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# The machinery of membrane protein assembly

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The SecY (bacteria) and Sec61 (eukaryotes) translocon complexes, or protein-conducting channels, work in concert with bound ribosomes to insert proteins into membranes during the first step of membrane protein assembly. The crystallographic structure of an archaeal SecY translocon provides dramatic new insights into the mechanism of translocon function. This structure suggests an explanation for how the translocon can aid in establishing membrane protein topology via the positive-inside rule. The folding of membrane proteins may begin in the ribosome exit tunnel, before entering the translocon, according to cryo-electron microscopy and biophysical studies.

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**Current Opinion in Structural Biology** 2004, **14**:397–404

This review comes from a themed issue on
Membranes
Edited by So Iwata and H Ronald Kaback

Available online 20th July 2004

0959-440X/$ – see front matter


**DOI 10.1016/j.sbi.2004.07.003**

**Abbreviations**

| | |
|---|---|
| **EM** | electron microscopy |
| **ER** | endoplasmic reticulum |
| **FRET** | Förster resonance energy transfer |
| **MP** | membrane protein |
| **SR** | SRP receptor |
| **SRP** | signal recognition particle |
| **TM** | transmembrane |

## Introduction

The prediction of the three-dimensional structure of a membrane protein (MP) from its sequence requires an understanding of two fundamental issues: mechanisms of the biological assembly of MPs and the principles behind the physical stability of MPs in their natural lipid bilayer milieu. The physical principles behind MP stability have recently been addressed in several reviews [1–3], as have the mechanisms of assembly [4–6]. During the past year or two, exceptional progress has been made toward understanding important details of how the machinery of MP assembly works. This progress is the focus of this review, which emphasizes the assembly of α-helical MPs.

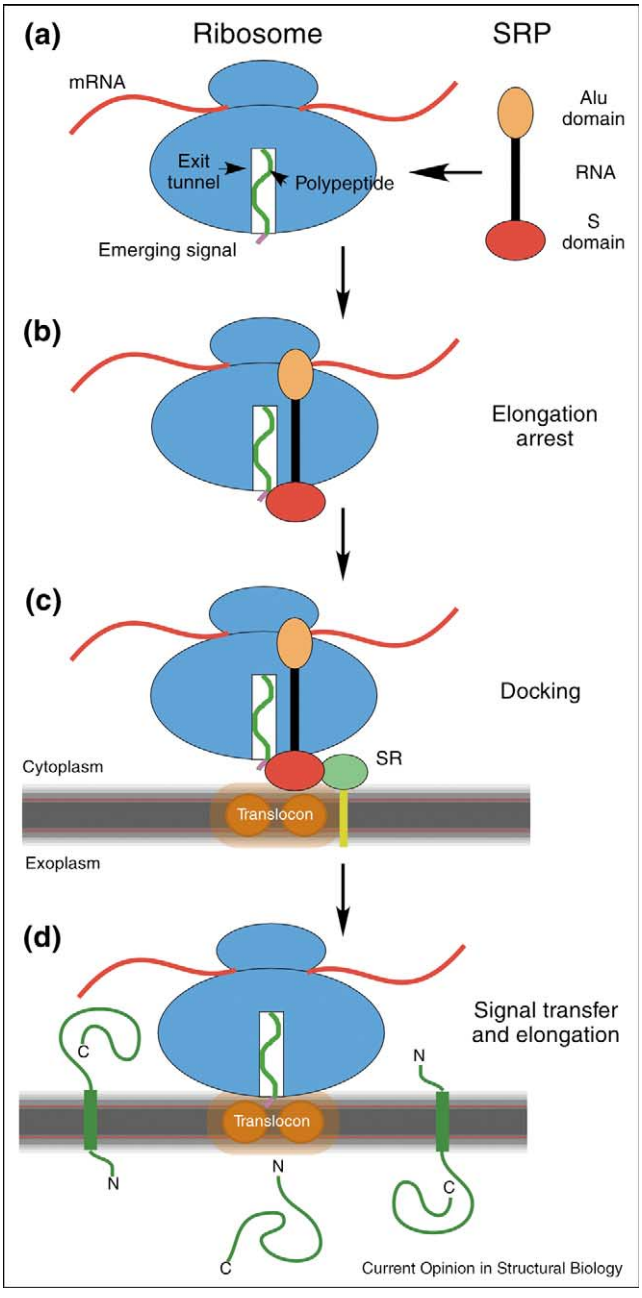
Proteins destined for export (translocation) across or insertion into membranes are generally managed by the concerted action of translating ribosomes in the cytoplasm and translocons located in the endoplasmic reticulum (ER) of eukaryotes or the plasma membrane of bacteria. The management principles are summarized in Figure 1. This highly schematic figure directs attention to several important advances discussed in this review. These advances concern three important questions: what is the structure of the translocon (also called the protein-conducting channel); how is the topology of MPs established; and to what extent are nascent proteins folded while in the exit tunnel of the ribosome? The logical starting point for addressing these questions is the structure of a protein-conducting channel, described for the first time at atomic resolution at the beginning of 2004.

