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THE TRANSLOCON: A Dynamic Gateway at the ER Membrane

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■ **Abstract** Cotranslational protein translocation across and integration into the membrane of the endoplasmic reticulum (ER) occur at sites termed translocons. Translocons are composed of several ER membrane proteins that associate to form an aqueous pore through which secretory proteins and lumenal domains of membrane proteins pass from the cytoplasm to the ER lumen. These sites are not passive holes in the bilayer, but instead are quite dynamic both structurally and functionally. Translocons cycle between ribosome-bound and ribosome-free states, and convert between translocation and integration modes of operation. These changes in functional state are accompanied by structural rearrangements that alter translocon conformation, composition, and interactions with ligands such as the ribosome and BiP. Recent studies have revealed that the translocon is a complex and sophisticated molecular machine that regulates the movement of polypeptides through the bilayer, apparently in both directions as well as laterally into the bilayer, all while maintaining the membrane permeability barrier.

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INTRODUCTION

In every cell, a substantial fraction of the proteins synthesized by cytoplasmic ribosomes must be transported through or integrated into a membrane. Because the primary function of a membrane is to serve as a barrier and to separate two aqueous compartments, the movement of a macromolecule from one side of a membrane to the other raises a number of fundamental mechanistic questions. How are proteins moved across or into a membrane that normally prevents such movement? How is the permeability barrier of the membrane maintained while a macromolecule or a portion of it moves through the membrane? What provides the energy necessary to transport proteins at the membrane? How are transmembrane (TM) domains of integral membrane proteins recognized, selected, and oriented properly in the membrane before being moved laterally into the bilayer? How is the operational mode of the protein-sorting machinery converted from translocation to integration?

Even this minimal listing of basic mechanistic issues associated with protein movement across or into a membrane reveals the substantial intrinsic complexity of these processes at the molecular level. A wide spectrum of approaches has been used to explore various aspects of these processes, and the combined results from many laboratories over the past decade have shown that organisms have evolved complicated molecular machinery to satisfy the protein-sorting requirements at various membranes. A comprehensive review of all of this work is well beyond

the scope of this article. Thus, we have chosen to focus primarily on what happens during translocation and integration at the membrane of the eukaryotic endoplasmic reticulum (ER).

Blobel & Dobberstein (1975) hypothesized that secretory proteins are translocated through the ER membrane via aqueous channels formed by integral ER membrane proteins. For many years thereafter, the existence of aqueous channels and proteinaceous translocation sites was vigorously debated, but without resolution because of the absence of convincing experimental data on either side. New experimental approaches ultimately provided such data and led to a general agreement in the early 1990s that proteinaceous sites on membranes were involved in translocation. In their 1986 review of cotranslational translocation at the ER membrane, Walter & Lingappa (1986) coined the term "translocon" to identify the sites on the membrane at which secretory protein translocation and membrane protein integration occur. Variations of this term have been used to describe the sites of translocation and integration in other systems (e.g. translocase for bacterial secretion), but we shall here refer to all such sites as translocons to emphasize the similarity in their job descriptions and, to a lesser extent, their components.

Recent experimental efforts have focused on defining the structure of the translocon and on identifying its functional roles in protein sorting. Many laboratories are contributing to those efforts, and a number of excellent reviews have documented the recent progress made in our understanding of protein targeting, translocation, and integration at the ER membrane (Corsi & Schekman 1996, Rapoport et al 1996, Hedge & Lingappa 1997, High & Laird 1997, Johnson 1997, Matlack et al 1998), mitochondrial membranes (Schatz 1996, Neupert 1997, Pfanner et al 1997), bacterial plasma membranes (Wickner & Leonard 1996, Driessen et al 1998, Economou 1998), chloroplast membranes (Robinson & Mant 1997, Schnell 1998, Keegstra & Cline 1999), and peroxisomal membranes (McNew & Goodman 1996, Subramani 1996, Titorenko & Rachubinski 1998). Here we focus primarily on translocon structure, function, and regulation during cotranslational translocation and integration at the ER membrane. Aspects of post-translational translocation and integration at the yeast ER and other membranes are also considered, but not in detail.

TRANSLOCON STRUCTURE

Composition

Translocon-mediated translocation is accomplished in every membrane by a complex of different polypeptides that associate to create a protein-conducting channel. The translocon components that form this channel in the ER membrane were first identified by photocross-linking with an approach that incorporated photoreactive probes directly into the nascent chain. When these photoprobes were positioned inside the ER membrane and then photolyzed, the nascent chain was

photocross-linked to specific ER membrane proteins that were adjacent to the nascent chain throughout its translocation or during its integration (Krieg et al 1989, Wiedmann et al 1989, High et al 1991, Thrift et al 1991). Then, as reported in a very important series of papers, the translocon proteins that formed photoadducts with the nascent chain were purified, reconstituted into proteoliposomes, and shown to successfully carry out translocation and integration (Nicchitta & Blobel 1990, Görlich et al 1992a,b, Görlich & Rapoport 1993). One component of the mammalian translocon, the translocation-associated membrane protein (or TRAM) (Görlich et al 1992a), was found (by reconstituting proteoliposomes with or without TRAM) to be required for the translocation or integration of most, but not all, proteins (Görlich & Rapoport 1993, Oliver et al 1995, Voigt et al 1996). Another protein was designated Sec61α (Görlich et al 1992b) because it was homologous to the yeast Sec61p that had been identified in early genetic screens for secretion mutants (Deshaies & Schekman 1987, Stirling et al 1992). Two other polypeptides were purified as a heterotrimer with Sec61α and were termed Sec61β and Sec61γ (Görlich & Rapoport 1993). Because translocation and integration activity can be successfully reconstituted with only these four proteins, the heterotrimeric Sec61 and TRAM are considered to be the core components of the mammalian translocon.

