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Topical Review

Biogenesis of CFTR and other Polytopic Membrane Proteins: New Roles for the Ribosome-Translocon Complex

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Abstract. Polytopic protein biogenesis represents a critical, yet poorly understood area of modern biology with important implications for human disease. Inherited mutations in a growing array of membrane proteins frequently lead to improper folding and/or trafficking. The cystic fibrosis transmembrane conductance regulator (CFTR) is a primary example in which point mutations disrupt CFTR folding and lead to rapid degradation in the endoplasmic reticulum (ER). It has been difficult, however, to discern the mechanistic principles of such disorders, in part, because membrane protein folding takes place coincident with translation and within a highly specialized environment formed by the ribosome, Sec61 translocon, and the ER membrane. This ribosome-translocon complex (RTC) coordinates the synthesis, folding, orientation and integration of transmembrane segments across and into the ER membrane. At the same time, RTC function is controlled by specific sequence determinants within the nascent polypeptide. Recent studies of CFTR and other native membrane proteins have begun to define novel variations in translocation pathways and to elucidate the specific steps that establish complex topology. This article will attempt to reconcile advances in our understanding of protein biogenesis with emerging models of RTC function. In particular, it will emphasize how information within the nascent polypeptide is interpreted by and in turn controls RTC dynamics to generate the broad structural and functional diversity observed for naturally occurring membrane proteins.

Key words: Polytopic protein — Topogenesis — Translocon — Membrane protein — Biogenesis — Cystic fibrosis — CFTR

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Abbreviations: AQP, aquaporin; CFTR, cystic fibrosis transmembrane conductance regulator; ECL, extracellular loop; EM, electron microscopy; ER, endoplasmic reticulum; ICL, intracellular loop; PTC, peptidyltransferase center; RNC, ribosome-nascent chain; RTC, ribosome-translocon complex; SRP, signal recognition particle; SR, SRP receptor; TM, transmembrane (segment); TMD, transmembrane domain. ABC, ATP binding cassette; BiP, heavy chain binding protein; FRET, Förster resonance energy transfer; NBD, nucleotide binding domain; SPC, signal peptidase complex; TrAF, translocation-associated factors; TRAM, translocating chain-associated membrane protein; TRAP, translocon-associated protein.

Mechanism of Polypeptide Translocation and Membrane Integration

A major challenge in membrane protein biogenesis is to understand how the ribosome translocon complex (RTC) establishes topology of TM segments and their hydrophilic connecting loops during the initial stages of protein folding. While recent advances in RTC structure and function have addressed many long-standing questions regarding basic translocation mechanisms, important details of this process remain unresolved. This is particularly the case for polytopic proteins where even our primitive understanding of biogenesis events cannot be satisfactorily explained by current models. In the first part of this review, we discuss various ways in which the RTC is thought to facilitate nascent polypeptide translocation across and integration into lipid bilayer. We then address how prevailing models of RTC function might be used to explain recent observations of polytopic protein biogenesis using the ATP binding cassette (ABC) transporter CFTR as a primary example. Particular emphasis is given to areas where refinements are required in order to understand and predict protein topogenesis at a molecular level.

Most membrane proteins in mammalian cells are synthesized and folded at specialized sites in the rough endoplasmic reticulum (ER). This process begins when an ER signal sequence emerges from an actively translating ribosome and binds the signal recognition particle (SRP). SRP binding transiently stalls translation and docks the ribosome-nascent chain complex (RNC) at the SRP receptor (SR) on the ER membrane (Walter & Blobel, 1981; Keenan et al., 2001) (Fig. 1). Coordinated GTP hydrolysis by SRP and SR releases the signal sequence and transfers the RNC to the Sec61 ER translocation channel (translocon) (Song et al., 2000). As translation resumes, the signal sequence engages Sec61α (Plath et al., 1998), triggers a tight association between the ribosome and translocon (Jungnickel & Rapoport, 1995; Belin et al., 1996; Rapiejko & Gilmore, 1997), and opens the lumenal gate of the translocon channel by perturbing interactions with the lumenal chaperone BiP (Hamman, Hendershot & Johnson, 1998). This creates a continuous aqueous translocation pathway that extends from the ribosome exit tunnel through the translocon pore into the ER lumen (Crowley, Reinhart & Johnson, 1993; Crowley et al., 1994).

The eukaryotic translocon consists of a large protein complex in which the Sec61αβγ heterotrimer forms a central aqueous pore for protein translocation (reviewed in Johnson & van Waes, 1999; Schnell & Hebert, 2003). Early freeze fracture and subsequent cryo-EM studies of purified and ribosome-associated Sec61 complexes revealed the translocon to be an irregular ovoid disc ~100 Å (diameter) by 50 Å with an extensive (50 Å) lumenal projection and a large central pore (Hanein et al., 1996; Menetret et al., 2000; Beckmann et al., 2001; Morgan et al., 2002). These results supported fluorescence-quenching studies, which demonstrated that the translocating polypeptide moved through a large pore >40 Å in diameter (Hamman et al., 1997). In contrast, recent X-ray crystal structure has revealed that the archibacterial homolog of Sec61, SecYEβ, from *Methanococcus jannaschii* forms a ~45–50 Å cuboidal structure that contains a central 5–8 Å pore partially lined with flexible hydrophobic residues and a single lateral opening into the lipid bilayer (van den Berg et al., 2004). In its closed conformation, the pore is partially occluded by a short helix, which is proposed to be displaced outward during translocation. Molecular modeling has further suggested that lateral displacement of helices could increase pore size to 10–12 Å, which would be sufficient to accommodate a translocating nascent polypeptide (Clemons et al., 2004; van den Berg et al., 2004). Hence it has been proposed that the translocation pathway may be much smaller than previously thought.

Although basic aspects of translocation require only Sec61αβγ, SRP and SR (Gorlich & Rapoport, 1993), functional translocon complexes contain multiple Sec61 heterotrimers (Snapp et al., 2004) as well as additional proteins that modulate translocation and protein topology (Schroder et al., 1999; Wang & Dobberstein, 1999). Oligosaccharyltransferase (OST), is responsible for the lumenal attachment of N-linked sugars, whereas the translocating chain-associated membrane protein (TRAM) is involved in facilitating translocation (Gorlich et al., 1992; Gorlich & Rapoport, 1993; Hegde, Voigt & Lingappa, 1998b), formation of the ribosome-translocon junction (Voigt et al., 1996; Hegde et al., 1998a, c) and integration of TM segments (High et al., 1993; Martoglio et al., 1995; Do et al., 1996). The translocon-associated protein (TRAP) complex, originally called the signal sequence receptor (Prehn et al., 1990), was recently demonstrated to influence nascent chain orientation within the translocon and facilitate post-targeting translocation initiation (Fons, Bogert & Hegde, 2003). In detergent-solubilized RTCs, the translocon pore is directly aligned with the ribosome exit tunnel (Beckmann et al., 1997). However, the ribosome and translocon are separated by a 15–20 Å gap (Menetret et al., 2000; Beckmann et al., 2001; Morgan et al., 2002), which contradicts the tight ribosomal binding observed in biochemical studies (Jungnickel & Rapoport, 1995; Belin et al., 1996) and the presence of an ion-impermeant seal demonstrated by fluorescence quenching (*discussed below*) (Crowley et al., 1993, 1994). One possibility is that loss of lipids and/or translocon-associated proteins during detergent solubilization might account for the presence of such a gap. Growing evidence also suggests that stability of the ribosome-translocon junction may be physiologically regulated depending on specific properties of

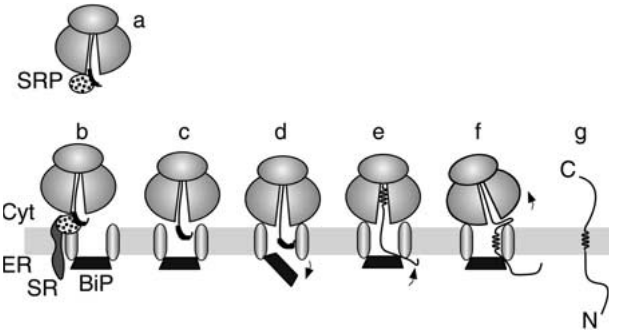

Fig. 1. Bitopic protein biogenesis. SRP (*checkered circle*) targets the RNC to the SRP receptor (SR) at the ER membrane (*a-b*). Translation resumes and the signal sequence (*bold line*) is released to engage and open the translocon lumenal gate maintained by BiP (*c-d*). N-terminal signal sequences are cleaved by the signal peptidase complex. The TM segment (*squiggle*) is recognized in the ribosomal tunnel where it first initiates closure of the lumenal side of the translocon (*e*) and then relaxes the ribosome-translocon junction to expose the polypeptide within the ribosome to the cytosol (*f*). Based on the mechanism of translocon gating described by Crowley et al. (Crowley et al., 1994; Liao et al., 1997).

the signal sequence and its dependence on translocation-associated factors (TrAFs) such as TRAM, or TRAP (Hegde & Lingappa, 1997, 1999; Hegde et al., 1998b). Further studies are needed to establish the structural details of ribosome binding to functional translocons actively engaged in protein translocation.

