Current Topics

Topogenesis of Membrane Proteins at the Endoplasmic Reticulum†

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ABSTRACT: Most eukaryotic membrane proteins are cotranslationally integrated into the endoplasmic reticulum membrane by the Sec61 translocation complex. They are targeted to the translocon by hydrophobic signal sequences, which induce the translocation of either their N- or their C-terminal sequence. Signal sequence orientation is largely determined by charged residues flanking the apolar sequence (the positive-inside rule), folding properties of the N-terminal segment, and the hydrophobicity of the signal. Recent in vivo experiments suggest that N-terminal signals initially insert into the translocon head-on to yield a translocated N-terminus. Driven by a local electrical potential, the signal may invert its orientation and translocate the C-terminal sequence. Increased hydrophobicity slows down inversion by stabilizing the initial bound state. In vitro cross-linking studies indicate that signals rapidly contact lipids upon entering the translocon. Together with the recent crystal structure of the homologous SecYEβ translocation complex of *Methanococcus jannaschii*, which did not reveal an obvious hydrophobic binding site for signals within the pore, a model emerges in which the translocon allows the lateral partitioning of hydrophobic segments between the aqueous pore and the lipid membrane. Signals may return into the pore for reorientation until translation is terminated. Subsequent transmembrane segments in multispanning proteins behave similarly and contribute to the overall topology of the protein.

Few membranes in a eukaryotic cell are competent to translocate and integrate proteins synthesized by the ribosomes in the cytoplasm: mitochondria, chloroplasts, peroxisomes, and—most prominently—the endoplasmic reticulum (ER).[1] The ER serves as the gateway for proteins destined for all compartments of the secretory pathway, for the plasma membrane and the cell exterior, and for the endocytic organelles. The signals for protein targeting to the ER are highly degenerate. Their essence is an uncharged, predominantly hydrophobic stretch of 7−25 amino acids (*1*). They not only are important for targeting to the ER membrane but also play a role in protein topogenesis. In some cases, they also anchor the polypeptide as a transmembrane domain and assemble into helix bundles, contributing to the structure and function of complex membrane proteins.

In cotranslational targeting, which is the predominant mode of ER sorting in mammalian cells, a signal sequence is first recognized by signal recognition particle (SRP). As it emerges from the ribosome, it binds to a hydrophobic groove or saddle created by a cluster of methionines on the 54-kD subunit (SRP54) (*2*−*4*). The ribosome−nascent chain−SRP complex is directed to the ER membrane by interaction with the SRP receptor (*5*). Both SRP and SRP receptor are GTPases that interact in a unique manner by forming a shared catalytic chamber for the two GTP nucleotides (*6, 7*). Reciprocal GTPase activation upon the release of the signal from SRP triggers disassembly of the targeting complex. The ribosome docks onto the translocon aligning the ribosomal exit tunnel with the protein-conducting channel (*8, 9*). The signal enters the translocon and is oriented with respect to the membrane to initiate translocation of its N- or C-terminal sequence across the membrane. The respective hydrophilic portion of the polypeptide is transferred through the channel into the ER lumen, and the signal is released laterally into the lipid bilayer. Additional hydrophobic segments may stop or reinitiate protein transfer and integrate as transmembrane domains into the membrane to generate multispanning helix-bundle proteins. These processes determine the topology of proteins in the lipid bilayer.

In the 30 years since the discovery of ER signals (*10, 11*), determinants of protein topology have been characterized by mutagenesis of substrate proteins (*12*), and the components of the translocation machinery have been discovered by genetic and biochemical studies (*13*). Sophisticated cross-linking experiments identified molecules in contact with various parts of substrate proteins (*14*). These mostly static data together with the recent first crystal structure of a protein-conducting channel (*15*) lead to new insights into the highly dynamic process of protein topogenesis and membrane integration.

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[1] Abbreviations: ER, endoplasmic reticulum; N_{cyt}/C_{exo}, cytoplasmic N- and exoplasmic C-terminus; SRP, signal recognition particle; TRAM, translocating chain-associated membrane protein.