Although translocation at the mammalian ER membrane occurs cotranslationally, translocation at the ER membrane in yeasts can proceed either co- or post-translationally. Sec61p, Sbh1p, and Sss1p (the yeast homologs of Sec61α, Sec61β, and Sec61γ) are required to reconstitute post-translational translocation in proteoliposomes, as are Sec62p, Sec63p, Sec71p, Sec72p, and BiP (Panzner et al 1995, Rapoport et al 1996). The soluble Kar2p protein, the yeast homolog of the mammalian BiP, is localized at the post-translational translocon by binding to the DnaJ-like domain of Sec63p (Brodsky & Schekman 1993, Corsi & Schekman 1997). The photocross-linking of Sec61p to proteins being translocated post-translationally reveals that the protein-conducting channel in this translocon is formed, at least in part, by Sec61p (Müsch et al 1992, Sanders et al 1992), just as Sec61α is involved in the formation of the cotranslational channel. The latter four proteins (except BiP) may perform pathway-specific roles in the post-translational translocon involved in targeting, maintaining the permeability barrier, and/or actively transporting the secretory protein across the bilayer (Rapoport et al 1996). However, mutations in Sec63p and Kar2p/BiP have been shown to interfere with both post- and cotranslational translocation in yeasts (Brodsky et al 1995). Genetic studies in yeasts have also identified a second set of translocon homologs whose role in protein sorting is not well characterized (Finke et al 1996, Toikkanen et al 1996). SecY, the bacterial homolog of the yeast Sec61p, was also initially identified by genetic criteria as being important for secretion, as were SecA and SecE (Schatz & Beckwith 1990). When purified, these three bacterial proteins plus SecG were later shown to accomplish post-translational translocation when reconstituted into proteoliposomes (Brundage et al 1990, Nishiyama et al 1993), and cross-linking studies showed

that the translocating polypeptide was adjacent to SecY and SecA (Joly & Wickner 1993).

These combined results strongly indicate that the protein-conducting channel is formed by similar proteins in prokaryotic and eukaryotic membranes for both the co- and post-translational pathways. Some aspects of translocon structure and function have therefore been strongly conserved. However, each system uses unique components, and hence interactions and mechanisms, to accommodate its specific needs. For example, no structural homologs of SecA have been identified in yeasts or mammals, and no yeast or bacterial homolog of TRAM has been reported to date.

Translocon-Associated Proteins

The signal peptidase (SP) and oligosaccharyltransferase (OST) enzymes appear to be adjacent to the translocon because each acts on a nascent chain as it is being translocated, SP to cleave off the signal sequence and OST to glycosylate the polypeptide. Because *N*-glycosylation can occur near either end of a nascent protein, the OST remains adjacent to the translocon throughout translocation. Thus, it seems reasonable to consider the OST as an integral component of the functioning translocon, even if the OST does not contribute to forming the protein-conducting channel. Less clear is the case of calnexin, an ER membrane protein that acts as a chaperone during the folding of nascent membrane proteins (Chen et al 1995), apparently regulated by the extent of nascent chain glucosylation (Helenius et al 1997), and can be cross-linked to nascent chains (Degen & Williams 1991, Oliver et al 1996, Tatu & Helenius 1997). Calnexin therefore appears to be positioned adjacent or proximal to the translocon, but there is currently no evidence that calnexin forms a specific complex with translocon proteins. Other soluble lumenal proteins such as calreticulin, protein disulfide isomerase, BiP, and ERp57 also interact with the nascent chain cotranslationally (Nicchitta & Blobel 1993, Helenius et al 1997, Oliver et al 1997, Wang et al 1997), but we have arbitrarily chosen to consider only membrane proteins as translocon associated.

Other proteins are located transiently at the translocon. The heterodimeric signal-recognition particle (SRP) receptor is required for the targeting of a ribosome-nascent chain/signal sequence-SRP complex to the mammalian ER membrane, but the SRP receptor is then presumably released from the translocon because there are fewer SRP receptors than there are translocons in the ER membrane (Rapiejko & Gilmore 1997). Although only transiently present at the translocon, the SRP receptor may play a critical role in establishing the structure of the ribosome-free translocon. However, the ability to substitute the SRP and the SRP receptor with their respective bacterial analogs, Ffh-4.5S RNA and FtsY (Walter & Johnson 1994), in targeting reactions in vitro (Powers & Walter 1997) indicates that the SRP receptor may not reside within the ribosome-free translocon, but rather may bind to the outer periphery of the translocon to carry out its targeting function.

BiP is another protein found transiently at the translocon. BiP was first identified as a soluble lumenal protein bound to the immunoglobulin G heavy chain (Haas & Wabl 1983), and BiP has since been thought of as being primarily involved in protein folding and assembly (e.g. Gething & Sambrook 1992, Simons et al 1995, Hendershot et al 1996). Kar2p, the yeast homolog of BiP, has also been shown to be important in translocation, both cotranslational (Brodsky et al 1995) and post-translational (Vogel et al 1990, Nguyen et al 1991, Sanders et al 1992, Brodsky et al 1995, Panzner et al 1995). In the latter case, Kar2p (BiP) binds to Sec63p in the yeast post-translational translocon (Brodsky & Schekman 1993, Lyman & Schekman 1997) to facilitate protein translocation, perhaps by acting as the molecular motor that powers the translocating polypeptide across the membrane (Brodsky 1996, Lyman & Schekman 1997). Recently, BiP was also found to be responsible for sealing the lumenal end of the mammalian ribosome-free translocon, and to be released from the translocon shortly after ribosome-nascent chain targeting has been completed (Hamman et al 1998). The mechanism by which BiP closes the ribosome-free pore is not known, nor is (are) the translocon protein(s) with which BiP interacts. The simplest possibility is that BiP binds to the lumenal end of the translocon pore so as to plug the hole. It is also possible, however, that the pore is sealed indirectly if BiP binding to a translocon component (e.g. a Sec63p homolog; Skowronek et al 1999), perhaps through another soluble lumenal protein (Boisramé et al 1998), elicits a conformational change that seals the pore. Whatever the case, it is clear that BiP has multiple functions within the ER lumen.

Thus, proper translocon function requires both BiP and SRP receptor to interact with the translocon at specific stages during cotranslational translocation and integration. We therefore believe that it is reasonable to consider them as essential translocon components.

Four ER membrane proteins have been reported to act as ribosome receptors, a function that would presumably locate them at the translocon: p34 (Tazawa et al 1991), p180 (Savitz & Meyer 1990, 1993), ribophorin I (Marcantonio et al 1984, Yu et al 1990), and Sec61α (Görlich et al 1992b, Kalies et al 1994). Yet, as discussed in a recent review (Rapoport et al 1996), there is little agreement on which protein constitutes the ribosome receptor. In view of this uncertainty, we have chosen to simplify our Figure 1 (see color insert) by assuming that the ribosome binds to Sec61α and/or ribophorin I, which are already located at the translocon. But it remains to be seen whether any of the other putative ribosome receptors are positioned at the translocon.