A unique feature of the RTC is that it simultaneously facilitates axial translocation of polypeptide into the ER lumen and lateral transfer of TM segments into the membrane while maintaining the permeability barrier between ER and cytosol. How this occurs is controversial. Structure of Sec YEβ would suggest that a flexible "gasket" composed of 6 hydrophobic side chains in the center of the heterotrimer pore provides a self-sealing passage large enough to accommodate an alpha-helix while blocking ion movement. In contrast, collisional quenching studies of fluorescent probes incorporated into intact and functional RTCs have demonstrated that translocation is controlled via a signaling pathway that precisely coordinates sequential gating at the lumenal and cytosolic faces of the translocon. Consistent with this, FRET studies have demonstrated that formation of TM helices deep within the ribosome exit tunnel (Woolhead, McCormick & Johnson, 2004) triggers a sequence of events that first closes the lumenal side of the translocon via ATP-dependent interactions with BiP and then relaxes the ribosome-translocon junction to allow nascent polypeptide access to the cytosol (Liao et al., 1997; Haigh & Johnson, 2002) (Fig. 1). Thus, TM segments appear to terminate translocation by transmitting information indirectly to the translocon via the ribosome. This implies that the ribosome monitors structural features of the nascent chain during synthesis and, upon detection of a TM segment, triggers conformational changes within the RTC that direct regions of polypeptide into their appropriate compartments.

Two slightly different models have also been proposed to explain how the translocon facilitates integration of TM segments into the lipid bilayer. In the first, TM segments passively partition between the translocon pore and the membrane based on their hydrophobicity (Heinrich et al., 2000). This is consistent with theoretical considerations (White, 2003) and cross-linking experiments that demonstrate rapid access of TM segments to membrane phospholipids (Martoglio et al., 1995; Mothes et al., 1997; Heinrich et al., 2000). However, other studies indicate that TM segment integration is regulated via protein-protein interactions within the RTC (Do et al., 1996). Instead of moving directly into the bilayer, some TM segments reside for extended periods within the translocon at the interface between Sec61α and TRAM even during synthesis of very long C-terminal domains (Do et al., 1996; Meacock et al., 2002; McCormick et al., 2003). For such proteins, TM segments leave the translocon and enter the lipid bilayer at specific points of synthesis (Meacock et al., 2002; H. Sadlish and W. Skach, unpublished observations) and/or at the termination of translation (Thrift et al., 1991; Do et al., 1996; McCormick et al., 2003).

Clearly, current studies do not present a single cohesive mechanism to explain how the RTC facilitates membrane protein biogenesis. Differences in the nature of the translocation pathway, the mechanism of gating and the process of membrane integration must be reconciled before a relatively complete understanding is reached. Some discrepancies likely arise from the use of different substrates, which may exhibit unanticipated effects on RTC structure, while other differences may result from specific technical approaches. Because fluorescent quenching studies were performed using intact and functional RTC complexes actively engaged in productive translocation, at present, they appear to provide the best view of true physiologic translocation intermediates and thus serve as a starting point with which to view translocon function and regulation. However, also included in our discussion are implications of models derived from recent structural studies.

Role of the RTC in Polytopic Protein Biogenesis

Data from a variety of approaches indicate that biogenesis of even simple membrane proteins is controlled by complex regulation of the RTC. How then is the RTC regulated during polytopic protein biogenesis when multiple TMs are presented in rapid succession? The simplest model, proposed more than 20 years ago (Blobel, 1980) and supported by experimental studies of chimeric proteins (Rothman et al., 1988; Lipp et al, 1989), suggested that polytopic proteins could be generated through the sequential action of independent signal (anchor) and stop transfer sequences. Thus, one might expect that translocon gating mechanics, as demonstrated for secretory and bitopic proteins, would apply directly to polytopic proteins. In this case, the first signal anchor would target the RNC to the membrane, gate the translocon open by releasing BiP, and translocate the first peptide loop into the ER lumen (Fig. 2). The following stop transfer sequence would subsequently close the lumenal gate, relax the ribosome-translocon junction, and orient the next peptide loop in the cytosol. The next signal anchor would re-establish the ribosome-translocon junction, reopen the gate and so forth. Alternate gating of the translocon to the ER lumen and cytosol, as dictated by the sequential presentation of topological determinants, would thus fulfill the requirement of properly orienting TM segments while maintaining the ER permeability barrier. This model, however, raises a number of unanswered questions.

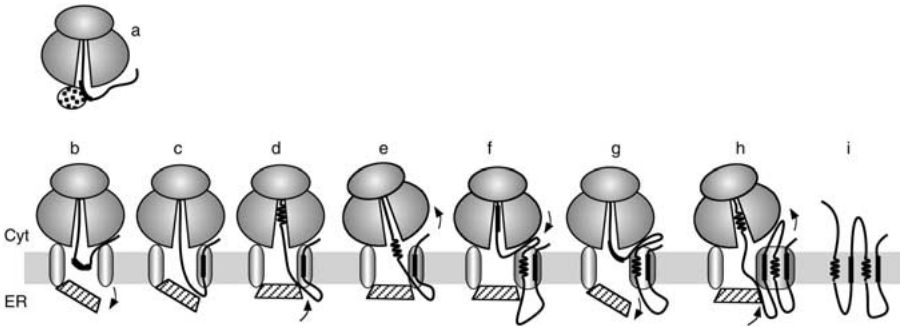

Fig. 2. Simple model of co-translational polytopic protein biogenesis. In the simplest model, ER targeting and translocation are initiated by a signal anchor sequence (*bold line*); (*a-b*). The second TM segment functions as a stop transfer (*squiggle*) to sequentially close the translocon from the lumen (*d*) and relax the ribosome translocon junction (*e*). This would establish topology of the ECL1 and allow ICL2 access to the cytosol. The next signal anchor sequence translocates ECL2 by re-opening the translocon, and re-establishing the ribosome-translocon seal (*f-g*). In this manner, signal anchor and stop transfer sequences could regulate sequential gating of the translocon to the lumen and cytosol, orient cytosolic and lumenal domains, maintain integrity of the ER permeability barrier, and position each TM segment sequentially in the bilayer.

First, we do not currently know how the presence of multiple topogenic determinants influences RTC function. For example, the model assumes that only one TM segment is present within the RTC at a time and that this segment dictates its own topology and hence topology of its flanking residues. For many native proteins, however, this is not the case because peptide loops connecting topogenic determinants are so short that synthesis of a TM segment (stop transfer sequence) would be completed before the preceding signal sequence (and peptide loop) could exit the RTC translocation pathway. In such cases, it is not clear when (or if) the translocon is gated closed to the lumen and how extracytoplasmic loops might pass through the translocon, particularly if TM segments cause translocon closure from within the ribosome exit tunnel. Alternatively, it is also unknown how the relatively small (10–12 Å) pore formed by the Sec61 heterotrimer could accommodate multiple TM segments that are closely tethered.

