Understanding integration of α-helical membrane proteins: the next steps

Reid Gilmore and **Elisabet C. Mandon**
Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, Massachusetts 01605-2324

Abstract

Integration of a protein into the endoplasmic reticulum (ER) membrane occurs through a series of multi-step reactions that include targeting of ribosome-nascent polypeptide complexes to the ER, attachment of the ribosome to the protein translocation channel, lateral partitioning of α-helical transmembrane spans into the lipid bilayer, and folding of the lumenal, cytosolic and membrane embedded domains of the protein. However, the molecular mechanisms and kinetics of these steps are still not entirely clear. To obtain a better understanding of the mechanism of membrane protein integration, we propose that it will be important to utilize *in vivo* experiments to examine the kinetics of membrane protein integration and *in vitro* experiments to characterize interactions between nascent membrane proteins, protein translocation factors and molecular chaperones.

Limitations to *in vitro* analyses of membrane protein integration

Integral membrane proteins account for roughly 25% of the open reading frames in eubacterial, archaebacterial and eukaryotic organisms [1]. With the exception of proteins destined for the mitochondria and chloroplast, almost all integral membrane proteins in eukaryotic cells are synthesized by membrane-bound ribosomes on the surface of the rough ER (RER). Tail-anchored (TA) membrane proteins, which have a single C-terminal α-helical TM span, are not synthesized by membrane bound ribosomes and so they are not discussed in this article (see [2] for a recent review). Current models describing the mechanism of membrane protein integration are primarily based upon *in vitro* biochemical and biophysical assays that monitor specific steps in this complex reaction pathway (as reviewed by [2, 3]). Transmembrane (TM) spans, like the cleavable signal sequences on secretory proteins, serve as RER-targeting signals that are recognized by the signal recognition particle (SRP, see Glossary) as the TM span emerges from the large ribosomal subunit (Figure 1a,b). Targeting of the SRP-ribosome-nascent polypeptide complex (RNC) to the SRP receptor (SR) on the ER leads to the formation of a high-affinity GTP stabilized complex between the SRP and SR [4] (Figure 1e). Dissociation of the SRP from the signal sequence then allows RNC to attach to the cytosolic face of the Sec61 complex, which is the eukaryotic cotranslational translocation channel (Figure 1f). The TM spans of integral membrane proteins adopt an α-helical conformation before passing through the lateral gate of Sec61 into the membrane bilayer, thereby minimizing the number of unsatisfied hydrogen bond donors and acceptors. In our opinion, a better understanding of the *in vivo* kinetics of

Corresponding author: Gilmore, R. (reid.gilmore@umassmed.edu).

membrane protein integration will provide novel insight into the mechanism of membrane protein synthesis, and might yield insight into integral membrane protein misfolding diseases.

An *in vitro* experimental strategy that has proven to be particularly useful to investigate protein translocation and membrane protein integration is to assemble RNC complexes by *in vitro* translating a truncated mRNA that lacks a termination codon. Incorporation of a photoreactive amino acid into the nascent polypeptide has led to the identification and characterization of the mammalian Sec61 complex as the protein translocation channel [5], consistent with evidence that Sec61p is the core subunit of the yeast protein translocation channel [6]. Site-specific incorporation of photoreactive amino acids [7] or fluorescent probes[8] has allowed a detailed examination of the integration pathway for multi-spanning membrane proteins [9–11]. Intramolecular fluorescence energy transfer (FRET) between fluorophores that flank a TM span indicate that the TM spans of membrane proteins adopt a more compact, perhaps α-helical, conformation before they emerge from the large ribosomal subunit, and upon doing so alter the junction between the RNC and the translocation channel [12, 13].

One limitation of *in vitro* analysis of membrane protein integration is that eukaryotic translation systems synthesize proteins at roughly 5–10% of the *in vivo* translation elongation rate (5–7 residues/second, [14]), hence *in vitro* experiments provide little information about the kinetics of membrane protein integration in cells. This caveat is compounded when one considers the time required to purify and analyze membrane protein integration intermediates assembled by translation of a truncated mRNA. Consequently, *in vitro* assembled integration intermediates might correspond to equilibrium conformations of the RNC-translocon complex rather than kinetically relevant reaction pathway intermediates.