## Translocon structure

The translocon is composed of a heterotrimeric protein called Sec61 in eukaryotes and SecY in bacteria. Early cryo-EM image reconstructions [7–9] of translocons and ribosome–translocon complexes revealed a translocon with a diameter of about 100 Å and, apparently, a central ~20 Å diameter transmembrane (TM) passageway for nascent proteins, suggesting that the functional translocon is composed of about three to four SecY/Sec61 complexes. A crucial issue arose regarding how a tight seal is maintained to prevent ion leakage across the membrane. Various biochemical and biophysical evidence suggested that the ribosome forms a tight seal with the translocon on the cytoplasmic side of the membrane; other proteins, such as BiP, provide a seal on the opposite side (reviewed in [4]). On the other hand, more recent low-resolution image reconstructions [10,11•] suggested, as did one of the earlier reconstructions [9], that the ribosome does not form a tight seal with the translocon. The lack of a tight seal raised the possibility that, somehow, the translocon is self-sealing. The apparent lack of an obvious large diameter tunnel in these reconstructions and in an 8 Å electron crystallographic structure [12•] supported this view.

The 3.8 Å crystallographic structure (Figure 2) of the SecY complex from *Methanococcus jannaschii*, published by van den Berg [13••], also supports this new view of translocon function. Furthermore, it suggests that translocon-mediated protein export and membrane insertion is a solo act, involving, at any particular time, only one of the SecY/Sec61 complexes that may, in a tetrameric arrangement (Figure 2a, upper right), form the ~100 Å

**Figure 1**




The machinery of membrane protein assembly. **(a)** A ribosome translating the mRNA of a protein targeted for export (translocation) across or insertion into membranes. An SRP, which is a GTPase, can bind to the ribosome and thereby arrest elongation. The structures of ribosomes are reviewed in [31–33] and the structure of SRP in [39,40]. **(b)** The ribosome and SRP recognize the emerging signal (purple), bind to each other and cause arrest of elongation. A cryo-EM structure of an elongation-arrested ribosome with bound SRP, solved to 12 Å resolution, has been recently published [41•]. **(c)** The ribosome–SRP complex binds to membrane-bound SR (green), another GTPase, which associates dynamically with the translocon (orange). Prokaryotes use a simplified SRP (Ffh) and SR (FtsY). Recent three-dimensional structures of the Ffh–FtsY complex [42•,43•] show that the two molecules form a quasi-twofold symmetrical dimer. The binding of SRP to SR causes reciprocal stimulation of their GTPase activities. As a result, the SRP disengages from the SR and the ribosome, the nascent protein signal is transferred to the translocon and elongation of the nascent peptide resumes. **(d)** Proteins targeted for translocation are secreted into the periplasm (bacteria) or ER lumen (eukaryotes), whereas the stop-transfer signals of MPs are transferred to the membrane bilayer. The direction of insertion of the signal sequence N terminus across the membrane determines the topology of the MP (see Figure 3a). However, this picture is not complete for bacteria, mitochondria and chloroplasts. An additional membrane protein (YidC, Oxa1 and Alb3, respectively) can be important for proper protein insertion and/or folding (reviewed in [44,45]). Nagamori *et al.* [46] recently showed that the membrane transport protein lactose permease (LacY) is inserted into the plasma membrane of *E. coli* in the absence of YidC, but with an incorrect fold. If YidC is present, then LacY folds correctly.