Second, translocation in most polytopic proteins is initiated by uncleaved signal anchor sequences, which start translocation like cleaved signals but also terminate translocation and integrate into the lipid bilayer (Lipp & Dobberstein, 1988). Signal anchors have the unique ability to translocate N- or C-terminal flanking residues (Spiess, 1995) and thus direct either an N_{lum}/C_{cyt} (type I) or an N_{cyt}/C_{lum} (type II) topology. Topology is in part governed by the ''positive-inside rule'' (von Heijne, 1986) as well as length and hydrophobicity of the TM segment and folding kinetics of flanking domains (Sakaguchi et al., 1992; Denzer, Nabholz & Spiess, 1995; Coder & Spiess, 2001). Because of the vectoral nature of translation, TM segments exit the ribosome in an N- to C-terminal direction. Recent studies have indicated that electrostatic interactions between charged residues flanking the signal anchor and charged residues within Sec61α are at least partially responsible for inverting TM segments after they have entered the translocon (Coder, Junne & Spiess, 2004). It is currently unknown how such an event would occur within the confines of the proposed Sec61αβγ pore. Similarly, models of translocon gating do not provide a satisfactory explanation as to how ''simultaneous'' translocation initiation and termination events would be carried out. Either lumenal and cytosolic gating of the RTC would have to be initiated by the signal anchor within the translocon itself, or the translocon channel would need to be sufficiently large to accommodate a 180° rotation of an entire TM helix and still maintain the ER membrane permeability barrier.

Third, if the translocation pore is formed by a single Sec61αβγ heterotrimer as proposed (van den Berg et al., 2004), then functional translocons, which contain multiple copies of Sec61, provide several pathways for translocation (Snapp et al., 2004). How then would a particular pathway be chosen? Electron microscopy of two-dimensional crystals have suggested that oligomers of the prokaryotic SecYEG (and by extension, Sec61αβγ) exist in a back-to-back configuration (Breyton et al., 2002; van den Berg et al., 2004). This places the lateral opening of each heterotrimer on opposite sides of the translocon nearly 80 Å apart, or alternatively, at least 40 Å apart in a side-side configuration. Thus, while sequential TMs could potentially enter into different Sec61 pores, short peptide loops would force native proteins to use a single translocation pathway during synthesis of most or all of its TM segments unless the functional architecture were assembled in a different configuration from that observed in the crystal lattice.

Mechanisms of CFTR Translocation and Topogenesis

To date, no studies have directly measured how topogenic determinants from native polytopic proteins regulate the RTC. Rather, RTC function has been inferred by the ability of determinants to direct translocation and integration of heterologous reporter domains as assayed by glycosylation, protease protection and membrane extraction. Although a relatively small number of native proteins have been examined to date, it is already clear that polytopic topology can be generated by diverse mechanisms. Not only are topogenic determinants arranged in unexpected patterns, but many determinants exhibit different properties from traditional signal (anchor) and stop transfer sequences (Audigier, Friedlander & Blobel, 1987; Skach and Lingappa, 1993; Lin & Addison, 1995; Xie et al., 1996; Lu et al., 1998; Moss et al., 1998). In this respect, ATP binding cassette transporters have been particularly informative in defining native protein biogenesis pathways. Many eukaryotic members exist as modular proteins that arose from gene duplication, whereby each half of the polypeptide is composed of a transmembrane domain (TMD) with 4 to 8 TM segments and a cytosolic nucleotide binding domain (NBD). In the case of CFTR and P-glycoprotein, the N- and C-terminal TMDs exhibit similar overall topology six TMs each, (Chen et al., 1986; Riordan et al., 1989) (Fig. 3), and this has allowed direct comparisons of their topogenic determinants and folding pathways. Because features of P-glycoprotein biogenesis have recently been described elsewhere (Anthony & Skach, 2002), this review will primarily focus on how topogenic determinants contained within CFTR direct its 12-TM-spanning topology, and address additional questions regarding general mechanisms of membrane protein topogenesis.

Studies of CFTR biogenesis have shown that even the initial stages of targeting and translocation do not follow the simple model outlined in Fig. 2. For example, TM1 and TM2 do not encode efficient signal and stop transfer determinants, respectively. Rather, TM1 functions very poorly as a type II signal anchor because two charged residues (Glu92 and Lys95) within the hydrophobic segment markedly impair its ability to engage SRP and/or Sec61α and initiate translocation (Xiong et al., 1997; Lu et al., 1998). As a result, TM1-2 topology is generated by two alternate translocation pathways illustrated in Fig. 4. In the first, TM1 initiates translocation in ~25% of nascent CFTR molecules. For this subset of polypeptides, TM2 terminates translocation to establish topology of the first extracellular loop (ECL1) and orients the first intracellular loop (ICL1) towards the cytosol (Fig. 4*A*). Although this topology is established cotranslationally, this process does not strictly follow the co-translational model. TM1

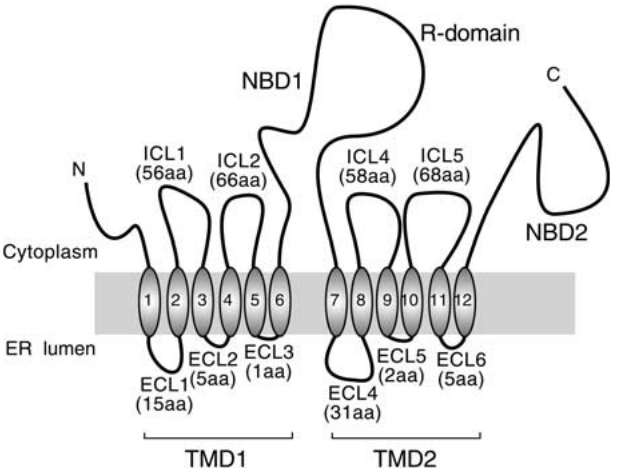

Fig. 3. Transmembrane topology of CFTR. Topology of transmembrane domains (*TMDs*), cytosolic nucleotide binding domains (*NBDs*) and the R-domain are indicated. TM segments are numbered from N-terminus. The predicted lengths of extracellular (ECL) and intracellular (ICL) loops are based on Riordan et al. 1989.

and TM2 are separated by only ~15 residues (Riordan et al., 1989), and ECL1 would therefore reside almost entirely within the 100 Å long ribosome exit tunnel when TM2 synthesis is completed. If TM2 triggered translocon closure from within the ribosome, then this action would prevent translocation of ECL1 through the aqueous Sec61 pore. If, on the other hand, ECL1 translocates through the pore, then closure of the translocon must be delayed until TM2 is entirely out of the ribosome exit tunnel and well within the translocon itself.

For the majority of CFTR polypeptides, translocation is not initiated by TM1, but rather by TM2, which functions as an efficient type I (N_{lum}/C_{cyt}) signal anchor sequence (Lu et al., 1998). This requires that TM2 must open the lumenal end of the translocon channel in order to direct translocation of its N-terminal hydrophilic flanking residues from the cytosol into the ER lumen (Fig. 4*B*). TM1 must then terminate translocation in order to span the membrane in its proper topology and keep the N-terminus in the cytosol (Lu et al., 1998). Remarkably, TM2 is able to direct this process even for naturally occurring CFTR mutants that completely lack TM1 signal sequence activity (Xiong et al., 1997). Because ECL1 (and TM1) enter the translocon only after initially emerging into the cytosol, this process has been referred to as a form of post-translational translocation because the nascent polypeptide does not enter the translocon directly from the ribosome exit tunnel (Perara, Rothman & Lingappa, 1986; Kanner et al., 2002). However, ECL1 translocation requires the presence of an attached ribosome and is hence likely SRP dependent (Lu et al., 1998). Importantly, this

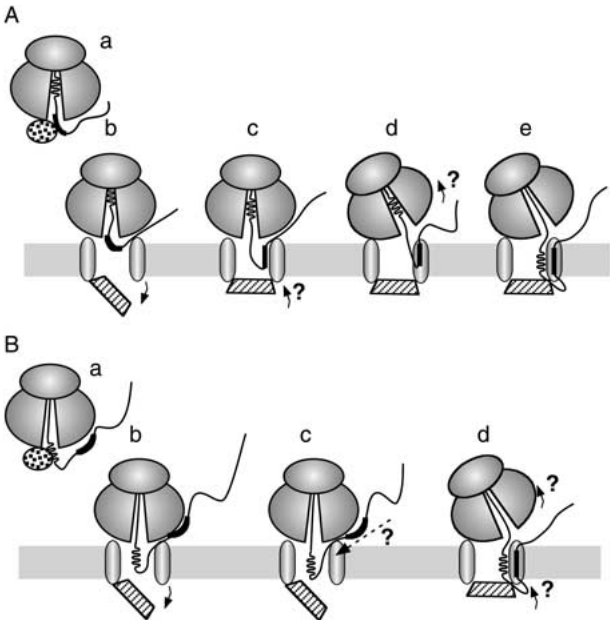

Fig. 4. Co- and post-translational mechanisms of CFTR N-terminus biogenesis. (*A*) In the cotranslational pathway, weak signal sequence activity of TM1 initiates translocation of ~25% of nascent CFTR polypeptides (*a-b*) and TM2 terminates translocation and establishes topology of ICL2 (*c-e*). (*B*) In the posttranslational pathway, TM2 initiates translocation of N-terminal-flanking residues after TM1 has already emerged from the ribosome (*a-b*), and subsequently directs TM1 into the translocon where it posttranslationally terminates translocation to span the membrane (*c-d*).Possible scenarios for translocon gating are shown, although details of this process are not known (adapted from Lu et al., 1998).

post-translational mechanism appears to be commonly used for other polytopic proteins where specific residues (band 3 protein, Ota et al., 1998), or structural constraints (Kv1.3 potassium channel, Tu et al., 2000) decrease signal (anchor) sequence activity of certain TM segments.