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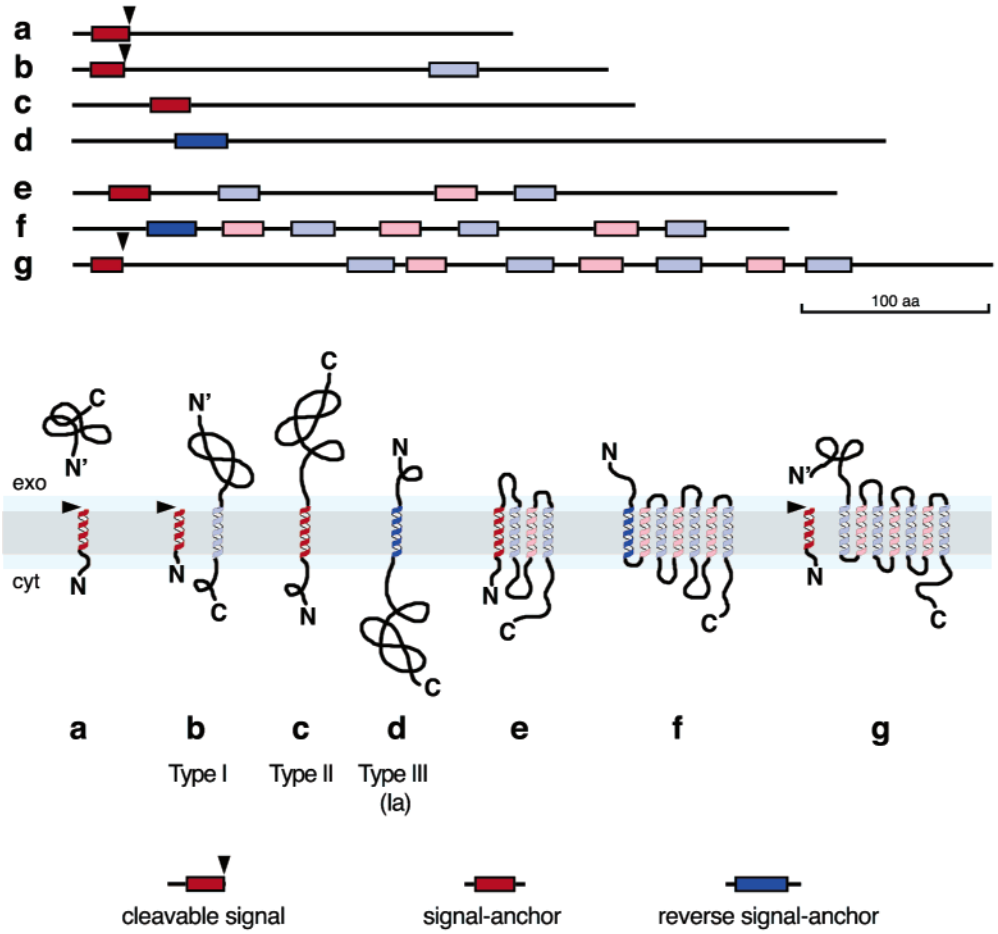

FIGURE 1: Three types of signals initiate cotranslational protein topogenesis. Cleavable signals (red with arrowhead indicating the signal peptidase cleavage site) and uncleaved signal-anchor sequences (red without arrowhead) induce translocation of the C-terminal sequence and assume an N_{cyt}/C_{exo} orientation. Reverse signal anchors (blue) insert with the opposite N_{exo}/C_{cyt} orientation and translocate their N-terminus. More complex topologies are produced by the combination of the signal with additional transmembrane segments inserting in alternating orientations (light red for N_{cyt}/C_{exo} and light blue for N_{exo}/C_{cyt}). The distribution of hydrophobic signal and transmembrane segments and their orientation in the membrane are shown for a secretory protein (a, preprolactin), a type I membrane protein (d, cation-dependent mannose-6-phosphate receptor), a type II membrane protein (b, asialoglycoprotein receptor), and a type III membrane protein (c, synaptotagmin I), and for examples of multispanning membrane proteins with corresponding initial signal sequences (e, gap junction protein α6; f, vasopressin receptor V2; g, glucagon receptor).

Orienting Signal Sequences in the Membrane

In secretory and single-spanning membrane proteins, topology is determined by the orientation of the signal sequence in the membrane. Cleavable signals of secretory proteins (Figure 1a) or type I membrane proteins (Figure 1b) initiate translocation of their C-terminal sequence. Signal peptidase cleaves off these signals and generates new lumenal N-termini (*16*). Signal anchors of type II membrane proteins (Figure 1c) similarly translocate their C-terminus. They are not necessarily at the very N-terminus of the protein, remain uncleaved, and have a longer apolar segment to span the hydrophobic core of the bilayer with an N_{cyt}/C_{exo} orientation (cytoplasmic N-, exoplasmic C-terminus) in the completed protein. Very likely, even cleaved signals integrate into the lipid membrane. Signal peptide peptidase, an intramembrane protease, was shown to process signal peptides within the membrane producing soluble fragments with potential signaling function (*17*−*19*). In contrast to cleavable signals and signal anchors, reverse signal anchors of type III proteins (also classified as type Ia) insert with an N_{exo}/C_{cyt} orientation and induce translocation of the N-terminus (Figure 1d).