diameter tunnel-like structure seen in image reconstructions. The authors present strong arguments supporting the arrangement of SecY complex monomers shown in the figure, if indeed the structure is a tetramer. They note that the probable passageways out of the monomers into the bilayer (see below) face away from the tetramer's center of symmetry and that, at the symmetry axis of the tetramer, the surfaces of the monomers are highly hydrophobic. They suggest that any 'hole' at the center of symmetry is likely to be filled with lipids rather than water.

Figure 2a shows top (viewed normal to the membrane), front (viewed parallel to the membrane) and back views of the α, β and γ subunits comprising the SecY protein-conducting channel. No nascent peptide is observed in the structure, which is assumed to be in the closed state. The ten TM helices of the α subunit are arranged to form an inverted 'U' (Figure 2a, top view), with TM helices 1–5 (colored green in Figure 2a) forming one leg and helices 6–10 (colored orange) forming the other. The two sets of helices have a pseudo-symmetric arrangement around a front-to-back twofold rotation axis in the plane of the membrane and are connected at the back by an external loop. This loop and the single TM helix of the γ subunit, which runs diagonally across the back of the α subunit, largely prevent lipids contacting the helices of the α subunit from the backside. The unrestricted front of the molecule thus provides the only possible opening from the interior into the lipid bilayer. This opening is protected by TM helices 2 and 7 (light green and light orange, respectively), which form the edges of the so-called lateral gate; this is hypothesized to control the passage of nascent TM helices into the bilayer. Biochemical evidence discussed by van den Berg *et al.* [13••] implicates helices 2 and 7, and parts of adjacent helices 3 and 8 as the binding site for signal sequences and, presumably, stop-transfer sequences (see below). A modest rearrangement of these helices permitted the authors to model, rather easily, in this location a nascent chain as an α helix. Interestingly, a short helical segment (helix 2A) that immediately precedes helix 2 acts as an apparent 'plug' to block the passage of small molecules through the

translocon in the closed state. The authors hypothesize that the plug is displaced by nascent protein translocation. The plug sits just below the waist of an hourglass-shaped vestibule that provides access from either side of the protein-conducting channel into the protein interior. The waist of the hourglass is formed by a ring of hydrophobic residues (hydrophobic collar, Figure 2b) that is hypothesized to form a seal around translocating nascent chains.

The structures of both the protein-conducting channel and the hypothesized lateral gate that opens to allow the passage of TM helices into the lipid membrane are consistent with cross-linking studies [14–16] of nascent chains, and suggest that TM helices integrate into the membrane by simple partitioning between the translocon and the membrane. In such a scheme, sufficiently hydrophobic helices would prefer the bilayer, whereas more polar helices would favor the translocon and ultimately the aqueous phase. That is, the translocon and lipid bilayer would work in concert to decipher the code for TM helices embedded in the amino acid sequence. If this view is correct, then the next big question concerns the code and the details of the deciphering process. Answers to this question should lead to major improvements in the prediction of membrane protein structure.