In vivo experiments are also particularly informative for proteins that are either targeted to multiple organelles or are targeted to an unexpected cellular location. A recent noteworthy example concerns the biosynthesis of peroxisomal membrane proteins, which were previously thought to be synthesized by free polysomes in the cytosol. Recent *in vivo* evidence indicates that all peroxisomal membrane proteins are initially integrated into the RER in yeast cells [15]. In this article, we review recent conceptual advances in the analysis of membrane protein integration, many of which have been made possible by *in vivo* experimental approaches. We also highlight some of the major open questions in this research field, which we believe can best be addressed by *in vivo* studies.

In vivo targeting of mRNAs and RNC complexes to the RER

The SRP and the SR mediate targeting of RNCs that are synthesizing membrane proteins to the Sec61 complex. However, a growing body of evidence suggests that trafficking of mRNAs to the vicinity of the RER can also occur by a translation-independent process (Figure 1c). In mammalian cells, many mRNAs encoding resident proteins of the exocytic and endocytic membrane systems are bound to the ER by a ribosome-independent mechanism, suggesting that mRNA targeting to the vicinity of the RER might precede SRP-SR mediated RNC targeting to the Sec61 complex [16] (Figure 1d). Additionally, mRNAs encoding a subset of nucleocytoplasmic proteins were recovered in the membrane bound polysome fraction [17]. Additional research might lead to the identification of cis-acting mRNA motifs that interact with novel RER-associated trans-acting factors to mediate mRNA targeting or binding to the ER.

A panel of secretory protein reporter constructs consisting of a cleavable signal sequence and a transcription factor reporter domain were expressed in cultured cells to evaluate the *in*

vivo efficiency of protein translocation [18]. Interestingly, translocation of the reporter constructs varied considerably (60 – 95% translocation) depending upon the signal sequence and showed additional variability between different mammalian cell lines, implying that trans-acting factors could impact translocation efficiency.

In vivo experiments conducted using the budding yeast *Saccharomyces cerevisiae* have provided insight into the physiological role of the SRP-SR targeting pathway. Despite the well-characterized role for SRP and the SR in RNC targeting to the Sec61 complex, budding yeast can tolerate disruption of the genes that encode the SR or SRP subunits, or the RNA component of the SRP [19, 20]. Adaption of budding yeast to the loss of the SRP-SR targeting pathway is accompanied by a 4-fold decrease in growth rate, a loss of the ability to grow on a non-fermentable carbon source and a global change in gene expression profile [21]. A reduction in ribosome biogenesis in SRP-deficient cells reduces the protein synthesis rate several fold [21]. Integration of membrane proteins in SRP or SR-deficient yeast cells occurs by partitioning of nascent integral membrane proteins between a posttranslational translocation pathway and a SRP-SR independent cotranslational pathway [22]. Direct binding of RNCs to the Sec61 complex, via the an interaction between the cytosolic loops of Sec61p and the large ribosomal subunit [23], is the most likely mechanism to explain SRP-independent targeting of RNCs to the Sec61 complex. Unlike budding yeast, the fission yeast *Schizosaccharomyces pombe* cannot tolerate disruption of the genes that encode the RNA or protein subunits of the SRP [24, 25] highlighting the importance of conducting *in vivo* experiments using several model systems.

In vivo kinetics of precursor targeting to the ER

Differential accessibility of nascent polypeptides to protein modification enzymes that are either localized in the cytosol or within the ER lumen has allowed investigators to devise assays that monitor the *in vivo* kinetics of targeting of precursor proteins to the ER. For example, coexpression of the BirA biotin ligase in the cytosol, and a precursor polypeptide appended with a 13-residue biotinylation tag, can be used to identify cytosolically exposed domains of membrane proteins [26]. Recently, this method was used to analyze posttranslational translocation of a very small secretory protein in mammalian cells [27]. Asparagine linked (e.g. N-linked) glycosylation of proteins by the oligosaccharyltransferase in the RER lumen has been widely used to detect lumenally exposed domains of integral membrane proteins [28, 29]. N-linked glycosylation is primarily cotranslational in mammalian cells [30, 31], therefore acquisition of an N-linked oligosaccharide can provide kinetic information about membrane protein integration. The *in vivo* kinetics of SRP-SR mediated targeting of RNCs to the ER has also been monitored using a protein modification technique. In this case, a protein kinase A phosphorylation site (LRRA$\underline{S}$LG) was inserted into the N-terminal lumenal domain of a model type 1 membrane protein [32]. Phosphorylation of the serine residue, as detected using ^{32}P orthophosphate, can only occur when the protein kinase A site is in the cytosol. Transient exposure of the lumenal domain in the cytosol allowed the calculation of the time required for SRP-mediated RNC targeting (~3–4 sec) and translocation of the N-terminal 85 residue lumenal domain (~10 sec).