Have all TM components of the translocon been identified? A likely site of BiP binding to the ribosome-free translocon is a mammalian homolog of yeast Sec63p, and such a protein has recently been described (Skowronek et al 1999). However, its function in cotranslational translocation remains to be fully characterized. A *Drosophila* cDNA with homology to the yeast Sec62 gene has also been reported (e.g. Noel & Cartwright 1994). In addition, a small ER membrane protein has been cross-linked to a nascent chimeric protein that contains the apolipoprotein

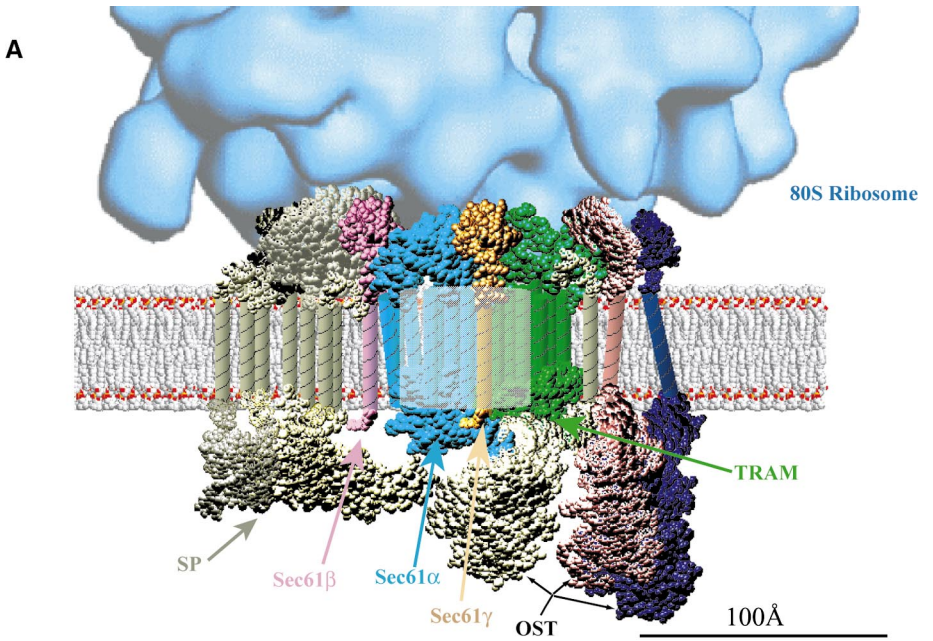


Figure 1 Protein packing in the mammalian translocon. (*A*) The components of the translocon are depicted approximately to scale in this cross section that is perpendicular to the plane of the membrane. Transmembrane segments are represented by a cylindrical volume with the dimensions of an average α-helix (12-Å diameter), whereas the cytoplasmic and lumenal domains of each protein are modeled using the dimensions of globular proteins or of portions of proteins with the same number of amino acids (the three-dimensional structures of ubiquitin, phospholipase, and bacteriorhodopsin were used as models for globular domains and α-helices). The shape of each domain is arbitrary and is shown merely to indicate the relative amounts of space occupied by translocon components on each side of the membrane. The relative positions of the polypeptides were chosen to be consistent with current models of a working translocon, so proteins known to interact with each other were placed in mutual proximity. Where no data regarding location are available, the placement was arbitrary. For clarity, only a single Sec61 heterotrimer is depicted. A ribosome is also shown to scale (From the surface reconstruction in Beckmann et al 1997) to indicate its size relative to the translocation machinery and the pore, depicted here as a 50-Å box with faded coloration.

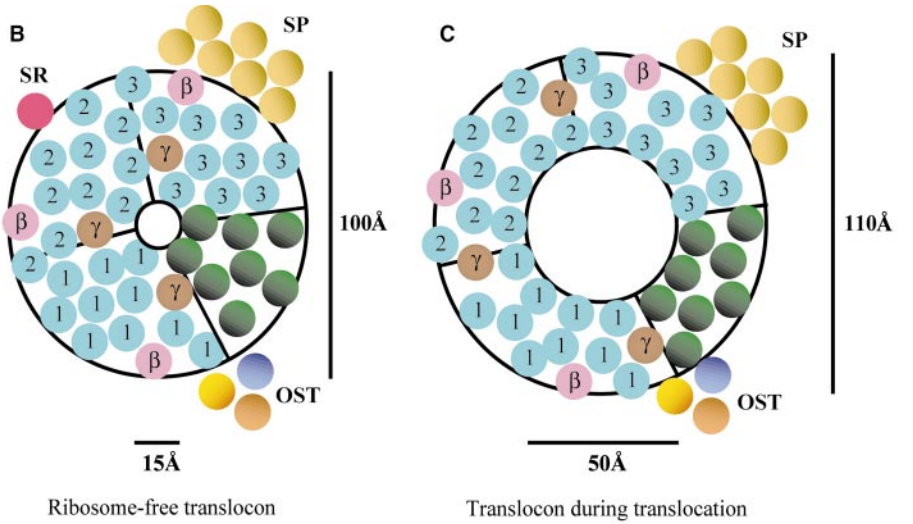


Figure 1 (continued) (*B*) A ribosome-free translocon is viewed in cross section parallel to the plane of the membrane and within the bilayer. Each circle represents a TM segment (12-Å average diameter) of a translocon protein, with the same color coding as in *A*, and their placement is arbitrary. The inner- and outer-pore dimensions correspond to those reported by Hamman et al 1998 and Henein et al 1996. The number of Sec61 heterotrimers was arbitraily set to three, and they are identified by number. A single molecule of TRAM was included per translocon. (*C*) The cross section of a translocon functioning in translocation is depicted here by using the same color scheme as in *A* and *B*. The inner diameter corresponds to that necessary to maintain the same translocon cross-sectional surface area as in *B*. SR, SRP receptor; SP, signal peptidase; OST, oligosaccharyltransferase; β, Sec61β; γ, Sec61γ.

