the details of how the SAII helices initially orient from an SRP bound cytosolic location to a TM orientation are unresolved.

Recently it has been demonstrated that tight folding of small domains C-terminal to an SS (not an SA) can occur within the ribosome, inhibiting translocation, and that this inhibition can be reversed upon relaxation of folding (Conti et al., 2014). These observations raise numerous questions about the mechanism of translocation of amino acids following an SS (and perhaps SAII) including the possibility that translocation is not coupled to translation, at least for secreted proteins.

Reorientation

So far we have considered the orientation of the first TM domain as being determined by the initial insertion event. However, there is good evidence that an initial insertion topology can be reversed (Goder and Spiess, 2003). This has been demonstrated in the assembly of aquaporin-4 where its first TM domain initially inserts with its N-terminus in the ER lumen, at short nascent chain lengths, before reversing to its final N-cytoplasmic/C-lumenal orientation as the nascent chain elongates (Devaraneni et al., 2011). These findings corroborate initial observations of model SS flipping that could be influenced by nascent polypeptide length, charge difference and signal sequence hydrophobicity (Goder and Spiess, 2003). Reorientation of the first TM domain in this way may be a more widespread phenomenon than one might intuitively expect as it has also been observed to occur for short polar N-domains (24 or fewer amino acid residues) preceding a stretch of 16 leucine residues (Kocik et al., 2012). In the assembly of a potassium channel subunit (TASK-1) and a viral potassium channel protein it seems that even N-terminal residues with oligosaccharides attached (engineered and not present in the natural proteins) can be reoriented from the lumen to the cytosol (Watson et al., 2013). However, it should be mentioned that glycosylation has been demonstrated to prevent return of luminal domains of proteins to the cytoplasm (Goder et al., 1999) in other studies. Reorientation of TM domains following initial insertion not only highlights the functional flexibility of the translocon in facilitating assembly of membrane proteins, but also raises the issue as to the route (presumably through the translocon) N-terminal residues take in returning to the cytosol (see Figure 3, dotted arrow).

Integrating the second TM domain (making the second stitch)

Making the assumption that the most N-terminal TM domain is inserted first; the orientation of the second TM

segment is necessarily dependent on how the first TM segment has inserted.

Assembling TM2 into an N-lumen C-cyt orientation following an SAII

Following transmembrane disposition of an SAII in the translocon/lateral gate, nascent chain synthesis pushes the elongating chain into the ER lumen. If a second, sufficiently hydrophobic stretch of amino acids is positioned within the translocon there is strong evidence that it will exit the translocation channel, driven by thermodynamic partitioning into a more hydrophobic environment, possibly through the lateral gate of the translocon (Hessa et al., 2005, 2007; Ojemalm et al., 2011). TM domains that autonomously (independently) exit the translocation channel in this way are also known as 'stop transfer' (ST) sequences, as translocation of C-terminal residues of the nascent polypeptide is halted (Figure 3, bottom).

A scenario may also be envisaged in which partitioning of the second TM domain into a transmembrane location is dependent on specific interactions with the first TM domain. This may be particularly important for marginally hydrophobic TM domains that would fully translocate into the ER lumen in the absence of a preceding 'capture helix'.

Helical hairpin (consisting of two closely spaced helical TM segments separated by a short extra-membrane or surface turn) insertion is another possible mechanism for integrating marginally hydrophobic TM domains that relies on TM1 and TM2 achieving transmembrane location in a concerted manner rather than sequentially (Meacock et al., 2002; Sauri et al., 2005, 2007; Cross et al., 2009; Pitonzo et al., 2009). More recently, charge-pair interactions between a TM1 and a TM2 together with turn-promoting residues have been found to favour helical hairpin insertion (Bano-Polo et al., 2013), while neither the TM1 or the TM2 inserted efficiently independently of one another (Martinez-Gil et al., 2011a).