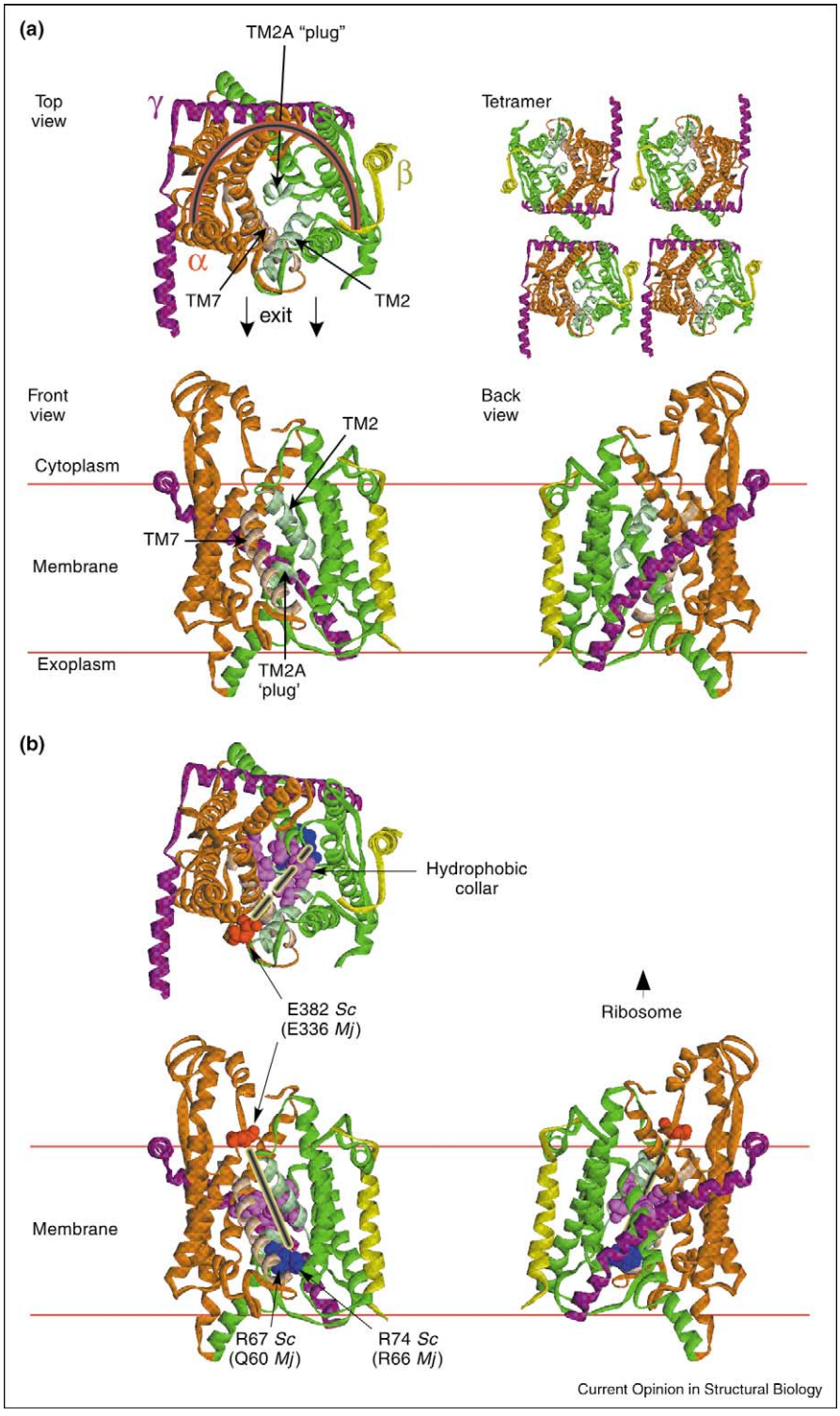
## Establishing membrane protein topology

The topology of α-helical MPs, whether single-span or multispan, is apparently established during the passage of nascent chains through the translocon (reviewed in [17]). The three principal types of single-span MPs are shown in Figure 3. In very broad terms, the topology follows the so-called positive-inside rule [18,19]. For bacterial MPs, there is a very strong statistical preference (about threefold) for positively charged residues to be present in cytoplasmic loops rather than periplasmic loops. This has generally been attributed to the transmembrane potential (negative in the cytoplasm relative to the periplasm; however, a TM potential is apparently not required for membrane synthesis [20]) and to the fact that negatively charged lipids are more abundant in the cytoplasmic bilayer leaflet. A similar bias is seen for ER, mitochondria and chloroplasts [21,22]. Hartmann *et al.* [23] found that the net charge difference between sequence segments lying on either end of a hydrophobic TM signal-sequence segment correlates best with the orientation for ER MPs and studies in yeast support this contention [24]. As summarized in Figure 3a, the flanking segment with the greater positive charge is generally cytoplasmic. Importantly, changing the flanking charges by site-directed mutagenesis can reverse the topology of a segment (reviewed in [17]). However, other features of signal-sequence segments, such as the length and hydrophobicity, are also important.