Note that while the same final topology is achieved via either the co-translational (Fig. 4*A*) or post-translational (Fig. 4*B*) pathways, the underlying mechanisms are dramatically different. Type I signal anchors such as TM2 must facilitate the post-translational movement of a hydrophilic, cytosolic N-terminus domain through the ribosome-translocon junction after ribosome docking and translocation have been initiated. Thus, the direction of polypeptide movement through the translocon directed by TM2 (C- to N-terminus) is opposite of that directed by TM1 (N- to C-terminus). TM2 must also facilitate movement of an upstream TM segment (TM1) into the translocon and ultimately into the bilayer. It is possible that translocation of ECL1 and TM1 occurs through the 20 Å gap between the ribosome and translocon (Menetret et al., 2000; Beckmann et al., 2001; Morgan et al., 2002). If so, then an alternate mechanism (such as the hydrophobic gasket) is required to maintain the ER membrane permeability barrier. It is also important to note that ECL1 and TM1 would be entering a translocon pore that already contains TM2. Accommodation of both TMs would require significantly more space than that provided by the 10–12 Å Sec61αβγ pore, even in the open and "extended" conformation. At present, the mechanistic details of how this posttranslational translocation process might be coordinated by the RTC remain unknown.

CFTR TM3 and TM4 independently exhibit weak signal anchor activities, and efficiently initiate ECL2 translocation only when both segments are present simultaneously (Zhang et al., 1998; Chen & Zhang, 1999; Carveth et al., 2002). Because the TM2-3 loop is ~56 residues in length, TM3 should enter the ribosome exit tunnel after topology of TM1-2 is established. TM3 and TM4, however, are connected by only 5 residues (ECL2) that could only reach the ER lumen after both TM segments are properly oriented within the translocon and/or membrane (Fig. 5). During this process the ribosome-translocon junction must also be relaxed to allow ICL2 (66 residues) access to the cytosol. One possibility is that ECL2 moves into the ER lumen during a brief opening of the translocon channel initiated by the combined presence of TM3-4. Alternatively, ECL2 translocation may not actually require an open translocon if the lumenal connecting loop is simply carried laterally out of the translocon during membrane integration. If this were true, then a minimum number of residues may be required to separate adjacent TM segments in order for translocon gating by BiP to occur. If, however, translocation proceeded through a single Sec61αβγ heterotrimer, it is difficult to imagine how the small translocon pore could simultaneously accommodate TM3 and TM4 in the hairpin configuration shown in Fig. 5. Similarly, adjacent Sec61 molecules could not engage separate helices with such a short connecting peptide loop in their proposed back-to-back arrangement.

The problem of accommodating multiple TM segments simultaneously is one faced by many native polytopic proteins. TM3 and TM4 of P-glycoprotein are also connected via a very short loop (4 residues, Chen et al., 1986) and appear to use a similar cooperative mechanism to translocate ECL2 (Skach & Lingappa, 1994; Zhang et al., 1998). CFTR TM5-6 are also closely spaced, being separated by only a single charged lysine residue (Riordan et al., 1989). In the latter case, TM5 exhibits independent type II signal anchor activity, and TM6 (together with its C-terminal flanking residues) efficiently terminates polypeptide translocation (Tector & Hartl, 1999; Carveth et al., 2002; and W. Skach, *unpublished observations*). While TM5 has the capacity to gate open the translocon and TM6 could potentially gate

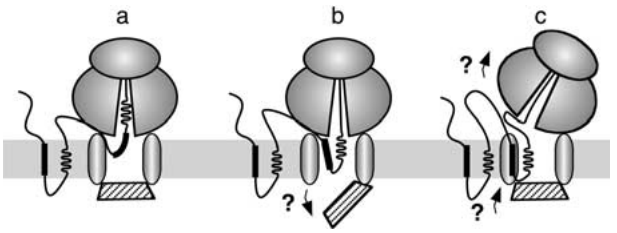

Fig. 5. Possible mechanism of CFTR TM3-4. TM3 and TM4 exit the ribosome in rapid succession and are simultaneously positioned within the RTC. It is unknown whether TM3 and TM4 play distinct roles in this process or act in unison to effect translocation and integration. Given that the TM3-4 loop is only 5 residues in length, it is unknown whether translocon opening is required.

it closed, this scenario is unfeasible, again because TM5-6 must establish topology simultaneously in a manner similar to TM3-4. Topogenic information from two very closely spaced TM segments may therefore act more like a single determinant and direct translocation through as yet undefined mechanisms. Given these considerations, additional refinements in translocon architecture are needed to satisfactorily explain how efficient translocation of TM pairs in CFTR and other proteins is carried out.

CFTR N- and C-terminal TM Domains Utilize Different Biogenesis Pathways

Despite their conserved topology, corresponding TM segments within CFTR TMD1 and TMD2 encode dissimilar topogenic information and use different translocation mechanisms to acquire their topology (Carveth et al., 2002). In contrast to TM1, TM7 functions as an efficient type II signal anchor sequence to initiate membrane targeting, translocation and integration. TM8, which is separated from TM7 by ~31 residues, terminates translocation to establish ECL4 topology and orient ICL4 in the cytosol. However, when TM8 was isolated and examined in a chimeric protein containing a cleaved N-terminal signal sequence, it was unable to independently stop translocation (Carveth et al., 2002). This was surprising because TM8 would be expected to span the membrane regardless of whether it followed TM7 or a cleaved signal sequence. Yet it only functioned as a stop transfer sequence when TM7 was present. It is possible that direct interactions with TM7 might influence TM8 stop transfer activity, although it is unknown how such an event might be mediated within the translocon itself. Alternatively, if TM7 and TM8 act at different locations within the translocation pathway, then this would indicate that a TM segment in the translocon (TM7) can influence recognition of a potential TM segment (TM8) within the ribosome. This raises the intriguing possibility that communication between the ribosome and translocon could be bidirectional.

A second unusual feature of TM7-8 topogenesis is that an N-linked glycosylation consensus site introduced just 4 residues from the predicted end of TM8 (Asn908) is efficiently utilized by oligosaccharyltransferase (Hammerle et al., 2000; Carveth et al., 2002). Because glycosylation is sterically hindered when consensus sites are less than 12–14 residues from the lipid bilayer (Nilsson & von Heijne, 1993; Popov et al., 1997), the N-terminus of TM8 appears to extend significantly farther into the ER lumen than expected. This could occur by one of three scenarios: i) if TM8 transiently passed through the translocon before integrating into the membrane, thus exposing Asn908 transiently to the active site of OST; ii) if the predicted boundaries of TM8 were incorrect; or iii) if TM8 entered the translocon lumen in an extended rather than helical conformation. Interestingly, removal of a single charged residue from TM8 (D924V) prevented Asn908 from being glycosylated and at the same time conferred TM7-independent stop transfer activity onto the TM8 hydrophobic segment (Carveth et al., 2002). The RTC therefore exhibits remarkable specificity in interpreting topogenic information. Individual residues within the translocation pathway (e.g., Asp924) can significantly impact how the RTC integrates information from multiple topogenic determinants as it directs early steps of protein folding and topology.