B (apoB) pause-transfer sequence (Hegde & Lingappa 1996). It is conceivable that this ER protein is located at the translocon to regulate the translocation of apoB, the major protein component of the liver-derived, very low-density lipoprotein and low-density lipoprotein (VLDL and LDL, respectively). The orientation (N terminus in cytoplasm or lumen) of signal-anchor sequences inserted into the bilayer (uncleaved signal sequences that enter the lipid bilayer to create a membrane protein) is thought by some to be regulated by proteins, as is the 180° rotation of every second TM sequence that occurs during the integration of multiple-spanning membrane proteins. However, no protein candidates for these functions have been identified as yet.

Protein Stoichiometry and Arrangement

Little information is available about the numbers of individual proteins in each translocon. Because the mammalian Sec61 complex is purified as a heterotrimer (Görlich & Rapoport 1993), it seems likely that there are equal numbers of Sec61α, Sec61β, and Sec61γ in each cotranslational translocon. The number of Sec61α proteins per translocon has been estimated to be 3–4 (Hanein et al 1996) or 2 (Beckmann et al 1997) based on electron microscopy data. TRAM has been estimated to be present at a level of 1–2 copies per ribosome (Görlich et al 1992a), whereas estimates of stoichiometry indicate that rough ER microsomes contain approximately equimolar numbers of ribosomes and SP (Evans et al 1986) and of ribosomes and ribophorin I (Kelleher et al 1992), one of the subunits of OST. It has therefore been assumed that one SP and one OST are associated with every translocon. But in no case has the number of copies of a protein in the translocon been firmly established. A new experimental approach will be required to determine the stoichiometry accurately for each of the components in an intact, fully assembled translocon.

Similarly, information about the arrangement of proteins within the translocon is fragmentary. Sec61α (e.g. High et al 1993, Mothes et al 1994, Do et al 1996), TRAM (e.g. High et al 1993, Mothes et al 1994, Do et al 1996), and Sec61β (Laird & High 1997, Mothes et al 1997, Knight & High 1998, Mitchell et al 1998) have each been cross-linked to nascent chains, so domains of these components are adjacent to the nascent chain pathway through the translocon. A systematic examination of photocross-linking targets as a function of probe location within the nascent chain revealed that Sec61α is the primary target within the pore (High et al 1993, Mothes et al 1994). These results strongly indicate that the walls facing the aqueous interior of the translocon pore are formed largely by the α-helices of Sec61α, a conclusion that is consistent with both the electron microscopy results discussed below and the unusually limited hydrophobicity of some of the Sec61α TM segments (Wilkinson et al 1996).

Photoreactive probes at various positions within nascent secretory proteins reacted covalently with the TRAM glycoprotein (Krieg et al 1989, Görlich et al 1992a). These results indicate that TRAM is exposed to the aqueous pore,

although it is not yet clear whether this exposure is transient or constant. Because signal sequences photocross-link efficiently to TRAM, TRAM has been proposed to play a role in targeting at the translocon (Jungnickel & Rapoport 1995, Voigt et al 1996). In addition, TRAM has been implicated in apoB pause-transfer by comparing the translocation of apoB in purified proteoliposomes that were reconstituted either in the presence or absence of TRAM (Hegde et al 1998c). TRAM also appears to be involved in integration. Photoreactive probes positioned in the center of a TM sequence of a nascent membrane protein (and hence in the middle of the bilayer) can react with either Sec61α or TRAM when the TM sequence initially reaches the translocon, which is consistent with the TM sequence moving out of the pore and into the translocon via an interface between Sec61α and TRAM (Do et al 1996). As integration proceeds, the TM sequence moves away from (no longer cross-links to) Sec61α, but remains adjacent to TRAM until translation terminates and the TM sequence is released from TRAM, presumably at the outer periphery of the translocon (Do et al 1996). TRAM may therefore extend from the inner surface of the pore to the outer periphery of the translocon.

The existence of a stable Sec61 heterotrimer shows that there are direct protein-protein interactions between the Sec61 polypeptides. In addition, Sss1p (Sec61γ) has been chemically cross-linked to a section of Sec61p that contains TM segments 6–8 (Wilkinson et al 1997). Furthermore, Sec61β has been cross-linked chemically to both Sec61α and the 25-kDa SP subunit (Kalies et al 1998). The close proximity between Sec61β and the SP subunit suggests that the positioning of the SP at the translocon is mediated, as expected, by specific protein-protein interactions. This cross-linking is ribosome dependent, which indicates that the presence of the ribosome alters the juxtaposition of the cross-linked sites, either via conformational changes or via recruitment of SP to the translocon.

No direct interactions between OST subunits and translocon components have been identified to date. Yet the OST is nearby, because one of the ribophorin subunits of the OST cross-links to ribosomes (Kreibich et al 1978) and also because the active site of the OST is close to the translocon (Nilsson & von Heijne 1993, Popov et al 1997). By inserting *N*-glycosylation sites into a nascent chain at various distances from the lumenal end of a TM sequence, it was found that OST only glycosylates sites that are 12–14 residues from the end of the TM sequence (Nilsson & von Heijne 1993, Popov et al 1997). Although the distance of the OST active site from the pore cannot be determined in Å from these experiments (the TM sequence has moved laterally an undetermined distance into the translocon, the height of the OST active site above the membrane surface is unknown, and the conformation of the nascent chain is unknown), the OST active site is $<$49 Å (14 $\times$ 3.5 Å fully extended) from the end of the TM segment, a distance that is about the estimated outer radius of a translocon (Hanein et al 1996, Beckmann et al 1997). The OST therefore appears to be closely juxtaposed to translocon components.

Is the translocon entirely proteinaceous? Based on the high efficiency of photocross-linking of probes in signal sequences and signal-anchor sequences to phospholipids (Martoglio et al 1995, Mothes et al 1997), phospholipids must be present

at or near the binding sites for these sequences in the translocon. Yet the nature of phospholipid exposure to the nascent chain remains ill defined. It is possible that the nonpolar interior of the bilayer itself is exposed to the aqueous pore, as has been proposed (Martoglio et al 1995). Whether any such exposure is transient or constant has yet to be determined. It is also conceivable that individual phospholipid molecules either associate with specific sites on Sec61α and TRAM or intercalate between the α-helices of the translocon proteins and thereby form part of the binding site in the translocon that interacts with signal sequences, signal-anchor sequences, and/or TM sequences. It is interesting that phospholipids have been shown to play a role in translocation across the bacterial membrane (de Vrije et al 1988, Lill et al 1990).