Spiess and colleagues have recently reported experiments that help clarify two questions about how MP topology is established within the translocon. The first question addressed is whether the translocon itself dictates the orientation of the TM segments. Hypothesizing that negatively charged residues in the cytoplasmic loops and/or positively charged residues in the exoplasmic loops of *Saccharomyces cerevisiae* Sec61p (equivalent to the Sec61 α subunit in mammals) interact directly with the flanking charges of a hydrophobic signal segment, Goder *et al.* [25••] mutated numerous charged residues in these regions to residues of the opposite charge. Using a model protein with an N-terminal signal anchor (type II, Figure 3a), they found that several charge inversions (R67E, R74E, E382R) caused a strong shift of the signal anchor orientation from the normal $N_{cyt}/C_{exo}$ orientation to $N_{exo}/C_{cyt}$, consistent with their hypothesis. Where are these residues located in the context of the *M. jannaschii* SecY α subunit structure of van den Berg *et al.* [13••]? To find out, we first established from the Sec61/SecY sequence alignments of van den Berg *et al.* (see supplement S1 of [13••]) the corresponding *M. jannaschii* SecY α subunit residues: Q60 (~R67), R66 (~R74) and E336 (~E382). These equivalent residues are identified in Figure 2b, where they have been connected by a broad yellow-and-black line. This line runs rather precisely from E336 at the top of TM helix 8 (part of the lateral gate), through the hydrophobic collar, and terminates at Q60 and R66, which are in close proximity to one another on TM helix 2A. The distance between E336 and Q60/R66 is 33 Å, which is about the thickness of the hydrocarbon core of a lipid bilayer. This path corresponds closely to the axis of the hydrophobic signal sequence modeled into the SecY α subunit by van den Berg *et al.* (see Figure 5 in [13••]). It is intriguing that Q60 and R66 are part of the so-called plug that obscures the presumed exit of the translocon in the absence of a nascent protein. Is it possible that these two residues participate in the displacement of the plug by nascent helices?

The second question addressed by Spiess and colleagues concerns getting the signal anchor to orient in the correct direction within the translocon and thus set MP topology. The natural direction of movement of a nascent chain is for the N terminus to move from cytoplasm toward exoplasm. So, the orientations of type I and type III MPs seem expected. But what about the type II MPs and even the cleavable sequence of type I MPs? Their orientations seem contrary to the natural direction of movement. Goder and Spiess [26••] hypothesized that all signal sequences enter the translocon N terminus first, meaning that type II signal anchors must reorient in the course of translocation. To study this hypothesis, Goder and Spiess used a collection of chimeric model type II signal anchor proteins with C-terminal sequences ranging in length from 100 to 580 residues. They found that the percentage of proteins with translocated C termini increased linearly from about 20% for a C-terminal length of 100 residues to a plateau of about 55% for C-terminal

**Figure 2**




Structure of the translocon (SecYαβγ) from *M. jannaschii* (*Mj*), determined by van den Berg *et al*. [13••] to a resolution of 3.2 Å. The images shown are based upon PDB coordinates 1RHZ and were prepared using DS ViewerPro$^{TM}$ 5.0 by Accelrys, Inc. No nascent peptide is seen in the structure, which is assumed to be in the closed state. **(a)** General structural features. The top view (along the membrane normal) shows the inverted 'U' arrangement (broad red and black line) of TM helices 1–5 (green) and TM helices 6–10 (orange). An external connecting link between the two sets of helices and the TM helix of the γ subunit at the back of the structure largely prevent contact of lipids with the α-subunit helices from the backside. The unrestricted front of the subunit provides the only possible opening into the bilayer. However, in the closed state shown here, the opening is protected by TM helices 2 and 7 (light green and light orange, respectively), which form the edges of the so-called