TM9-10 and TM11-12 encode signal anchor and stop transfer pairs, have very short extracellular loops, and likely orient simultaneously within the translocon in a manner similar to TM3-4 and TM5-6 (Carveth et al., 2002). CFTR therefore utilizes at least three distinct variations on cotranslocational translocation to establish topology of its 12 TMs. TM1-2 acquire their topology via a combination of co- and post-translational translocation pathways. Because of their short connecting loops, TMs 3-4, 5-6, 9-10 and 11-12 must either insert simultaneously into the translocon as anti-parallel helices, or both TMs must be accommodated within the translocon as the upstream TM segment flips into its N_{cyt}/C_{lum} topology. Finally, TM7-8 utilize a sequential signal-anchor stop transfer arrangement in which determinants function synergistically within the RTC to achieve their proper topology. In each case, current structural and functional models of the RTC provide only a partial explanation as to how the observed translocation and integration events might be carried out. A basic question in these studies is how the translocation pathway accommodates CFTR TM segments and how the translocon is gated when multiple determinants are presented in rapid succession.

Mutagenesis studies have raised some intriguing possibilities as to why CFTR utilizes several different translocation mechanisms to acquire its topology.

Removal of charged residues (Glu92 and Lys95) converts CFTR TM1 to a strong signal anchor sequence such that TM1-2 topogenesis occurs solely through the co-translational pathway (Lu et al., 1998). However, these CFTR mutants failed to generate chloride currents when expressed in *Xenopus* oocytes (K. Foskett and W. Skach, *unpublished observations*). Similarly, the D924V mutation converts TM8 to a strong, independent stop transfer sequence but dramatically decreases CFTR chloride conductance (D. Dawson and W. Skach, *unpublished observations*). Mutations that influence biogenesis pathways of P-glycoprotein (Moss et al., 1998) and aquaporin 1 (Foster et al., 2000) also have deleterious effects on protein function. Thus, sequence requirements needed to direct cotranslational translocation events appear to frequently conflict with sequence requirements needed for function in the final folded structure. By providing alternative folding pathways that maintain proper topology, the translocon appears to have provided a means to increase sequence diversity in native polytopic proteins. The intimate relationship between topogenesis and the RTC also suggests a remarkable co-evolutionary process that enables the translocon to efficiently interpret increasingly diverse determinants in native polytopic proteins.

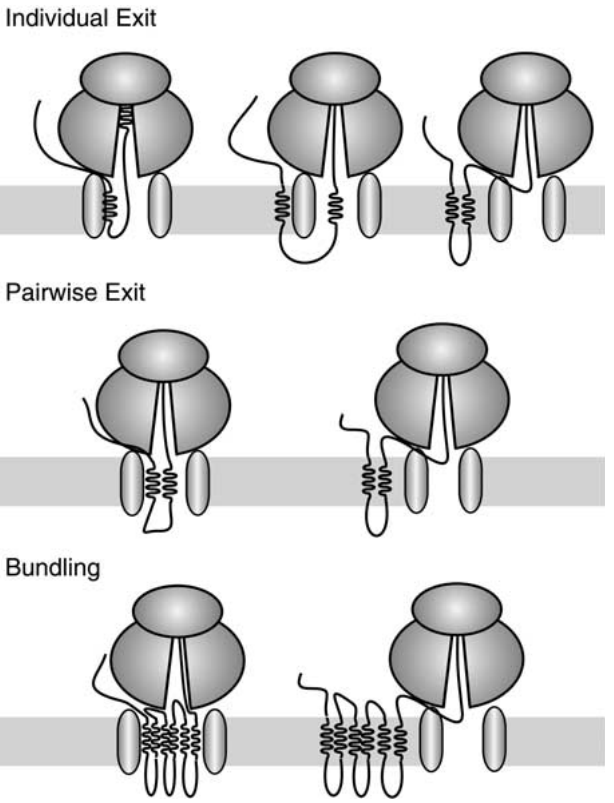

Fig. 6. Models of polytopic protein membrane integration. The integration of TM segments within a polytopic protein may occur individually as each segment is synthesized, in a pair-wise fashion, or in groups if one or more segments require intermolecular stabilization prior to integration into the lipid bilayer.

Polytopic Protein Membrane Integration

Once properly oriented within the translocon, TMs must laterally exit and integrate into the lipid bilayer before final stages of folding can be completed (Popot & Engelman, 2000). While little is currently known regarding mechanisms of CFTR integration, membrane extraction experiments of other polytopic proteins have revealed that not all TM segments can integrate into the membrane as pairs (Skach & Lingappa, 1993; Lin & Addison, 1995; Ota et al., 2000; Tu et al., 2000) or bundles, and multiple TMs can remain in an aqueous environment until the entire protein is released from the ribosome (Fig. 6) (Borel & Simon, 1996). Cross-linking studies have provided a more direct assessment of integration because they can be carried out in the context of intact programmed translocation intermediates. Using heterobifunctional cross-linking agents, TM1 and TM2 of opsin were shown to interact with Sec61α and Sec61β (Laird & High, 1997), and subsequently both TMs were observed to move through very specific molecular environments prior to entering the lipid bilayer (Meacock et al., 2002). Incorporated photo-active crosslinkers have also revealed that TM helices are not randomly oriented with respect to Sec61α, but rather are held in a fixed position within the translocon (Do et al., 1996; McCormick et al., 2003). Integration of a weakly hydrophobic TM segment can also be facilitated by an adjacent TM segment (Heinrich & Rapoport, 2003). Preliminary cross-linking studies from our group have extended these observations to show that all six TM helices from aquaporin 4 move through the translocon in an ordered and sequential manner (H. Sadlish, A. Johnson & W. Skach; *unpublished observations*). Remarkably, each TM segment was sequentially displaced from its initial site of entry by the next TM. After displacement, however, TMs did not move directly into the bilayer, but rather entered into multiple secondary and tertiary sites such that up to four TMs were simultaneous adjacent to Sec61α prior to their coordinated release. These results raise the possibility that the translocon may function as a scaffold to provide a sequestered and/or flexible environment for helices to sample different folding states until sufficient polypeptide has been synthesized for stable integration. If so, then the translocon provides not only a lateral passage to the lipid bilayer, but also a specific controlled environment that facilitates early folding interactions. This may explain why only a subset of TMs interacts with associated translocon components such as TRAM and PAT10 (Meacock et al., 2002; McCormick et al., 2003), while essentially all

TMs contact Sec61α during the initial stages of translocation.

Other Variations on Co-translational Protein Biogenesis

Topogenic determinants that regulate the RTC in a conflicting or inefficient manner often generate proteins with multiple or ambiguous topologies. This phenomenon was first described for artificially engineered polytopic proteins by von Heijne's group and referred to as "topological frustration" (Gafvelin & von Heijne, 1994). However, several examples have now been reported in native proteins (Dunlop, Jones & Finbow, 1995; Hegde et al., 1998b; Moss et al., 1998), and are also referred to by the more descriptive term, "topological heterogeneity". One well-characterized example of topological heterogeneity occurs in the multidrug transporter, P-glycoprotein. Studies in bacterial, cell-free, *Xenopus* and mammalian expression systems have revealed that the TM segments 7-10, within the C-terminal half of P-glycoprotein, can achieve two distinct conformations in the ER membrane (Fig. 7) (Zhang & Ling, 1991; Bibi, Gros & Kaback, 1993; Skach, Calayag & Lingappa, 1993; Loo & Clarke, 1999). This occurs because TM7 contains two cryptic topogenic determinants distributed over an extended hydrophobic region of 38 residues (Beja & Bibi, 1995; Moss et al., 1998). The N-terminal half of TM7 (TM7a) functions as an efficient signal sequence that initiates ER targeting and translocation, while the C-terminal half (TM7b) encodes an inefficient stop transfer sequence (Skach, Calayagg & Lingappa, 1993; Moss et al., 1998). In the presence of TM7a, TM7b terminates translocation and generates a hairpin topology (Fig. 7*B*). TM8 can function either as a stop transfer, or a type II signal anchor sequence. Thus, in cases when TM7b fails to terminate translocation, TM8 functions as a stop transfer sequence and spans the membrane in its predicted type I orientation (Fig. 7*A*). However, when TM7b stops translocation, TM8 signal anchor activity re-initiates translocation of its C-terminal flanking residues to generate an alternative topology (Fig. 7*B*).