The kinetics of translocation channel gating can be monitored through the processing of an ubiquitin precursor protein by a cytosol-localized ubiquitin specific protease (UbP) [33]. The ubiquitin translocation assay (UTA) reporter consists of an N-terminal ER signal sequence, a spacer segment preceding a ubiquitin domain, followed by a UbP processing site and a C-terminal reporter domain appended with an epitope tag for immunoprecipitation of the pulse-labeled protein. If the C terminus of the ubiquitin domain emerges from the ribosome before the N terminus enters the translocation channel, rapid folding of ubiquitin in the cytosol leads to UbP cleavage of the UTA reporter. The UTA reporters were initially

used to provide *in vivo* evidence documenting the yeast posttranslational translocation pathway [33]. More recently, UTA reporters with variable length spacer segments have been used to analyze the kinetics of membrane protein integration [34]. The translocon gating assay revealed that translocation of the lumenal domain initiates within 15 sec after the signal sequence emerges from the large ribosomal subunit in yeast cells [34]. During integration of more complex model integral membrane proteins, lumenal domains and transmembrane spans are transiently exposed on the cytosolic surface of the membrane [34]. We speculate that cytosolic chaperones or accessory translocation components might prevent aggregation or premature folding of these segments within the cytosol. The UTA assay reporters have also been used to analyze partitioning of precursors between cotranslational and posttranslational pathways in yeast strains bearing mutations in the Sec61 complex, the SRP or the SR [22, 34, 35]. The *in vivo* approaches described above have confirmed a central role for the SRP-SR targeting pathway for membrane protein integration, and have provided insight into the kinetics and mechanism of membrane protein biosynthesis. After targeting of SRP-RNCs to the membrane has been completed, the next event in the biosynthesis of a membrane protein is the recognition and subsequent integration of TM spans by the Sec61 complex.

Recognition and orientation of transmembrane spans

The orientation of membrane proteins is primarily determined by the distribution of charged residues that flank the first TM span in accord with the 'positive-inside' rule [36, 37]. A net positive charge in the N-terminal flanking sequence favors the type 2 (N_{cyt}-C_{exo}) orientation, whereas a net positive charge in the C-terminal flanking sequence favors the type 1 (N_{exo}- C_{cyt}) orientation. Using model membrane proteins that were designed to yield mixed topologies *in vivo*, Goder and Spiess obtained evidence that type 2 (N_{cyt}-C_{exo}) membrane proteins are initially inserted into the Sec61 complex in a type 1 orientation (Figure 1f), but invert to obtain a type 2 topology within the first 50 sec of biosynthesis [38] (Figure 1g). TM span inversion occurred more rapidly when positively charged residues precede the TM span and less rapidly as the hydrophobic core of the TM span was increased from 13 residues to 25 residues, consistent with previous evidence that longer TM spans favor a type 1 orientation in model proteins that have similarly charged N-terminal and C-terminal flanking regions [39]. Based upon this analysis, a typical type 2 membrane protein is expected to adopt the correct orientation rapidly (< 5 sec) after insertion into the Sec61 complex. Consistent with the hypothesis that TM span orientation occurs after contact between the nascent membrane protein and Sec61, mutations in yeast Sec61 influence the orientation of model proteins with ambiguous topologies [40]. A recent *in vitro* analysis of a model type 2 membrane protein provided support for the hypothesis that the TM span of a type 2 protein is initially inserted into the Sec61 complex in a type 1 orientation [41].