Two critical aspects of translocon structure have not yet been addressed experimentally, despite their importance to any mechanistic considerations of translocon involvement in targeting, translocation, and integration. First, if the pore contains multiple copies of the Sec61 heterotrimer—as seems likely based on electron microscopy data (Hanein et al 1996, Beckmann et al 1997) and the size of the functioning pore (Hamman et al 1997)—and the Sec61 multimer is the core on which other proteins assemble via specific protein-protein interactions, then how does the system limit some components to one copy per translocon? For example, each translocon is thought to associate with one SP enzyme (Evans et al 1986). But if Sec61β binds to the 25-kDa SP subunit (Kalies et al 1998), then what prevents there being as many SP enzymes per translocon as there are Sec61β molecules? Similarly, if TRAM interacts directly and specifically with Sec61α, one would expect as many TRAMs as Sec61as in the translocon. What limits the number of SRP receptor molecules to one per translocon? If a signal sequence binds to a specific site on Sec61α, as was shown by photocross-linking to occur in a post-translational translocon (Plath et al 1998), does this mean that there are multiple such binding sites in a translocon? If so, are these sites functionally equivalent? One possible explanation is that any multiple subunits in the translocon are not structurally symmetrical and equivalent and that each subunit is therefore functionally unique within the context of a fully assembled translocon. For example, the Sec61α closest to the SRP receptor may become the chosen one for signal sequence binding. Alternatively, perhaps only a single copy of one protein (e.g. TRAM) is accommodated sterically within the translocon, and the assembly of other putative single-copy proteins (e.g. SP, OST, or SRP receptor) into the translocon is mediated through interactions with this initial single-copy protein.

Second, with so many proteins having been identified as being part of the translocon or as interacting with a nascent chain during translocation or integration, one wonders how these proteins are organized within the translocon so as to overcome the steric constraints of locating multiple proteins in a limited space. One approach that could have evolved to minimize this problem is to alter translocon composition and/or conformation as a function of time. By dynamically moving proteins in and out of the translocon or by altering the locations of domains within the translocon, nascent chain access to different proteins could be regulated as

needed. Examples of such changes include the release of SRP receptor from the translocon after the completion of targeting and the interaction of BiP with the lumenal side of ribosome-free, but not functioning, translocons (Hamman et al 1998).

Despite the above caveats and our relative lack of information about protein stoichiometry and organization, we have boldly prepared cartoons to depict three views of translocon structure (Figure 1). At this stage of our ignorance, the primary point of these cartoons is to marvel at the space occupied by the large amount of protein that is concentrated at the translocon, rather than to be concerned with the accuracy of protein placement. For example, the TM segments of three Sec61 heterotrimers and one TRAM per translocon occupy a substantial fraction of the available cross-sectional area (Figure 1*B*, 1*C*, see color insert).

The Pore

The most striking structural feature of the mammalian ER translocon is the aqueous pore that spans the membrane. Transmembrane aqueous channels in the ER membrane were first detected by conductivity measurements, and the puromycin-dependence of the channels suggested that nascent chains had to be released from the ribosomes before TM ion flow could occur (Simon & Blobel 1991). Because the channels closed when ribosomes were washed off of the membrane, the existence of these ion-conducting channels was both nascent chain- and ribosome-dependent.

To determine directly whether the nascent proteins are in an aqueous milieu as they pass through the bilayer, water-sensitive fluorescent probes were incorporated into the nascent chains of translocation intermediates. The fluorescence lifetimes of the resulting samples revealed that all of the probes inside the membrane-bound ribosome and the translocon were in an aqueous environment (Crowley et al 1993, 1994) (Figure 2*A*).

Independent confirmation that the nascent chain occupies an aqueous pore in the translocon was obtained by use of iodide ions as hydrophilic collisional quenchers of fluorescence. Iodide ions will reduce the fluorescence intensity of a sample whenever they can collide with the fluorescent probes in the sample, but the iodide ions are unable to pass through the hydrophobic interior of the ER membrane (Cranney et al 1983, Crowley et al 1994). When I^- was added to the cytoplasmic side of translocation intermediates containing fluorescent-labeled nascent chains, no fluorescence quenching was observed (Figure 2*B*). The I^- therefore could not contact the fluorescent dyes in the nascent chains, and hence the nascent chains in the translocation intermediates were not accessible to the cytoplasm (Crowley et al 1993, 1994). However, when I^- was introduced into the lumenal compartment of the microsomes (Figure 2*C*), dyes located inside the ribosome on the cytoplasmic side of the membrane were quenched. This result could be obtained only if there was an aqueous pathway that completely spanned the ER membrane and extended into the ribosome (Crowley et al 1994).