Several factors have been shown to determine the orientation of the signal in the membrane. Most prominently, charged residues flanking the hydrophobic core of the signal influence orientation: the more positive end is generally cytosolic, a phenomenon known as the "positive-inside rule" (*20*−*23*). Since there is no general electrical potential across the ER membrane, local charges at the translocation apparatus must be involved in orienting the signal sequence (see below). In addition, folding of hydrophilic sequences N-terminal to a signal sterically hinders N-terminal translocation irrespective of the flanking charges (*24*). The polypeptide needs to be unfolded to be transferred through the translocation channel. A third determinant is the hydrophobicity of the core of the signal sequence (the h-domain) itself. Strongly hydrophobic signals were observed to insert with N_{exo}/C_{cyt} orientation even when the flanking charges were more positive at the N-terminus (*25*−*27*). How hydrophobicity exerts its topogenic effect was less obvious.

The mechanism by which hydrophobicity affects signal orientation was explained in a recent in vivo study by Goder and Spiess (*28*). An N-terminal signal-anchor with a generic h-domain of 22 leucine residues inserted with mixed orienta-

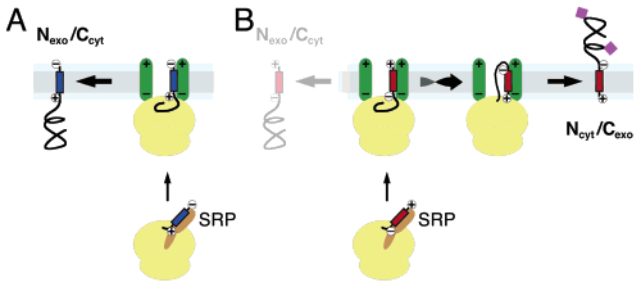

FIGURE 2: Insertion mechanism for N-terminal signal-anchor and reverse signal-anchor sequences. Reverse signal-anchor (panel A, blue) and signal-anchor sequences (panel B, red) initially insert into the translocation apparatus to yield an N_{exo}/C_{cyt} orientation (*28*). The charge distribution of signal anchors (positive N-terminus, negative C-terminus) drives their inversion to an N_{cyt}/C_{exo} orientation resulting in C-terminal translocation (and potentially glycosylation, shown by purple diamonds). Inversion is accelerated by increased charge difference ($\Delta N-C$), slowed by increased hydrophobicity of the signal core, and stopped upon translation termination or after ~50 s (*28*). Signal anchors that have not inverted when reorientation is blocked result in products with the "wrong" N_{exo}/C_{cyt} orientation (grayed-out portion). The process is illustrated schematically. The SRP receptor was omitted for simplicity.

tions despite a positive N-terminus. Surprisingly, the topology depended on the total length of the protein: the fraction of polypeptides with an N_{cyt}/C_{exo} orientation was lowest for a short protein and increased up to ~300 residues following the signal sequence. This result indicated that N-terminal signals initially insert to yield an N_{exo}/C_{cyt} orientation (schematically shown in Figure 2). Driven by electrostatic forces, a signal anchor is inverted until protein synthesis is completed or until further reorientation is stopped after approximately 40−50 s by an as yet unknown mechanism. Increased N-terminal positive charge accelerated the kinetics of signal inversion, whereas reduced charge slowed them. Increased hydrophobicity of the h-domain, however, diminished the rate of inversion, whereas reduced hydrophobicity accelerated it. Thirteen or more consecutive leucines were necessary to trap a fraction of the polypeptides before they had oriented themselves according to their flanking charges. Most or all natural cleavable signals and signal anchors are less hydrophobic and thus invert within seconds, long before translation is completed. Hydrophobicity inhibits inversion because the signal cannot reorient when bound at the translocation apparatus, but only when dissociated. The more hydrophobic the signal, the higher is the affinity to the bound state and the lower the inversion rate.