The TM spans of multi-spanning membrane proteins are often less hydrophobic than the TM spans of type 1 or type 2 membrane proteins. Nonetheless, the protein translocation channel must be able to recognize *bona fide* TM spans and partition these segments into the membrane bilayer, while allowing complete translocation of marginally hydrophobic segments from exoplasmic domains of membrane proteins. To obtain a better understanding of TM span recognition, the von Heijne lab devised a multi-TM span reporter protein to systematically test the integration efficiency of a large series of synthetic transmembrane spans that are flanked by diagnostic N-linked glycosylation sites [42]. The synthetic TM spans were derived from a 19-residue polyalanine test segment, wherein variable numbers of alanine residues were replaced by leucine residues to increase the hydrophobicity of the synthetic TM span, or by combinations of leucine and more hydrophilic amino acids to decrease the hydrophobicity of the synthetic TM span. The results of *in vitro* experiments using mammalian microsomes suggest that lateral exit of TM spans from the Sec61 complex

is primarily governed by partitioning of the TM span between the relatively polar interior of the Sec61 translocation pore and the more hydrophobic environment of the membrane bilayer [42]. Importantly, expression of these multi-TM span constructs in yeast cells yielded similar results to those obtained using the mammalian *in vitro* system [43].The *in vitro* analysis of the synthetic TM spans allowed von Heijne and colleagues to derive an *in vivo* hydrophobicity scale for amino acids, wherein the experimentally determined ΔG_{app} values correspond to the free energy of membrane insertion. Subsequent studies from von Heijne and colleagues have shown that the orientation of the tested TM span (N_{cyt} vs. C_{cyt}) does not significantly alter the experimentally determined ΔG_{app} values for each of the amino acids [43].

von Heijne and colleagues have now applied this strategy to investigate integration of proteins into the mitochondrial inner membrane by the Tim23 complex. In this case, the *in vivo* assay exploits processing of the GTPase Mgm1p by the rhomboid protease Pcp1p [44]. Although the biological hydrophobicity scales for Sec61- and Tim23- mediated integration of TM spans are in general similar, certain aromatic residues (tryptophan and tyrosine) that favor TM span integration into the ER are less favorable for integration into the inner mitochondrial membrane. It is not clear whether these differences in hydrophobicity scale are explained by differences in membrane phospholipid composition or by structural differences in the import channel (Sec61 versus Tim23). Although the recognition and correct orientation of TM spans can be viewed as an initial step in the folding of an α-helical membrane protein, acquisition of the native folded structure will depend upon interactions between the individual TM spans within the membrane bilayer and upon folding of lumenal or cytoplasmic domains.

Folding and misfolding of integral membrane proteins

Misfolding of integral membrane proteins, and their subsequent degradation via the ER associated degradation (ERAD) pathway, is an area of great research interest because certain disease-causing mutations result in the misfolding of membrane proteins, such as the cystic fibrosis transmembrane conductance regulator (CFTR; see [45] for a recent review of protein misfolding diseases).

Comparing the topology diagram of a membrane protein, derived from a hydropathy plot, to the three-dimensional structure, determined by crystallography, strikingly demonstrates that the final fold of a multi-spanning membrane protein is not readily predictable based upon the location of hydrophobic segments within the protein sequence. Transmembrane spans are often tilted with respect to the phospholipid bilayer, and have kinks. Membrane proteins that serve as channels can have hydrophilic reentrant loops that are within the plane of the membrane, but do not contact the lipid bilayer. A recent analysis of the *Pyrococcus hirokoshi* glutamate transporter Glt$_{Ph}$ by the von Heijne lab [46] revealed that transmembrane span boundaries calculated using the biological hydrophobicity scale [42], or detected in the context of a reporter protein, do not match the final folded structure of Glt$_{Ph}$. These results indicate that TM spans in multi-pass membrane proteins can be repositioned with respect to the membrane bilayer as the protein folds [46].

Concluding remarks

The combination of *in vitro* and *in vivo* assays to monitor specific reaction steps in membrane protein integration has led to a better understanding of how transmembrane spans are recognized by the translocation channel and partitioned between the relatively hydrophilic environment of the Sec61 pore and the hydrophobic environment of the membrane bilayer. In some cases, *in vitro* and *in vivo* approaches have yielded conflicting

rather than complementary results, so it clear that there are many open questions (Box 1) that remain to be addressed. In our opinion, further progress in this research field will be aided by the continued use of a multi-faceted experimental approach that incorporates both *in vitro* and *in vivo* methods.