lengths of 300 or more residues. Is this effect due to the length of the C-terminal sequence or to time (nascent polypeptides elongate at a constant rate of about five amino acids per second)? This question was answered by administering the reversible elongation inhibitor cyclohexamide, which caused the edge of the plateau to shift downward to C-terminal lengths of 150 residues and the percentage of C termini translocated to increase to about 80 from 55. The bottom line from the studies is that time rather than C-terminal length is the actual variable and that about 50 s are required for reorientation (inversion) of the signal anchor.

Additional studies showed that the inversion rate depends upon the hydrophobic length of the segment and the flanking charges: short segments (16–19 leucines) inverted faster than long ones (20–23 leucines); reducing the charge at the N terminus slowed down inversion. Importantly, regardless of the properties of the signal and the consequent rate of inversion, changes in topology were always complete in about 50 s. That is, the final topology is fixed immutably after ~50 s regardless of inversion rate, implying that the time required for fixing the topology is an inherent property of the translocation machinery.

An interesting feature of these studies is that, generally, topology is mixed. For example, in the time-course studies described above [26••], typically ~75% of the C termini inverted (but this can be pushed nearly to 100% by reducing the hydrophobic length and increasing the flanking charges appropriately). Does this mean that the topologies of some MPs can be mixed under native physiological conditions? One tantalizing case is provided by the recently determined three-dimensional structure of the EmrE multidrug resistance transporter from *Escherichia coli* [27•]. This protein appears to be a homotetramer comprising two conformational heterodimers related by a pseudo-twofold symmetry axis perpendicular to the cell membrane (Figure 3b). This arrangement creates a structure with a mixed topology not previously seen in three-dimensional structures of MPs: two of the dimers have their N termini on the cytoplasmic side of the membrane, whereas the other two have them on the periplasmic side (green arrows, Figure 3b). If the crystal structure is faithful to the *in situ* structure, and it may not be [28–30], then the question arises as to how the mixed topology could come about. One possibility is that both topologies are produced by the translocation machinery. Alternatively, it could be that the mixed topology is produced post-translationally by strong physical interactions amongst the monomers. Our basic understanding of MP folding will be challenged regardless of which explanation is correct.