Topological heterogeneity illustrates that information encoded within topogenic determinants, even in native polytopic proteins, is not necessarily absolute. Rather, topology is dependent upon the manner in which specific sequence determinants are presented to the RTC. For example, if TM7b terminates translocation, then TM8 is recognized by the ribosome and/or translocon as a signal anchor and re-initiates translocation. This is precisely what occurs when TM8 is presented to a closed translocon in the absence of TM7 (Moss et al., 1998) (Fig. 7*A*). On the other hand, when the RTC is in an actively translocating state, either opened by an N-terminal signal sequence or when TM7b fails to terminate translocation, TM8 functions as a stop transfer. The behavior of TM7 therefore, has a major impact on TM8 topology, but in a very different manner from that described above for CFTR. By initiating translocation of ICL4, TM8 actually antagonizes TM7b stop transfer activity, thereby contributing to the mixture of topological outcomes observed. The multiple topologies of P-glycoprotein arise from a delicate balance of topogenic information. Addition of basic residues to the C-terminus of TM7b or increasing TM7b hydrophobicity creates a better stop transfer sequence and overcomes TM8 signal anchor activity (Moss et al., 1998). In contrast, removal of the β-turn separating TM7a and TM7b inhibits TM7 from adapting a double-spanning topology and forces TM8 into its conventional type I orientation.

Examples of topological heterogeneity argue against a simple model in which complex topology is dictated by the independent function of local sequence determinants. Rather, topogenic information appears to be integrated over significant regions of the polypeptide that involve multiple determinants. At present we do not know whether such determinants exert their antagonistic (or cooperative) influences locally within the translocon channel, as would be predicted by a relatively passive model of translocation and integration (Clemons et al., 2004; van den Berg et al., 2004), or whether they act at different positions within the translocation pathway, which would imply close coordination of ribosome and translocon function, as demonstrated by fluorescence quenching studies (Johnson & van Waes, 1999). While the data seem to support this latter model, additional experiments are needed to determine precisely how and where topogenic determinants function during the biogenesis process.

Conclusions

CFTR and other native polytopic proteins utilize multiple variations in the mechanics of translocation to generate similar topologies for pairs and groups of TM segments. It would appear that these alternative mechanisms exist because TM segments must play dual roles in directing early events of topogenesis while later providing key structural elements for protein function. These two roles are often at odds, and as a result, topology is achieved through a variety of mechanisms that include co- and post-translational translocation events, co-operative translocation, co-operative integration of multiple TM segments, alternate topological outcomes, and post-translational re-orientation. It is likely that flexibility inherent in the ER translocation machinery has co-evolved with the sequence and functional diversity of

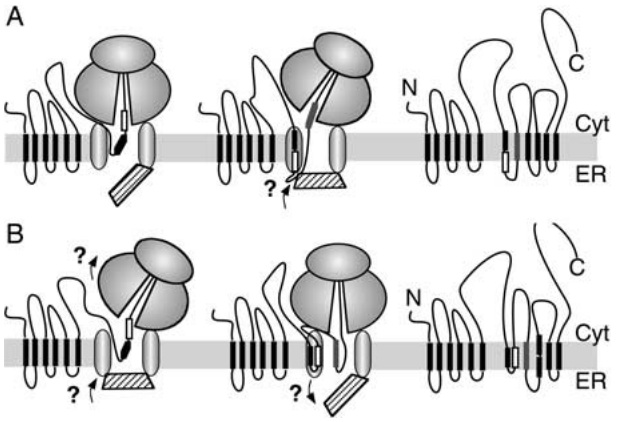

Fig. 7. Topological heterogeneity of P-glycoprotein. (*A*) TM7a/b (*black* and *white rectangles*, respectively) function as a signal sequence to form a single membrane-spanning segment. TM8 (*gray rectangle*) stop transfer activity terminates translocation to adopt a type I, conventional, topology. (*B*) When TM7a initiates translocation and TM7b terminates translocation, TM8 (*gray rectangle*) functions as a type II signal anchor sequence to generate an alternate topology. Note that like TM7a/b, TM9/10 can adopt a single- or double-spanning conformation. Depiction of translocon gating by TM8 is based on current models although the actual mechanism of gating remains unknown.

polytopic proteins, in order to provide a significant evolutionary advantage for the cell.

No single model can fully encompass the highly dynamic interplay observed between polytopic topogenic determinants and the RTC. In one view, the ribosome and translocon function as a cohesive machine in which gating is precisely coordinated at lumenal and cytosolic faces to maintain the ER permeability barrier. However, the mechanism by which these events are carried out in the case of polytopic proteins needs to be elucidated. A second view is that the translocon channel provides a self-sealing conduit relatively independent of the ribosome that allows the nascent polypeptide to passively orient and partition into the lipid bilayer based on local structural features. But such a model does not satisfactorily explain the translocation events observed for native polytopic proteins. These apparent paradoxes identify several important questions for future work. When and where do TM helices form within the translocation pathway? How do closely spaced TM pairs translocate and orient within the translocon prior to entering the membrane? How do signal anchor sequences facilitate their own entry and translocation of flanking residues within the translocon pore? When a determinant can function as both a stop transfer or a signal anchor sequence, how does the RTC decide the appropriate response? Further studies in these areas are likely to bring new insights and surprises as we expand our understanding of unique variations on mechanisms of protein translocation across the ER membrane.

References

Anthony, V., Skach, W.R. 2002. Molecular mechanism of P-glycoprotein assembly into cellular membranes. *Curr. Prot. Pept. Sci.* **3:**485–501

Audigier, Y., Friedlander, M., Blobel, G. 1987. Multiple topogenic sequences in bovine opsin. *Proc. Natl. Acad. Sci. USA* **84:**5783–5787

Beckmann, R., Bubeck, D., Grassucci, R., Penczek, P., Verschoor, A., Blobel, G., Frank, J. 1997. Alignment of conduits for the nascent polypeptide chain in the ribosome-Sec61 complex. [*see* comment]. *Science* **278:**2123–2126

Beckmann, R., Spahn, C.M., Eswar, N., Helmers, J., Penczek, P.A., Sali, A., Frank, J., Blobel, G. 2001. Architecture of the protein-conducting channel associated with the translating 80S ribosome. *Cell* **107:**361–372

Beja, O., Bibi, E. 1995. Multidrug resistance protein (Mdr)-alkaline phosphatase hybrids in Escherichia coli suggest a major revision in the topology of the C-terminal half of Mdr. *J. Biol. Chem.* **270:**12351–12354

Belin, D., Bost, S., Vassalli, J.-D., Strub, K. 1996. A two step recognition of signal sequences determines the translocation efficiency of proteins. *EMBO J.* **15:**468–478

Bibi, E., Gros, P., Kaback, H.R. 1993. Functional expression of mouse mdr1 in *Escherichia coli. Natl. Acad. Sci. USA* **90:**9209–9213

Blobel, G. 1980. Intracellular protein topogenesis. *Proc. Natl. Acad. Sci. USA* **77:**1496–1500

Borel, A.C., Simon, S.M. 1996. Biogenesis of polytopic membrane proteins: membrane segments assemble within translocation channels prior to membrane integration. *Cell* **85:**379 –389

Breyton, C., Haase, W., Rapoport, T., Kuehlbrandt, W., Collinson, I. 2002. Three-dimensional structure of the bacterial protein-translocation complex SecYEG. *Nature* **418:**662–665

Carveth, K., Buck, T., Anthony, V., Skach, W.R. 2002. Cooperativity and flexibility of cystic fibrosis transmembrane conductance regulator transmembrane segments participate in membrane localization of a charged residue. *J. Biol. Chem.* **277:**39507–39514

Chen, C.J., Chin, J.E., Ueda, K., Clark, D.P., Pastan, I., Gottesman, M.M., Roninson, I.B. 1986. Internal duplication and homology with bacterial transport proteins in the mdr1 (P-glycoprotein) gene from multidrug-resistant human cells. *Cell* **47:**381–389

Chen, M., Zhang, J.T. 1999. Topogenesis of cystic fibrosis transmembrane conductance regulator (CFTR): regulation by the amino terminal transmembrane sequences. *Biochemistry* **38:**5471–5477

Clemons, W., Menetret, J.-F., Akey, C., Rapoport, T. 2004. Structural insight into the protein translocation channel. *Curr. Opin. Struct. Biol.* **14:**390–396

Crowley, K.S., Liao, S., Worrell, V.E., Reinhart, G.D., Johnson, A.E. 1994. Secretory proteins move through the endoplasmic reticulum membrane via an aqueous, gated pore. *Cell* **78:**461–471