Acknowledgments

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Glossary Box

Endomembrane	The membranes in a eukaryotic cell that are biosynthetically derived from the ER. These membranes include the nuclear envelope, the peroxisome, the lysosome, the Golgi, the plasma membrane, endosomes and vesicles that traffic between these destinations.
FRET	Fluorescence resonance energy transfer between donor and acceptor fluorophores. FRET efficiency is dependent upon the distance between the two fluorescent probes and the overlap between the emission and excitation spectra of the donor and acceptor fluorophores. FRET measurements can be used to estimate distances between two sites on a protein.
ΔG_{app}	The ΔG_{app} can be viewed as the equilibrium for partitioning of an amino acid between an aqueous environment and the hydrophobic core of the membrane bilayer. The calculated ΔG_{app} values of the *in vivo* hydrophobicity scale include TM-span location specific differences in the partitioning of amino acid side chains into the membrane bilayer.
Positive-inside rule	The cytosolically exposed loops of membrane proteins have a net positive charge relative to the exoplasmic loops of membrane proteins. As a predictive method, one determines the net charge on the 10 amino acid residues on both sides that flank the first TM span in a protein. The flanking segment that has the greater net positive charge will face the cytosol.
RNC	Ribosome-nascent chain complex. This term is used to refer to ribosomes synthesizing full length polypeptides and RNCs assembled *in vitro* by translation of a truncated mRNA that lacks a termination codon.
Sec61 complex	Eukaryotic protein translocation channel in the rough RER. The Sec61 complex, and associated proteins, is often referred to as the translocon.

SR	SRP receptor. A heterodimeric RER protein composed of two GTPases. The GTP-bound form of SRβ anchors SRα to the membrane. SRα and SRP54 hydrolyze GTP in a cooperative manner to allow dissociation of the SRP-SR complex.
SRP	Signal recognition particle. The 54kD subunit of the signal recognition particle (SRP). SRP54 is a GTPase that has a C-terminal methionine-rich domain (M-domain) that is the site for signal sequence binding.
Tim23 complex	The Tim23 complex is a protein translocase located in the inner membrane of the mitochondria that is primarily involved in transporting proteins with mitochondrial presequences into the matrix. A subset of mitochondrial inner membrane proteins, including Mgm1p, are integrated by the Tim23 complex.
UbP	Ubiquitin specific protease that is responsible for processing the polyubiquitin precursor into mature ubiquitin.
UTA	ubiquitin translocation assay. The ubiquitin translocation assay can be used to analyze the *in vivo* kinetics of protein translocation channel gating. Quantification of the cleaved and uncleaved forms of the UTA reporter indicates the fraction of RNCs that gate the Sec61 complex before the complete ubiquitin domain emerges from the large ribosomal subunit. Increasing the length of the spacer segment that precedes the Ub domain provides additional time (~0.125 sec per added residue) for translocon gating.
TRAM	The translocon-associated membrane protein is an accessory component of the mammalian translocation channel that is in contact with nascent TM spans that exit the Sec61 complex. The exact role of TRAM in membrane protein integration is not well understood. Budding yeast do not have an obvious homologue of TRAM.

Box 1. Outstanding questions

- Are mRNAs targeted to the RER by a translation-independent mechanism? Are there mRNA binding proteins on the RER that tether mRNAs to the organelle to facilitate translation in the vicinity of the RER?

- Does the 80S ribosome, or just the 40S small subunit, detach from the Sec61 complex following termination of protein synthesis? The high affinity between the ribosome and the Sec61 complex (~5 nM) raises the possibility that the large ribosomal subunit needs to be actively detached from Sec61.

- Are there membrane-embedded chaperones that assist protein folding of membrane proteins? A potential candidate for a general membrane protein chaperone in mammalian cells is the translocon-associated membrane (TRAM) protein [47], which contacts integral membrane proteins as they exit the translocation channel [48].