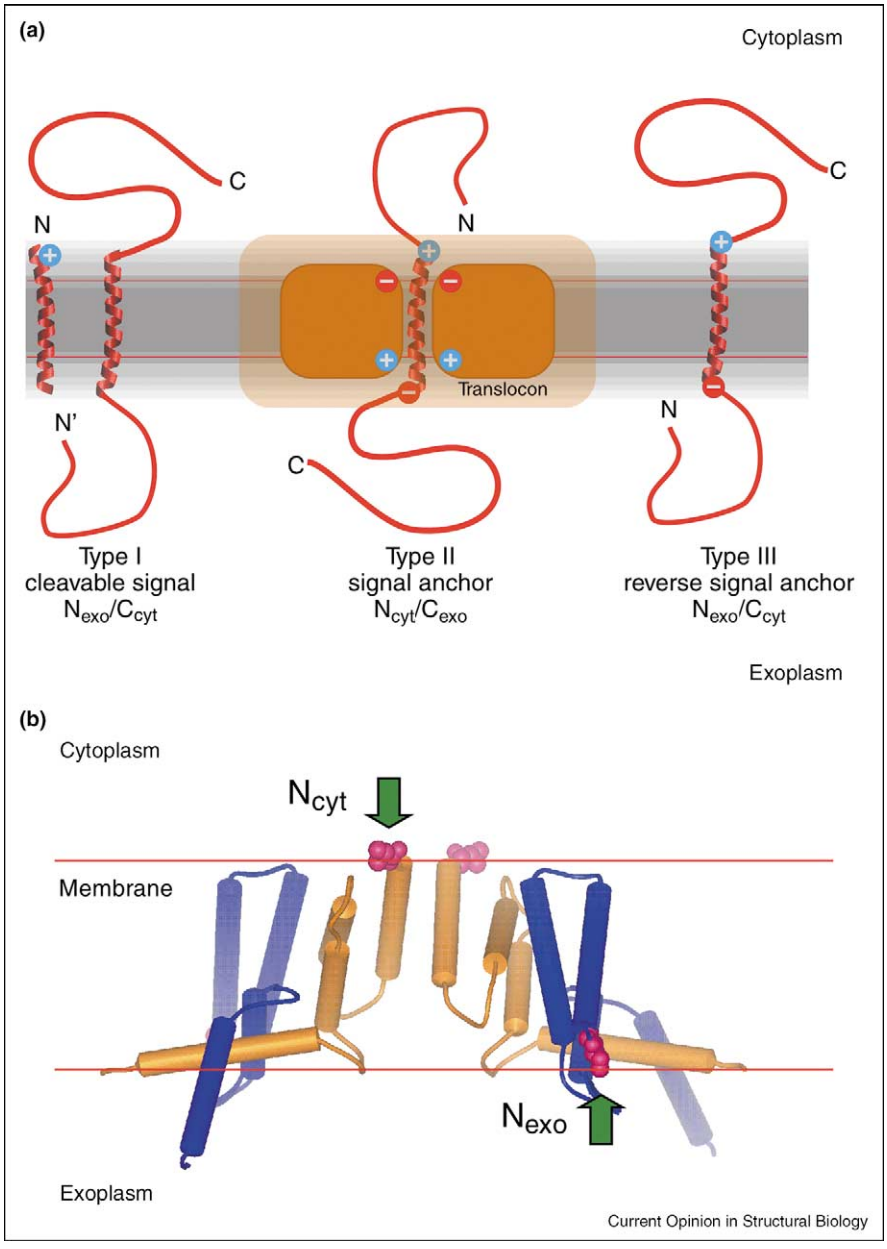
## Folding in the ribosome exit tunnel

A question that bears strongly on translocon function is the state of the nascent chain as it emerges from the ribosome. Is the chain extended or folded? Does the translocon receive nascent polypeptides as extended chains or as α helices? These questions can be meaningfully addressed now that high-resolution crystallographic structures of ribosomes are available (reviewed in [31–33]). Two papers, recently reviewed [34], provide insights, but also some confusion. Gilbert *et al.* [35•] have examined by cryo-EM the three-dimensional structures of ribosomes caught in the act (by using truncated mRNA lacking an in-frame stop codon) of translating three different cytosolic proteins. By comparing these images with control images, the authors were able to construct difference maps that revealed additional density in the exit tunnels of stalled translating ribosomes that could convincingly be interpreted as nascent proteins. The dimensions of these densities were consistent not with extended but rather with partially compacted chains, suggestive of preliminary folding in the tunnel of the proteins, all of which had β-barrel folds (Ig, Ig2 and green fluorescent protein). Initial interpretations of ribosome structures [36] concluded that the average tunnel diameter of 15 Å was too small to accommodate a folded protein, but later structural measurements suggested that the ribosome is highly dynamic, switching between compact and looser states with accompanying rearrangements of ribosomal proteins [37]. This may explain how the ribosome tunnel can provide space for nascent chain folding in the course of translation.

Because no α-helical proteins were used in the study of Gilbert *et al.* [35•], one cannot know if the compaction of nascent proteins in the tunnel is a general phenomenon applicable to all proteins or if MPs are handled differently to soluble proteins. Woolhead *et al.* [38•] have approached these issues by using Förster resonance energy transfer (FRET) to examine the in-tunnel folding of TM sequences and nascent secreted sequences. The idea of the experiments is straightforward. Donor–acceptor