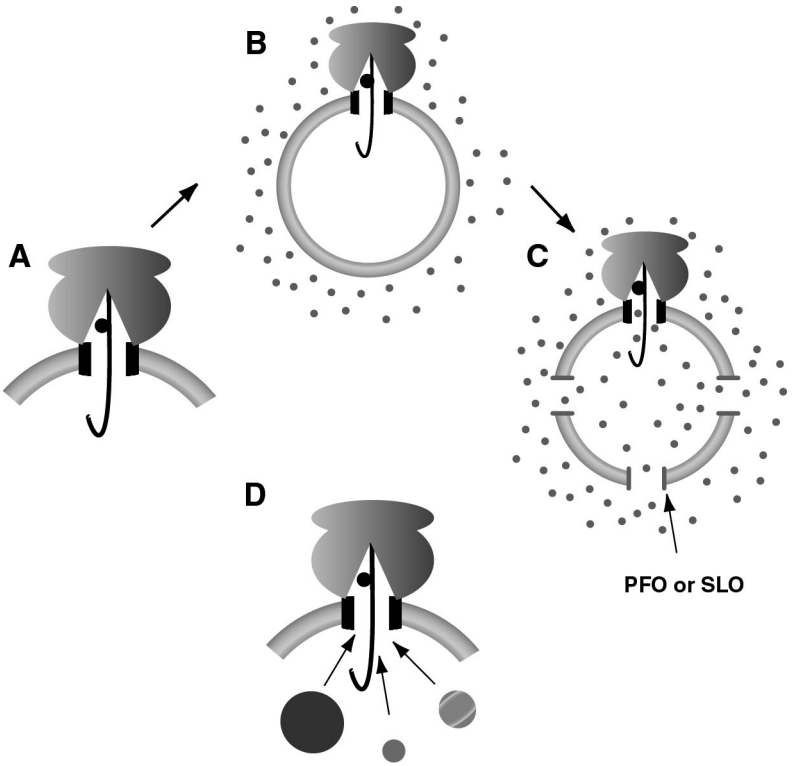


Figure 2 Assessing nascent chain exposure to the cytoplasm or endoplasmic reticulum lumen. (*A*) A translocation intermediate is formed by in vitro translation in the presence of SRP, ER microsomes, and a truncated mRNA lacking a stop codon. A fluorescent probe (•) is incorporated into the nascent chain by use of a Lys-tRNA analog (Crowley et al 1993). After purification, the fluorescence intensity of the sample is measured. (*B*) Hydrophilic collisional quenchers that cannot cross the membrane (e.g. iodide ions) are added to the sample (the cytoplasmic side of the membrane), and the fluorescence intensity of the sample is again measured to determine to what extent, if any, it has been reduced. (*C*) To examine accessibility from the lumenal side of the membrane, quenchers are introduced into the microsomes by forming large pores in the membrane by use of the bacterial cytolytic toxins perfringolysin O (PFO) or streptolysin O (SLO). If quenchers in the lumen can move through an aqueous pore that spans the membrane and can collide with the probe located inside the ribosome on the cytoplasmic side of the membrane, the fluorescence intensity of the sample is decreased. (*D*) The size of the pore can be estimated by using quenchers of different sizes and determining which are too large to move through the pore and quench the probe.

The Permeability Barrier

The molecular origin of the permeability barrier was also revealed by the collisional quenching experiments and their ability to assess exposure of the nascent chain to one side of the membrane or the other. Because the lumen, but not the cytoplasm, is continuous with the translocon pore during translocation, the ribosome itself must be forming the permeability barrier by binding tightly to the cytoplasmic surface of the translocon and sealing off the pore from the cytoplasm (Crowley et al 1994) (Figure 2*A*). Furthermore, because the nascent chain inside the ribosome is not exposed to the cytoplasm, the nascent chain moves through an aqueous tunnel in the ribosome that directs the nascent chain to the translocon (Crowley et al 1993, 1994). A nascent secretory protein need not be exposed to the aqueous cytoplasm during translocation, except in special circumstances (see below).

If the ribosome is responsible for the permeability barrier, what happens when the ribosome leaves the translocon at the termination of translation? Fluorescence quenching experiments revealed that extracting the soluble lumenal proteins from ER microsomes allowed cytosolic iodide ions to enter the lumenal interior of the microsomes (Hamman et al 1998). The holes exposed in these extracted microsomes were translocons because iodide ion passage was blocked by affinity-purified antibodies to either Sec61α or TRAM. To determine which soluble lumenal protein(s) was (were) responsible for sealing these ribosome-free translocon pores, extracted microsomes were reconstituted with various combinations of purified lumenal proteins, and it was discovered that purified BiP in the presence of ATP or ADP was sufficient to prevent quencher movement across the ER membrane (Hamman et al 1998). BiP is therefore required to maintain the permeability barrier at nonfunctioning translocons that lack ribosomes (Hamman et al 1998). As noted above, it is not yet clear whether this important function of BiP is accomplished directly or indirectly.

This set of experiments also showed that the translocon does not disassemble when the ribosome leaves (Hamman et al 1998). Instead, the translocon components remain assembled and form a pore—even in the absence of a ribosome. However, the structure of the translocon is altered, conformationally (see below) and compositionally (the return of the SRP receptor to a ribosome-free translocon), when the ribosome leaves.

The Size of the Pore

To estimate the size of the aqueous pore, hydrophilic quenching agents of different sizes were added to the lumenal side of intact, fully assembled translocation intermediates with fluorescent-labeled nascent chains (Figure 2*D*) to determine at what point the quenchers were too large to enter the aqueous translocon pore and contact the fluorophores. This experimental approach revealed that the aqueous pore in a functioning, ribosome-bound translocon is 40–60 Å in diameter (Hamman et al 1997). This hole is much larger than anyone expected and, in fact, is the largest

pore so far detected in a membrane that must maintain a permeability barrier. For this reason (its size), we refer to the hole in a translocon as a pore rather than a channel.

The pore was recently visualized in electron microscopic images of detergent-solubilized Sec61 heterotrimers as a ring with an outer diameter of ~110 Å (Hanein et al 1996, Beckmann et al 1997). In stark contrast to the 40- to 60-Å diameter hole determined by using fluorescence, electron microscopy indicated an inner pore diameter of ~20 Å (Hanein et al 1996). Rings of similar size were also visualized in images of both canine and yeast ER membranes (Hanein et al 1996), as well as SecYE complexes reconstituted into proteoliposomes (Meyer et al 1999). A ring of nearly the same size (~15-Å inner diameter) was also visualized in electron microscopic images of a ribosome-Sec61 complex (Beckmann et al 1997). It is interesting that the ribosomal nascent chain tunnel was coaxially oriented over the Sec61 pore (cf Figure 2*A*), even though the absence of a nascent chain prevented the formation of a tight seal (Beckmann et al 1997).

The apparent discrepancy in pore diameters determined by using fluorescence and electron microscopy was later resolved when it was discovered that the size of the aqueous pore in a ribosome-free translocon is much smaller than that in a functioning translocon (Hamman et al 1998). The ribosome-free translocon has a pore with an internal diameter of 9–15 Å, large enough to pass a hydrated iodide ion, but too small to pass a hydrated ADP molecule (Hamman et al 1998). The rings observed in electron microscopy studies therefore appear to be in the ribosome-free translocon conformation.

Because affinity-purified antibodies or Fab fragments specific for the C-terminal peptide of either Sec61α or TRAM inhibit iodide ion passage through the ribosome-free translocon (Hamman et al 1998), the C termini of Sec61α and TRAM are located close enough to the pore that binding to a Fab fragment completely (Sec61α) or partially (TRAM) blocks the pore.