- Do membrane protein sequences and topologies influence the kinetics of TM-span exit from the Sec61 complex? Are marginally hydrophobic TM spans retained in the Sec61complex for a longer time?
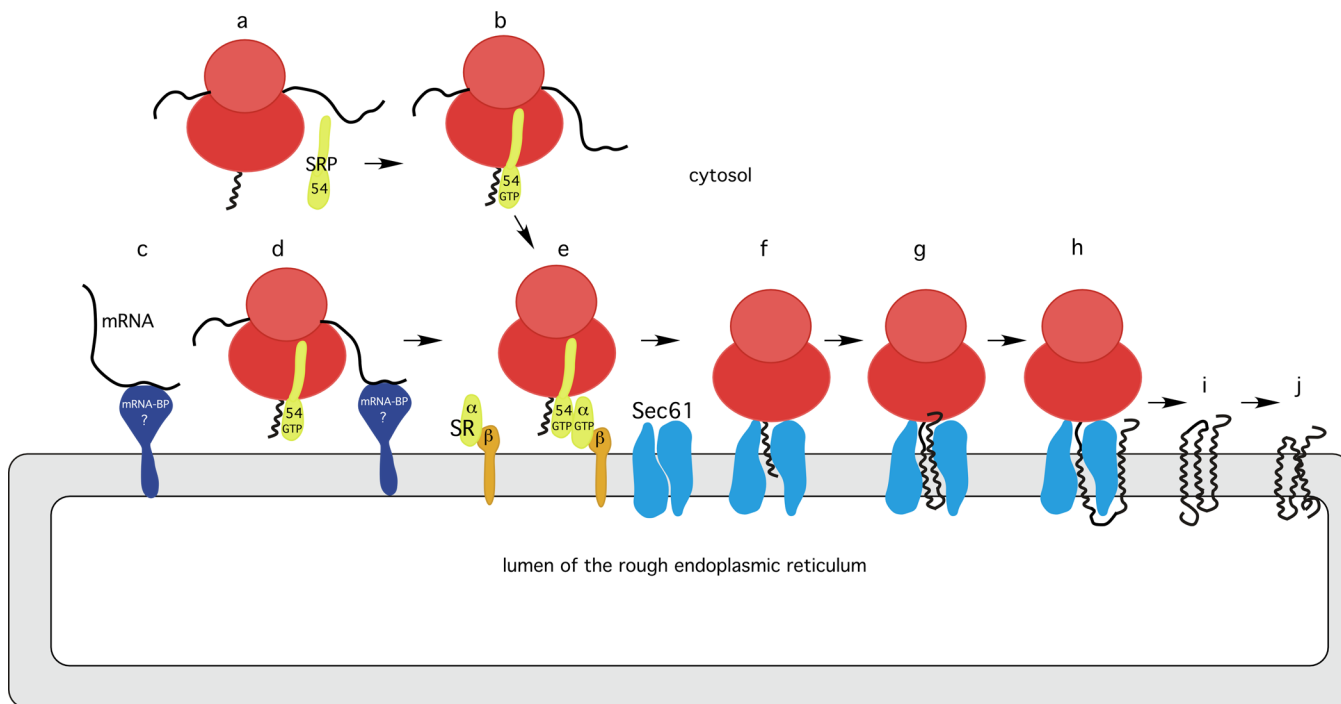
Figure 1.
Integration of α-helical membrane proteins. (**a**) Translation of an mRNA encoding an integral membrane protein probably initiates in the vicinity of the rough endoplasmic reticulum (RER). (**b**) The hydrophobic transmembrane span, like a cleavable signal sequence, is recognized by the 54 kDa subunit of the signal recognition particle (SRP) particle when the signal sequence emerges from the polypeptide exit tunnel on the large ribosomal subunit. (**c**) mRNA molecules encoding endomembrane resident proteins might be localized to the RER via interactions between an RER-localized mRNA binding protein (mRNA-BP) and sequence motifs in the mRNA. (**d**) SRP recognition of the signal sequence (transmembrane (TM) span) might occur on these RER-tethered mRNA molecules, thereby simplifying the targeting of ribosome nascent chain complexes (RNC) to the RER. (**e**) Both targeting pathways converge on the alpha subunit of the SRP receptor (SRα). The GTP-bound conformation of the beta-subunit of the SRP receptor (SRβ) anchors SRα to the RER. The GTP-stabilized SR-SRP-RNC does not hydrolyze GTP until the RNC is delivered to a vacant Sec61 complex. The Sec61 complex is in a closed conformation in the absence of the RNC. (**f**) Dissociation of SRP54 from the TM span allows the RNC to bind to the Sec61 complex. Current evidence indicates that the TM span is initially inserted in a 'head-first' orientation into the lateral gate region of Sec61, with the N terminus of the nascent polypeptide oriented towards the RER lumen. (**g**) If the N-terminal segment flanking the first TM span has a net positive charge relative to the C-terminal flanking segment, the first TM inverts as the nascent polypeptide elongates. (**h**) The first TM span passes through the lateral gate of Sec61 to be integrated into the membrane bilayer. (**i**) Upon termination of protein synthesis, the nascent integral membrane protein is in an incompletely folded, but membrane integrated conformation. (**j**) Folding of the membrane protein may be facilitated by the cooperative action of lumenal, cytosolic and membrane-embedded chaperones.