The Sec61 Complex: The Gateway through and into the Membrane

The machinery for the translocation and insertion of proteins into the membrane had first been identified genetically in yeast as Sec61p, a membrane protein with 10 transmembrane domains (*29, 30*). The mammalian homolog, Sec61α, was found to be part of a complex with two smaller components Sec61β (Sbh1p in yeast) and Sec61γ (Sss1p), which span the membrane only once (*31, 32*). Sec61α$\beta\gamma$ is evolutionarily homologous to the bacterial translocation complex SecYEG (*33*). The Sec61 complex is sufficient for translocation and membrane integration of some proteins in reconstituted liposomes, whereas others require an additional component, the translocating chain-associated membrane protein (TRAM), which spans the membrane eight times (*32*). Although not necessary in the minimal reconstituted system, the lumenal chaperone BiP/Kar2p and the ER membrane protein Sec63p are required for cotranslational translocation in yeast (*34*). Sec63p is part of a complex with Sec62p, Sec71p, and Sec72p, which together with the Sec61 complex constitute the machinery for posttranslational translocation in yeast (*35*). Sec63p contains a J domain that recruits BiP to the lumenal exit site of the translocon. BiP binds to translocating polypeptides and, acting as a molecular ratchet, drives translocation (*36*). Probably, this mechanism is also functional for efficient cotranslational translocation. In general, binding of chaperones to polypeptide segments emerging into the ER lumen is likely to trap them there and fix the topology of the protein accordingly.

To characterize the machinery components and the environment of nascent polypeptides at various stages of translocation and insertion, photo-cross-linking techniques proved extremely powerful (*14*). Photoreactive probes were incorporated by in vitro translation at defined positions into nascent chains programmed by truncated mRNAs. Upon photolysis, molecules in close proximity to the probe were cross-linked to the arrested polypeptides. As the signal emerged from the ribosome, it was found in contact with SRP54 (*37, 38*). Upon docking of the ribosome−nascent chain complex to the ER membrane, cleavable signals, signal anchors, and reverse signal anchors were all cross-linked to Sec61α (*39*). The N-terminus of a cleavable signal was shown to contact TRAM (*40*). The hydrophilic polypeptide arrested in translocation was also found close to Sec61α (*40*) while in an aqueous environment (*41*), indicating that Sec61α is the major component forming the protein-conducting channel through the bilayer. A stop-transfer sequence was cross-linked to Sec61α and TRAM in what appeared to be an ordered succession of proteinaceaous environments with increasing polypeptide length (*42*). This led to a model of specific proteinaceaous binding sites for signal and transmembrane domains in the translocation apparatus.

However, the signal in an arrested nascent chain that was just long enough to reach into the translocon was found to contact not only Sec61α but also lipids (*43, 44*). It was proposed that the signal might be bound at the lateral exit site of the channel, simultaneously exposed to the aqueous pore, Sec61α, and lipids. Cross-linking patterns obtained with a reactive side chain in different positions in the h-domain suggested that the signal was in a helical conformation in stable contact with transmembrane helices 2 and 7 of Sec61α on one side and with lipid on the other (*45*). However, these findings are also consistent with the signal leaving rapidly into the lipid bilayer upon entering the translocon. Because it is still tethered to the translocon, cross-linking to Sec61α persists. Position-dependent cross-linking may reflect preferred contact surfaces on the outside of the translocation complex. Indeed, stop-transfer sequences were similarly found to cross-link to Sec61α and lipid as soon as they extended into the channel (*45, 46*). Different sequences were detected in different positions, in some cases adjacent also to TRAM, suggesting that transmembrane segments tethered to the translocation complex associate at various places to the outside of the pore complex (*47*). It should also be considered that experiments with arrested nascent polypeptides do not truly represent a time course. In vivo experiments