The spectroscopically detected dynamic change in translocon pore diameter is substantial, from ~15 to ~50 Å (Hamman et al 1998). Yet this threefold increase in inner-pore diameter requires only a 10% increase in the outer diameter, from 100 to 110 Å, if this conformational change is accomplished without altering the cross-sectional surface area occupied by the translocon components (Figure 1*B*, 1*C*). Thus, the dramatic change in the inner diameter of the pore need not be accompanied by an equally dramatic change in the overall topography of the translocon. It will be interesting to see just how much protein movement actually occurs at the periphery during this structural transition.

Assembly, Post-Translational Modification, and Turnover

Among the most interesting aspects of the translocon are the mechanisms by which the cell regulates translocon number and activity. Yet to date, only two studies have dealt with translocon assembly, modification, or turnover. In the first study, photocross-linking experiments showed that Sec61α is cotranslationally integrated

into the ER membrane at translocons containing preexisting Sec61α (Knight & High 1998). Sec61α is therefore integrated into the ER membrane via the same pathway as other multispanning membrane proteins. In the second study, two translocon components, TRAM and Sec61β, were found to be phosphorylated, as was one subunit of the SRP receptor (Gruss et al 1999). Because these modifications are calcium dependent in vitro, they may play a role in regulating translocation.

Another interesting, but under-studied, aspect of the translocon is its localization within the cell. Translocons are found in the rough ER membrane of the eukaryotic cell, even though the translocon components do not have any of the known ER retention signals. However, Sec61 complexes have recently been observed in the ER-Golgi intermediate compartment, a result indicating that translocon components are retrieved from the intermediate by some unknown mechanism (Greenfield & High 1999).

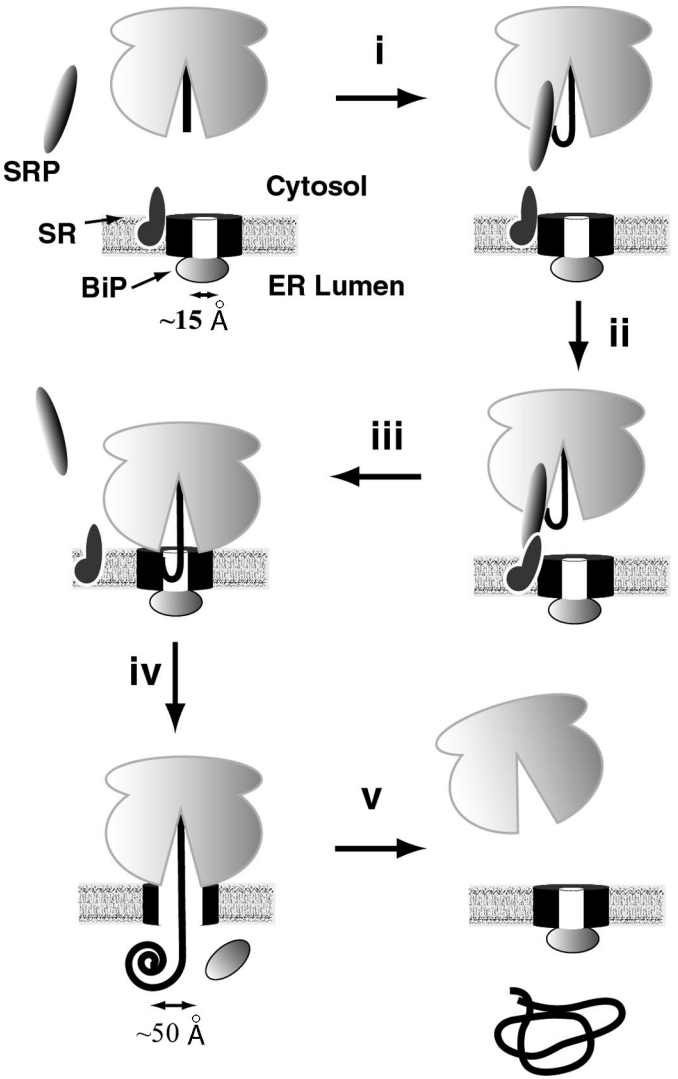
FUNCTIONAL MODES

Targeting

In mammalian cells, all nonorganellar protein biosynthesis is initiated on unbound cytoplasmic ribosomes (Figure 3). Those ribosomes synthesizing proteins destined for secretion or for integration into the ER membrane are identified by a 15- to 30-residue signal sequence at the N-terminal end of the nascent chain (reviewed by Walter & Johnson 1994). When the signal sequence emerges from the ribosome, SRP binds to the signal sequence and ribosome, thereby slowing or temporarily halting protein-chain elongation as the ribosome-nascent chain/signal sequence-SRP complex diffuses in the cytoplasm. The targeting of the SRP-ribosome complex to the ER membrane is then affected via a specific interaction between SRP and SRP receptor, a protein found only at the membrane of the rough ER.

Figure 3 Translocation across the endoplasmic reticulum membrane. Before targeting, the aqueous pore in the ribosome-free translocon (9- to 15-Å internal diameter) is sealed at its lumenal end directly or indirectly by BiP. After SRP binds to the signal sequence in a ribosome-nascent chain complex (i), an interaction between the SRP and SRP receptor (ii) leads to ribosome binding to the translocon and the insertion of the nascent chain into the translocon pore (iii). The nascent chain remains in an aqueous compartment inaccessible to either the cytoplasm or ER lumen while protein synthesis proceeds. When the nascent chain reaches $\sim$70 amino acids in length, BiP is released and the lumenal end of the pore is opened (iv). Cotranslational translocation then proceeds through an aqueous pore with a diameter of 40–60 Å that is sealed at its cytoplasmic end by an impermeable junction between the ribosome and the translocon. After termination of translation, the secretory protein is released into the ER lumen, the pore contracts, sealed on its lumenal side by BiP, and the ribosome is released into the cytoplasm.