Above, we have only considered insertion of a TM2 occuring from the cytoplasmic side of the membrane. There are also cases, notably aquaporin-1, in which the TM2 is initially fully translocated into the ER lumen prior to integration into the membrane from the lumenal side (Lu et al., 2000; Pitonzo and Skach, 2006). This re-insertion is frequently dependent on the subsequent insertion of downstream TM domains. The influence of neighbouring helices on membrane insertion of marginally hydrophobic

sequences has been elegantly demonstrated experimentally (Hedin et al., 2010).

Assembling TM2 into an N-cyt C-lumen orientation following an SAI

A TM2 downstream of an SAI sequence (Figure 3, top) should be able to re-initiate translocation of C-terminal amino acid residues in a similar manner to an SAII. We will therefore call these sequences 'internal SAII sequences'. However, whereas SAII sequences are presented to the translocon while bound to SRP (Figure 2) with translation stalled and 'handed over' to the translocon upon GTP hydrolysis, we are not aware of any evidence that internal SAII sequences are recognised by SRP. This could mean that internal SAII sequences initially engage with the translocon in a mechanistically different way to SAII sequences at the N-terminus of a nascent protein (Kocik et al., 2012). In order to prevent folding of the polypeptide chain region C-terminal to internal SAII helices, independently of SRP, translation may be slowed down by some mechanism, for instance by the translation of suboptimal codons 3′ to the SAII coding sequence on the mRNA. We would expect suboptimal codons to be positioned around 30–40 codons downstream of the end of an internal SAII as here they would be located at the ribosomal P-site when the SAII sequence emerges from the ribosomal tunnel (Mothes et al., 1994; Matlack and Walter, 1995; Whitley et al., 1996). Evidence for translational 'slowing' in the context of membrane protein assembly is lacking but it has been suggested that codon bias can influence translation kinetics facilitating co-translational protein folding (Pechmann and Frydman, 2013). Ribosomes may also have a more direct role in preparing the translocon in shifting its function from translocation to integration of *de novo* synthesised TM domains (Liao et al., 1997). TM domains positioned within the ribosome exit tunnel can act as a signal to recruit membrane protein RAMP4 to the vicinity of translocons (Pool, 2009), presumably having some implication for translocon function such as priming it to integrate a TM domain, thereby demonstrating that the two macromolecular machines (ribosome and translocon) are structurally coupled for functional purposes (Johnson, 2009).

Above, we have considered independent (autonomous) integration of a TM2 helix in an N-cyt/C-lumen orientation. Heteronomous insertion of an N-cyt/C-lumen

TM2, dependent on insertion of a third TM segment (TM3) may be required for some marginally hydrophobic TM domains (Sakaguchi, 2002). In this mode of insertion a TM3 integrating in an N-lumen/C-cyt orientation may force the preceding marginally hydrophobic domains to adopt a transmembrane disposition. A more complex scenario for heteronomously inserting the TM2 of aquaporin-1 has recently been reported (Virkki et al., 2014).

Concluding remarks

It is worth mentioning, while only the assembly of the first two TM domains is discussed in this review, that many of the situations described are applicable to the assembly of multispanning membrane proteins. We base many of our arguments on the assumption that the 'single translocon' model is correct and that ER translocons are structurally and functionally very similar to their prokaryotic equivalents. This view is almost certainly an oversimplification. Oligomerisation of translocons has been observed in many studies, which may provide functional flexibility beyond what has been considered here (Hanein et al., 1996). Furthermore accessory proteins in addition to core translocon components are likely to have substrate specific roles in membrane protein assembly, especially in the case of TRAM (translocating chain-associating membrane protein), which has been suggested to play a chaperoning role in this process by collecting poorly hydrophobic TM segments at a precise location within or adjacent to the translocon (Tamborero et al., 2011). We have not touched on the potential functions of other translocon accessory membrane proteins such as Sec62/Sec63 or TRAP, in complementing the role of the translocon in membrane protein insertion and folding (Shao and Hegde, 2011).

While possible ways of integrating two TM domains into the ER membrane may not have been exhausted we hope that this review has shown that stitching proteins into membranes is 'not sew' straightforward as one might imagine. In this sense, particularly intriguing is the phenomenon of large topological rearrangements of membrane helices during assembly. It remains to be determined whether specific chaperones and/or translocon accessory components facilitate TM-domain inversion.

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