**(Figure 2 Legend Continued)** lateral gate. A short helix, TM2A, seems to act as 'plug' that closes the secretion pathway into the exoplasm. Low-resolution cryo-EM images [7–9] of translocon–ribosome assemblies suggested a translocon with a diameter of ~100 Å, which may represent a tetrameter of SecYαβγ complexes. Because SecYαβγ apparently forms dimers by back-to-back contact, each SecYαβγ in a tetramer would have to open into the membrane bilayer rather than into the center of symmetry. **(b)** Notable features of the SecY α subunit [coloring and views as in (a)]. A closely juxtaposed group of aliphatic residues (mostly isoleucine) forms a hydrophobic collar (magenta) in the center. This collar may act as a seal around nascent polypeptides. The broad yellow-and-black line connects E336 to Q60/R66, which are the cognate residues of E382 and R67/R74 in Sec61p from *S. cerevisiae* (*Sc*). These residues apparently play a major role in determining the orientation of hydrophobic stop-transfer sequences [25••] and hence MP topology (see text and Figure 3). The yellow-and-black line, which passes directly through the hydrophobic collar, has a length of ~33 Å.

**Figure 3**




Types of single TM helix MPs and the structure of the EmrE multidrug efflux transporter. **(a)** Three types of MPs defined according to the location of the N terminus and the cleavability of the signal. Signals generally consist of a run of membrane-spanning hydrophobic residues flanked by charged residues. In eukaryotes, the balance of flanking charges determines the topology of the signal. The flank with the greater positive charge is generally on the cytoplasmic side of the membrane. Goder *et al.* [25••] present evidence that interactions of the flanking charges with charged residues in the loops of SecYα/Sec61p play a major role in orienting the signal (see Figure 2). **(b)** Structure [27•] of the EmrE multidrug resistance efflux transporter from *E. coli* shown in cartoon format with depth cueing. Image prepared from PDB coordinates 1S7B using DS ViewerPro$^{TM}$ 5.0 by Accelrys, Inc. The unusual feature of the structure is that the dimer-of-dimers configuration has N termini on both sides of the membrane (green arrows). If the structure *in vivo* corresponds to the crystallographic structure, then one must explain the origin of the dual topology (see text).

FRET partners were placed 24 residues apart by using fluorescent non-natural amino acids introduced into the nascent sequence. The FRET partners were chosen so that they would have high FRET efficiency if a helix formed, but low efficiency if extended. The authors present FRET data supporting the conclusion that TM segments form α helices in the tunnel, whereas secretory proteins do not. At face value, the apparent lack of folding of the secretory proteins suggests that the compaction of soluble proteins observed by Gilbert *et al.* [35•] is not

a universal phenomenon. Using cross-linking methods, Woolhead *et al.* [38•] found that two ribosomal proteins could cross-link to TM segments, but not to nascent secretory segments. This was interpreted as meaning that these proteins were able to recognize TM segments and thereby MPs. This raises the possibility that ribosomal recognition of nascent TM segments might control the translocon's 'operational mode'.

## Conclusions

The three-dimensional structure of a protein-conducting channel shows that SecY/Sec61 probably acts as a monomer in the incorporation of TM segments into membranes and that TM helix incorporation into membranes is a result of the simple partitioning of potential TM segments between the translocon and the membrane bilayer. The translocon helps establish MP topology through direct electrostatic interactions of some of its charged residues with flanking charges of stop-transfer sequences. Biochemical investigations of the kinetics of TM helix incorporation suggest that all stop-transfer sequences, regardless of final topology, enter the translocon N terminus first and then subsequently rearrange over a period of about 50 s to achieve their final TM orientation. Emerging evidence suggests that secondary structure may be formed during the passage of the nascent chain through the ribosome exit tunnel. Together, these structural and biochemical studies lay a solid foundation for an eventual detailed understanding of how the translocon and membrane bilayer work in concert to decipher the folding instructions embedded in the MP amino acid sequence. This will be a fruitful area for study in the next several years.

## Acknowledgements

This work was supported by grants from the National Institute of General Medical Sciences (GM46823 and GM68002) to SHW, and from the Swedish Cancer Foundation, the Swedish Research Council, and the Marianne and Marcus Wallenberg Foundation to GvH.

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