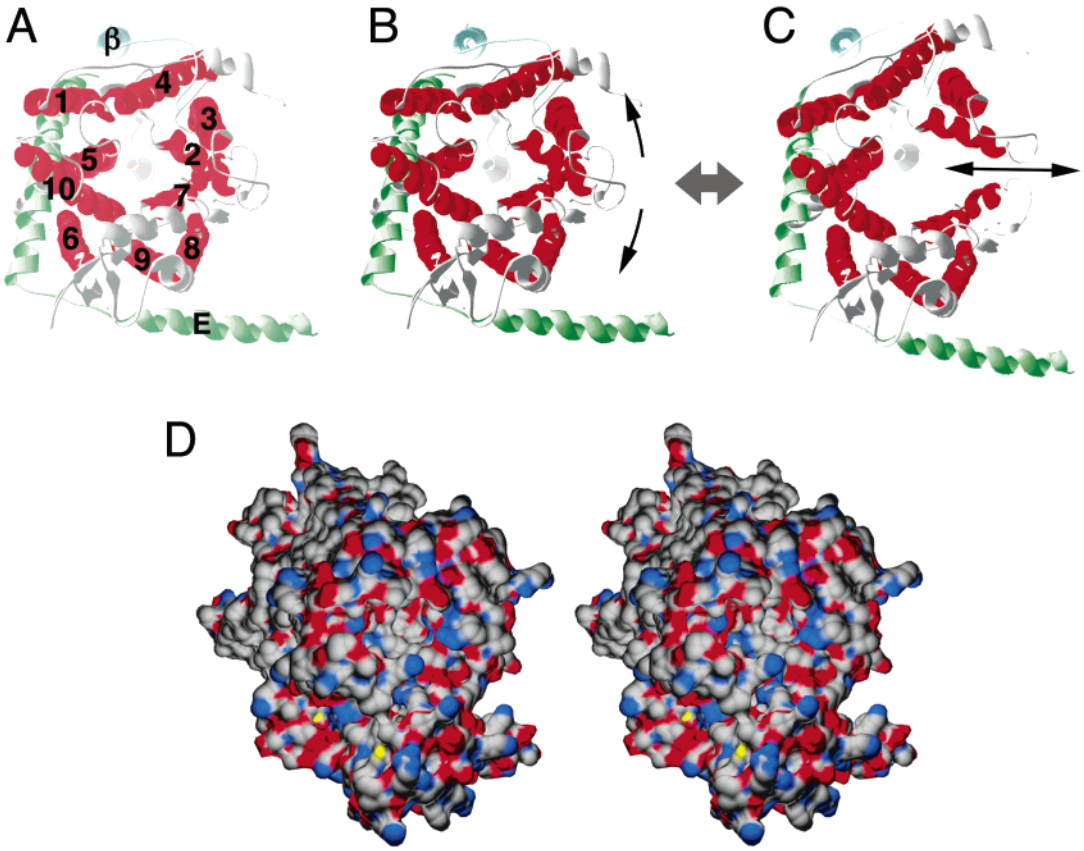

FIGURE 3: The translocation complex and its lateral exit site. The backbone structure of the *M. jannaschii* SecYEβ complex (Protein Data Base accession code 1RHZ) (*15*) is shown from the cytosolic side. SecY (corresponding to Sec61α) is shown in gray with its 10 transmembrane domains in red (numbered in panel A). The central hydrophilic pore is blocked by a short luminal helix that must move away to allow passage of a translocating polypeptide. SecE (Sec61γ) and the β subunit are shown in green and blue, respectively. To allow exit of a hydrophobic sequence into the lipid bilayer, the two pseudo-symmetric halves (helices 1−5 and 6−10) must open (arrows in panel B) hinging around the connection between helices 5 and 6 (schematically shown in panel C). Panel D shows a stereoview of the SecYEβ complex from the cytosolic side. The structure is slightly turned down in comparison to the view of panels A−C to better see into the hydrophilic pore, which in the closed state is blocked by the central constriction and the lumenal plug. Atoms are colored gray for C, blue for N, red for O, and yellow for S.

suggested that orientation of a signal anchor is terminated at the latest approximately 50 s after the signal emerged from the ribosome, even if translation is not yet completed (*28*). This period has certainly passed by the time of in vitro cross-linking. Cross-linking results are thus likely to reflect the situation of the signal after it has left the translocation pore for the lipid membrane, while still closely connected to the translocon via the nascent chain.

Recently, the crystal structure of the SecYEβ translocation complex of *Methanococcus jannaschii* has been determined (*15*). It suggests that a translocation pore is formed by a single SecYEβ complex rather than by three or four complexes as previously proposed based on electron microscopy of the yeast and mammalian translocons (*8, 9*). As a consequence, the hydrophilic pore is likely to be considerably less spacious than previously expected (*48*), even considering that the crystal structure is of the closed state. The 10 transmembrane helices of Sec61α form an aqueous channel with a central constriction of hydrophobic residues (Figure 3A). The channel is open to the cytosolic side, that is, to the ribosome, but plugged by a short helix inserted from the lumenal side. This plug has to move away, probably by turning out as a whole around a flexible hinge in the connecting sequences, to allow passage of a translocating polypeptide. The plug may also play a role in sealing the channel against ion loss from within the ER lumen while idle.