Crowley, K., Reinhart, G., Johnson, A. 1993. The signal sequence moves through a ribosomal tunnel into a noncytoplasmic aqueous environment at the ER membrane early in translocation. *Cell* **73:**1101–1115

Denzer, A., Nabholz, C., Spiess, M. 1995. Transmembrane orientation of signal-anchor proteins is affected by the folding state but not the size of the N-terminal domain. *EMBO J.* **14:**6311–6317

Do, H., Falcone, D., Lin, J., Andrews, D.W., Johnson, A.E. 1996. The cotranslational integration of membrane proteins into the phospholipid bilayer is a multistep process. *Cell* **85:**369–378

Dunlop, J., Jones, P.C., Finbow, M.E. 1995. Membrane insertion and assembly of ductin: a polytopic channel with dual orientations. *EMBO J.* **14:**3609–3616

Fons, R.D., Bogert, B.A., Hegde, R.S. 2003. Substrate-specific function of the translocon-associated protein complex during translocation across the ER membrane. *J. Cell Biol.* **160:**529–539

Foster W., Helm A., Turnbull, I., Gulati, H., Yang, B., Verkman, A.S., Skach, W.R. 2000. Identification of sequence determinants that direct different intracellular folding pathways for aquaporin-1 and aquaporin-4. *J. Biol. Chem,* **275:**34157–34165

Gafvelin, G., von Heijne, G. 1994. Topological 'frustration' in multispanning *E. coli* inner membrane proteins. *Cell* **77:**401–402

Goder, V., Junne, T., Spiess, M. 2004. Sec61p contributes to signal sequence orientation according to the positive inside rule. *Mol. Biol. Cell* **15:**1470–1478

Goder, V., Spiess, M. 2001. Topogenesis of membrane proetins: determinants and dynamics. *FEES Lett* **504:**87–93

Gorlich, D., Hartmann, E., Prehn, S., Rapoport, T.A. 1992. A protein of the endoplasmic reticulum involved early in polypeptide translocation. [see comment]. *Nature* **357:**47–52

Gorlich, D., Rapoport, T.A. 1993. Protein translocation into proteoliposomes reconstituted from purified components of the endoplasmic reticulum membrane, [see comment]. *Cell* **75:**615–630

Haigh, N.G., Johnson, A.E. 2002. A new role for BiP: closing the aqueous translocon pore during protein integration into the ER membrane. *J. Cell Biol.* **156:**261–270

Hamman, B.D., Chen, J.C., Johnson, E., Johnson, A.E. 1997. The aqueous pore through the translocon has a diameter of 40–60 Å during cotranslational protein translocation at the ER membrane. *Cell* **89:**535–544

Hamman, B.D., Hendershot, L.M., Johnson, A.E. 1998. BiP maintains the permeability barrier of the ER membrane by sealing the lumenal end of the translocon pore before and early in translocation. *Cell* **92:**747–758

Hammerle, M., Aleksandrov, A., Chang, X.B., Riordan, J.R. 2000. A novel CFTR disease-associated mutation causes addition of an extra N-linked oligosaccharide. *Glycoconjugate J.* **17:**807–813

Hanein, D., Matlack, K.E., Plath, K., Kalies, K.U., Miller, K.R., Rapoport, T.A., Akey, C.W. 1996. Oligomeric rings of the Sec61p complex induced by ligands required for protein translocation. [comment]. *Cell* **87:**721–732

Hegde, R.S., Lingappa, V.R. 1997. Membrane protein biogenesis: regulated complexity at the endoplasmic reticulum. *Cell* **91:**575–582

Hegde, R.S., Mastrianni, J.A., Scott, M.R., Defea, K.A., Tremblay, P., Torchia, M., Dearmond, S.J., Prusiner, S.B., Lingappa, V.R. 1998a. A transmembrane form of the prion protein in neurodegenerative disease. *Science* **279:**827–834

Hegde, R.S., Voigt, S., Lingappa, V.R. 1998b. Regulation of protein topology by trans-acting factors at the endoplasmic reticulum. *Mol. Cell* **2:**85–91

Hegde, R.S., Voigt, S., Rapoport, T.A., Lingappa, V.R. 1998c. TRAM regulates the exposure of nascent secretory proteins to the cytosol during translocation into the endoplasmic reticulum. *Cell* **92:**621–631

Heinrich, S.U., Mothes, W., Brunner, J., Rapoport, T.A. 2000. The Sec61p complex mediates the integration of a membrane protein by allowing lipid partitioning of the transmembrane domain. *Cell* **102:**233–244

Heinrich, S.U., Rapoport, T.A. 2003. Cooperation of transmembrane segments during the integration of a double-spanning protein into the ER membrane. *EMBO J.* **22:**3654–3663

High, S., Martoglio, B., Golrich, D., Andersen, S.S., Ashfore, A.J., Giner, A.E., Hartmann, E., Prehn, S., Rapoport, T.A., Dobberstein, B. 1993. Site-specific photocross-linking reveals that Sec61p and TRAM contact different regions of a membrane-inserted signal sequence. *J. Biol. Chem.* **268:**26745–26751

Johnson, A.E., van Waes, M.A. 1999. The translocon: a dynamic gateway at the ER membrane. *Ann. Rev. Cell Devel. Biol.* **15:**799–842

Jungnickel, B., Rapoport, T.A. 1995. A posttargeting signal sequence recognition event in the endoplasmic reticulum membrane. [see comment]. *Cell* **82:**261–2170

Kanner, E.M., Klein, I.K., Friedlander, M., Simon, S.M. 2002. The amino terminus of opsin translocates 'posttranslationally' as efficiently as cotranslationally. *Biochemistry* **41:**7707–7715

Keenan, R.J., Freymann, D.M., Stroud, R.M., Walter, P. 2001. The signal recognition particle. *Ann. Rev. Biochem.* **70:**755–775

Laird, V., High, S. 1997. Discrete cross-linking products identified during membrane protein biosynthesis. *J. Biol. Chem.* **72:**1983–1989

Liao, S., Lin, J., Do, H., Johnson, A.E. 1997. Both lumenal and cytosolic gating of the aqueous ER translocon pore are regulated from inside the ribosome during membrane protein integration. [comment]. *Cell* **90:**31–41

Lin, J., Addison, R. 1995. The membrane topology of the carboxyl-terminal third of the *Neurospora* plasma membrane H^+-ATPase. *J. Biol. Chem.* **270:**6942–6948

Lipp, J., Dobberstein, B. 1988. Signal and membrane anchor functions overlap in the type II membrane protein I gamma CAT. *J. Cell Biol.* **106:**1813–1820

Lipp, J., Flint, N., Haeuptle, M.T., Dobberstein, B. 1989. Structural requirements for membrane assembly of proteins spanning the membrane several times. *J. Cell Biol.* **109:**2013–2022

Loo, T.W., Clarke, D.M. 1999. Determining the structure and mechanism of the human multidrug resistance P-glycoprotein using cysteine-scanning mutagenesis and thiol-modification techniques. *Biochim. Biophys. Acta* **1461:**315–325

Lu, Y., Xiong, X., Helm, A., Kimani, K., Bragin, A., Skach, W.R. 1998. Co- and posttranslational translocation mechanisms direct cystic fibrosis transmembrane conductance regulator N terminus transmembrane assembly. *J. Biol. Chem.* **273:**568–576

Martoglio, B., Hofmann, M.W., Brunner, J., Dobberstein, B. 1995. The protein-conducting channel in the membrane of the endoplasmic reticulum is open laterally toward the lipid bilayer. *Cell* **81:**207–214

McCormick, P.J., Miao., Y., Shao, Y., Lin, J., Johnson, A.E. 2003. Cotranslational protein integration into the ER membrane is mediated by the binding of nascent chains to translocon proteins. *Mol. Cell* **12:**329–341

Meacock, S.L., Lecomte, F.J., Crawshaw, S.G., High, S. 2002. Different transmembrane domains associate with distinct endoplasmic reticulum components during membrane integration of a polytopic protein. *Mol. Biol. Cell* **13:**4114–4129

Menetret, J.F., Neuhof, A., Morgan, D.G., Plath, K., Radermacher, M., Rapoport, T.A., Akey, C.W. 2000. The structure of ribosome-channel complexes engaged in protein translocation. *Mol. Cell* **6:**1219–1232