Most interestingly for topogenesis, there is no obvious hydrophobic surface lining the inside of SecY/Sec61α that could serve as a static recognition site for signal sequences (Figure 3D). The translocon is organized in two halves (transmembrane helices 1−5 and 6−10; Figure 3A). To laterally leave the channel toward the lipid membrane, a polypeptide has to pass between helices 2/3 and 7/8. A hydrophobic environment is accessible to a signal sequence in the pore only when the channel laterally opens, for example, due to thermal motion within the structure (Figure 3B,C). As the h-domain of the signal exits into the surrounding lipid, it will form a helix, optimizing intramolecular hydrogen bonds. Similarly, the signal might return into the hydrophilic channel where the peptide as a flexible chain may invert its orientation due to the flanking charges. According to the model illustrated in Figure 4, the Sec61 complex allows lateral equilibration of the signal between an aqueous and a transmembrane environment. The translating ribosome may facilitate the transient lateral opening of the pore. Upon termination of translation, re-entry of the signal may be hindered, resulting in the observed block of further topology changes. For translocation of the C-terminal sequence, the lumenal plug is shifted out of the way. This is

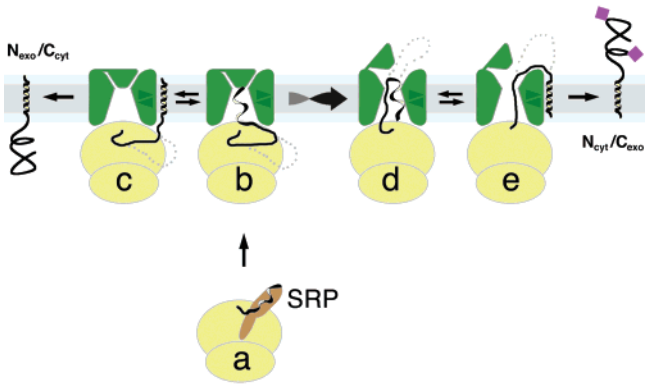

FIGURE 4: Model for signal orientation in the translocation complex. The signal of a nascent polypeptide is recognized by SRP (a) and targeted to the ER membrane via the SRP receptor (not shown). In the translocon, the signal reversibly partitions between the hydrophilic channel and the lipid environment via the lateral exit site (indicated by green arrowheads, b ↔ c and d ↔ e). The initial orientation is N_{exo}/C_{cyt} (b and c) because the polypeptide is too short to loop early in the process. Based on a local electrical potential and depending on the flanking charges, the signal may invert its orientation while in the channel (b → d) and enter the lipid in the N_{cyt}/C_{exo} topology (e). Translocation of hydrophilic sequences requires the opening of the lumenal plug (d and e). The kinetics of inversion are accelerated by increasing charge difference of the signal ($\Delta N-C$) and slowed by increasing hydrophobicity (shifting the equilibrium to the membrane-bound form). With further translation the growing peptide loops out (indicated by dotted lines) into the ER lumen or the cytoplasm depending on the signal orientation.

also necessary for translocation of the N-terminal domain of type III proteins with reverse signal anchors.

Charge interactions appear to provide the driving force for signal orientation. The role of conserved charged residues in Sec61p of yeast was tested by mutagenesis. Three mutations were identified that affect the topology of diagnostic substrates as expected when the positive-inside rule is weakened (*49*): two arginines at the lumenal plug and a glutamate at the cytosolic end of transmembrane domain 8. Although these three residues do not account for the entire charge effect in signal orientation, they show that Sec61p contributes to the positive-inside rule.

Topogenesis of Multispanning Membrane Proteins

In complex membrane proteins that span the membrane multiple times, it is generally the first hydrophobic sequence that targets the nascent protein to the ER membrane. This may be a cleavable signal, a signal anchor, or a reverse signal anchor, just as in single-spanning proteins (Figure 1e−g). Some members of the seven-transmembrane receptor family, for example, generate an exoplasmic N-terminus with a reverse signal anchor, whereas others (particularly those with large translocated N-terminal domains) employ a cleavable signal (f vs g). Subsequent transmembrane segments insert with alternating orientations. In the simplest case, their orientations are determined by that of the initial signal sequence. Indeed, signal anchors inserted downstream of a first cleavable signal or signal anchor can function perfectly as stop-transfer sequences. Artificial proteins spanning the membrane up to four times have been created by tandemly repeating identical copies of a signal anchor in a polypeptide separated by >100 amino acids from each other (*50*).

However, the topology of natural proteins is not just dictated by the first transmembrane domain but appears to be supported by contributions of downstream sequences. Statistically, internal transmembrane domains also follow the charge rule, although less stringently than the most N-terminal signal (*51*). Mutations designed to invert the orientation of the initial transmembrane domain of the glucose transporter Glut1 did not cause inversion of the entire protein but resulted in a local defect (*52*). Similarly, insertion of positive charge clusters into short exoplasmic loops of model proteins caused "frustration" of individual hydrophobic domains, showing that internal charges can be topogenically active, but did not affect the topology of the rest of the protein (*53*). In the case of the seven-transmembrane protein ProW, efficient translocation of the N-terminus required the presence of at least four transmembrane domains (*54*). The topology of multispanning proteins thus seems to be determined by a consensus of its segments.

Cooperation of topogenic determinants throughout the sequence could be accomplished by retaining and assembling the transmembrane domains within the translocation machinery before the completed protein is released as a whole into the membrane, as previously proposed (*55*). However, subsequent transmembrane domains could be cross-linked to lipid as soon as they reached the translocon (*43*, *44*, *46*), indicating that they exit into the membrane one-by-one. If downstream sequences overrule the initial topology of a transmembrane segment, it must be able to return back into the translocation pore to reorient itself.

Evidence for substantial polypeptide reorientation was obtained in in vivo experiments using a model protein with two conflicting topogenic sequences, a cleavable signal at the N-terminus and an internal signal anchor (*56*). When these two signals were separated by ≥80 residues, these spacer residues were translocated and the second hydrophobic segment functioned as a stop-transfer sequence. With shorter spacers, however, an increasing fraction of proteins inserted with a translocated C-terminus as dictated by the second signal. A glycosylation site in the spacer increased translocation of the spacer sequence. This indicates that the second hydrophobic sequence, by inserting in an N_{cyt}/C_{exo} orientation, forces the spacer of up to ~60 residues to return from the ER lumen to the cytosol, unless it is glycosylated. In the process, at least two hydrophilic polypeptide segments have to pass simultaneously through the translocation pore. A similar situation may underlie the generation of the various topologies of the prion protein (in particular those termed ^{Ntm}PrP and ^{Ctm}PrP, where a mildly hydrophobic sequence may integrate in either orientation) (*57*).

As one transmembrane segment after the other reversibly partitions into the lipid membrane, they may associate with each other and partially assemble before protein synthesis is completed. Membrane integration of weakly hydrophobic sequences was found to be stabilized by a preceding transmembrane segment, and the overall topology became more defined (*58*, *59*). Similarly, complementary charged residues in different transmembrane segments of the K^+ channel KAT1 were found to be required for the correct topology (*60*). Topogenesis and protein folding are thus not necessarily separable events. Helix bundling may start already during protein insertion and influence the resulting topology.

Outlook

The Sec61 translocon provides multiple functions: it constitutes a gated pore for the passage of hydrophilic polypeptides through the membrane barrier, it allows hydrophobic segments lateral access to the core of the lipid bilayer for integration as transmembrane helices, and it contributes to their orientation. It works with highly diverse substrate sequences and even de novo designed generic sequences. Other components are likely to act upon the translocation complex and regulate its properties. Regulatory roles have been suggested for the ribosome (*61*) and the lumenal chaperone BiP (*62*) in sealing the translocation pore either on the cytosolic or on the lumenal side to maintain ion gradients at the ER membrane (reviewed in more detail in ref *63*). It is likely that unassembled transmembrane domains of nascent proteins are taken care of by intramembrane chaperones (potentially TRAM or PAT-10 (*64*)). There are further indications that specific (particularly nonbilayer) lipids assist protein folding in the membrane as "lipochaperones" (*65*) and influence translocon function and topogenesis (*66*, *67*). The current challenge is to derive a molecular understanding of a highly dynamic process from relatively static experimental data such as cross-linking snapshots, endpoint topologies of model substrates, and structural data, the most recent milestone being the crystal structure of SecYEβ (*15*).

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