Morgan, D.G., Menetret, J.F., Neuhof, A., Rapoport, T.A., Akey, C.W. 2002. Structure of the mammalian ribosome-channel complex at 17Å resolution. *J. Mol. Biol.* **324:**871–86

Moss, K., Helm, A., Lu, Y., Bragin, A., Skach, W.R. 1998. Coupled translocation events generate topological heterogeneity at the endoplasmic reticulum membrane. *Mol. Biol. Cell* **9:**2681–2697

Mothes, W., Heinrich, S.U., Graf, R., Nilsson, I., von Heijne, G., Brunner, J., Rapoport, T.A. 1997. Molecular mechanism of

membrane protein integration into the endoplasmic reticulum. *Cell* **89:**523–533

Nilsson, I.M., von Heijne, G. 1993. Determination of the distance between the oligosaccharyltransferase active site and the endoplasmic reticulum membrane. *J. Biol. Chem.* **268:**5798–5801

Ota, K., Sakaguchi, M., Hamasaki, N., Mihara, K. 1998. Assessment of topogenic functions of anticipated transmembrane segments of human band 3. *J. Biol. Chem.* **273:**28286–28291

Ota, K., Sakaguchi, M., Hamasaki, N., Mihara, K. 2000. Membrane integration of the second transmembrane segment of band 3 requires a closely apposed preceding signal-anchor sequence. *J. Biol. Chem.* **275:**29743–29748

Perara, E., Rothman, R., Lingappa, V.R. 1986. Uncoupling translocation from translation: implications for transport of proteins across membranes. *Science* **232:**348–352

Plath, E., Mothes, W., Wilkinson, B.M., Stirling, C.J., Rapoport, T.A. 1998. Signal sequence recognition in posttranslational protein transport across the yeast ER membrane. *Cell* **94:**795–807

Popot, J., Engelman, D. 2000. Helical membrane protein folding, stability and evolution. *Ann. Rev. Biochem.* **69:**881–922

Popov, M., Tam, L.Y., Li, J., Reithmeier, R.A. 1997. Mapping the ends of transmembrane segments in a polytopic membrane protein. Scanning N-glycosylation mutagenesis of extracytosolic loops in the anion exchanger, band 3. *J. Biol. Chem.* **272:**18325–18332

Prehn, S., Herz, J., Hartmann, E., Kurzchalia, T.V., Frank, R., Roemisch, K., Dobberstein, B., Rapoport, T.A. 1990. Structure and biosynthesis of the signal-sequence receptor. *Eur. J. Biochem.* **188:**439–445

Rapiejko, P.J., Gilmore, R. 1997. Empty site forms of the SRP54 and SR alpha GTPases mediate targeting of ribosome-nascent chain complexes to the endoplasmic reticulum.[see comment]. *Cell* **89:**703–713

Riordan, J.R., Rommens, J.M., Kerem, B., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J.L. 1989. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA.[erratum appears in Science 1989;245:1437]. *Science* **245:**1066–1073

Rothman, R. E., Andrews, D.W., Calayag, M.C., Lingappa, V.R. 1988. Construction of defined polytopic integral transmembrane proteins. *Biol. Chem.* **263:**10470–10480

Sakaguchi, M., Tomiyoshi, R., Kuroiowa, T., Mihara, K., Omura, T. 1992. Functions of signal and signal-anchor sequences are determined by the balance between the hydrophobic segment and N-terminal charge. *Proc. Natl. Acad. Sci. USA* **89:**16–19

Schnell, D., Hebert, D. 2003. Protein translocons: multifunctional mediators of protein translocation across membranes. *Cell* **112:**491–505

Schroder, K., Martoglio, B., Hofmann, M., Holscher, C., Hartmann, E., Prehn, S., Rapoport, T.A., Dobberstein, B. 1999. Control of glycosylation of MHC class II-associated invariant chain by translocon-associated RAMP4.[erratum appears in *EMBO J.* 2002,**21:**6954]. *EMBO J* **18:**4804–4815

Skach, W.R., Calayag, M.C., Lingappa, V.R. 1993. Evidence for an alternate model of human P-glyocprotein structure and biogenesis. *J. Biol. Chem.* **268:**6903–6908

Skach, W.R., Lingappa, V.R. 1993. Amino-terminal assembly of human P-glycoprotein at the endoplasmic reticulum is directed by cooperative actions of two internal sequences. *J. Biol. Chem.* **268:**23552–23561

Skach, W.R., Lingappa, V.R. 1994. Transmembrane orientation and topogenesis of the third and fourth membrane-spanning regions of human P-glycoprotein (MDR1). *Cancer Res* **54:**3202–3209

Snapp, E.L., Reinhart, G.A., Bogert, B.A., Lippincott-Schwartz, J., Hegde, R.S. 2004. The organization of engaged and quiescent translocons in the endoplasmic reticulum of mammalian cells. *J. Cell. Biol.* **164:**997–1007

Song, W., Raden, D., Mandon, E.C., Gilmore, R. 2000. Role of Sec61alpha in the regulated transfer of the ribosome-nascent chain complex from the signal recognition particle to the translocation channel. *Cell* **100:**333–343

Spiess, M. 1995. Heads or tails—what determines the orientation of proteins in the membrane. *FEBS Lett* **369:**76–79

Tector Hartl, M.F.U. 1999. An unstable transmembrane segment in the cystic fibrosis transmembrane conductance regulator. *EMBO J.* **18:**6290–6298

Thrift, R.N., Andrews, D.W., Walter, P., Johnson, A.E. 1991. A nascent membrane protein is located adjacent to ER membrane proteins throughout its integration and translation. *J. Cell Biol.* **112:**809–821

Tu, L., Wang, J., Helm, A., Skach, W.R., Deutsch, C. 2000. Transmembrane biogenesis of Kv1.3. *Biochem.* **39:**824–836

van den Berg, B., Clemons, W.M. Jr., Collinson, I., Modis, Y., Hartmann, E., Harrison, S.C., Rapoport, T.A. 2004. X-ray structure of a protein-conducting channel.[see comment]. *Nature* **427:**36–44

Voigt, S., Jungnickel, B., Hartmann, E., Rapoport, T.A. 1996. Signal sequence-dependent function of the TRAM protein during early phases of protein transport across the endoplasmic reticulum membrane. *J. Cell Biol.* **134:**25–35

von Heijne, G. 1986. The distribution of positively charged residues in bacterial inner membrane proteins correlates with transmembrane topology. *EMBO J.* **5:**3021–3027

Walter, P., Blobel, G. 1981. Translocation of proteins across the endoplasmic reticulum. II. Signal recognition protein (SRP) mediates the selective binding to microsomal membranes of in-vitro-assembled polysomes synthesizing secretory protein. *J. Cell Biol.* **91:**551–556

Wang, L., Dobberstein, B. 1999. Oligomeric complexes involved in translocation of proteins across the membrane of the endoplasmic reticulum. *FEES Lett.* **457:**316–322

White, S.H. 2003. Translocons, thermodynamics, and the folding of membrane proteins. *FEBS Lett.* **555:**116–121

Woolhead, C.A., McCormick, P.A., Johnson, A.E. 2004. Nascent membrane and secretory proteins differ in FRET-detected folding far inside the ribosome and in their exposure to ribosomal proteins. *Cell* **116:**725–736

Xie, Y., Langhans-Rajasekaran, S.A., Bellovino, D., Morimoto, T. 1996. Only the first and the last hydrophobic segments in the COOH-terminal third of Na,K-ATPase alpha subunit initiate and halt, respectively, membrane translocation of the newly synthesized polypeptide. Implications for the membrane topology. *J. Biol. Chem.* **271:**2563–2573

Xiong, X., Bragin, A., Widdicombe, J.H., Cohn, J., Skach, W.R. 1997. Structural cues involved in endoplasmic reticulum degradation of G85E and G91R mutant cystic fibrosis transmembrane conductance regulator. *J. Clin. Invest.* **100:**1079–1088

Zhang, J.T., Chen, M., Han, E., Wang, C. 1998. Dissection of de novo membrane insertion activities of internal transmembrane segments of ATP-binding-cassette transporters: toward understanding topological rules for membrane assembly of polytopic membrane proteins. *Mol. Biol. Cell* **9:**853–863

Zhang, J.T., Ling, V. 1991. Study of membrane orientation and glycosylated extracellular loops of mouse P-glycoprotein by in vitro translation. *J. Biol. Chem.* **266:**18224–18232