Prior to targeting, the translocon is in its inactive, ribosome-free configuration: The SRP receptor is presumably located adjacent to the translocon; the translocon pore has a small diameter; and the lumenal end of the pore is sealed by a BiP-dependent process (Hamman et al 1998). A GTP-dependent interaction of SRP with its receptor triggers a series of events that includes the release of the signal sequence from the SRP, the binding of the ribosome to the translocon, and the release of the SRP and SRP receptor from the ribosome and translocon, respectively. The importance of GTP binding and hydrolysis in the targeting process is widely recognized, but a consensus has not yet been reached on the mechanism of GTP



involvement in targeting (Miller et al 1993, Powers & Walter 1995, Bacher et al 1996, Millman & Andrews 1997, Rapiejko & Gilmore 1997). Protein synthesis also resumes after the departure of the SRP, and the nascent chain proceeds to move into the translocon. Interestingly, BiP is still bound to the lumenal end of the pore at this point, and hence the nascent chain is in an aqueous compartment that is not contiguous with either the cytoplasm or the lumen (Crowley et al 1994, Hamman et al 1998) (Figure 3). The delayed release of BiP and opening of the pore must constitute a safety mechanism, evolved to ensure that the one end of the pore is not opened before the other end is firmly sealed. After the nascent chain reaches a length of ~70 residues (Crowley et al 1994), the pore is opened to the ER lumen. The release of BiP may be elicited after the unfolded nascent chain becomes long enough to bind to BiP, change its conformation, and release BiP from the translocon. At the end of the targeting process, after the departure of BiP, the ribosome-bound, functioning pore has a diameter of 40–60 Å (Hamman et al 1997).

Some aspects of the above scenario remain to be clarified experimentally. For example, the order and kinetics of the above events, such as signal sequence release and ribosome binding to the translocon, are not known. It is also not known whether the size of the pore increases after ribosome binding or after BiP release. In addition, it is not known whether BiP dissociates completely from the translocon or remains bound and simply moves to cause pore opening. The location of the signal sequence throughout the targeting process is also unclear (see below).

Another aspect of the targeting process that remains controversial is the role of the nascent polypeptide-associated complex (NAC). This protein was identified in photocross-linking experiments and originally proposed to bind to nascent chains before their association with SRP (Wiedmann et al 1994). These results led to a model in which NAC, rather than SRP, served as the primary regulator of targeting to the ER membrane by preventing ribosomes from binding to translocons in the absence of SRP, thereby serving to eliminate inadvertent ribosome binding to empty translocons (Lauring et al 1995a,b, Möller et al 1998a,b). Yet other groups found that neither the extent nor the kinetics of ribosome binding to microsomes were altered by the presence or absence of NAC, and they concluded that NAC has no direct role in the targeting process (Neuhof et al 1998, Raden & Gilmore 1998). It remains to be seen which of these opposing views of NAC function is correct.

Cotranslational Translocation

During the cotranslational translocation of secretory proteins across the ER membrane, the translocon must maintain the aqueous pore and the tight seal with the ribosome. Is the translocon also involved in moving the nascent chain across the bilayer? The topography of the translocon and ribosome would appear to obviate the need for such involvement because the newly synthesized nascent chain has only one direction to move—through the membrane and into the lumen (Figure 2*A*). The nascent chain is completely enclosed inside the ribosome in an aqueous tunnel, and the ribosome-translocon seal prevents nascent chain movement into the

cytoplasm (Crowley et al 1993, 1994). Thus, as the nascent chain grows in length, the entry of newly added amino acids into the nascent chain tunnel can be accommodated only by the movement of the rest of the polypeptide towards the lumen. The intrinsic topography of the ribosome-translocon complex therefore appears to eliminate the need for a ratcheting protein or other activity.

In the absence of ribosomes or a tight ribosomal seal, the above mechanism for achieving translocation does not apply, and the polypeptide must be actively transported from one side of the membrane to the other. Thus, BiP effects post-translational translocation in yeasts (Brodsky & Schekman 1993, Brodsky et al 1995, Matlack et al 1999), SecA powers protein export in bacteria (Economou & Wickner 1994), and Hsp70 powers import into the mitochondrial matrix (Glick 1995, Vosine et al 1999). Is such a mechanism also important during cotranslational translocation? The successful reconstitution of cotranslational translocation in the absence of soluble lumenal proteins suggests that BiP may not be required for cotranslational translocation (Görlich & Rapoport 1993). However, the low efficiency of the reconstituted system may be explained by the absence of some important protein components. In another study, the extraction of soluble lumenal proteins from microsomes reduced the translocation efficiency of secretory proteins that had been targeted to the ER membrane, thereby suggesting that lumenal proteins were required to complete the translocation of some nascent chains into the lumen (Nicchitta & Blobel 1993). Furthermore, studies with Kar2p mutants suggest that a functional BiP is required for optimal cotranslational translocation (Brodsky et al 1995, Boisramé et al 1998).

The ultimate resolution of this apparent discrepancy is of particular interest to those studying apoB translocation. At certain times during the cotranslational translocation of the very long 4536-residue apoB polypeptide, translocation is halted and the ribosome-translocon junction is broken, thereby exposing the apoB nascent chain to the cytoplasm (Hegde & Lingappa 1996, Rusinol et al 1998, Zhou et al 1998). This state is triggered by pause-transfer sequences in the nascent chain (Chuck & Lingappa 1992, 1993) and may lead to degradation of the nascent chain by proteasomes in the cytoplasm (Yeung et al 1996, Benoist & Grand-Perret 1997, Fisher et al 1997, Mitchell et al 1998), the primary mechanism by which apoB production is regulated. But when an apoB nascent chain is transiently exposed to the cytoplasm and not degraded, then how is the nascent chain moved back into the ER lumen to continue its translocation and processing? Is BiP or another lumenal protein involved? Concerning the latter possibility, both calnexin (Chen et al 1998) and microsomal triglyceride transfer protein (Du et al 1994, Benoist & Grand-Perret 1997) reduce the degradation of apoB, but the mechanisms of this protection are still unclear.

After translation terminates, the release of the ribosome apparently causes the translocon to contract, reducing the diameter of its pore from 40–60 Å to 9–15 Å (Hamman et al 1998) (Figure 3). This contraction presumably does not occur before the C-terminal end of the newly synthesized protein has moved through the translocon and into the lumen. Although the temporal ordering of ribosome

and nascent chain release has not yet been determined experimentally, it seems likely that the nascent chain must be completely translocated before the ribosome is released from the translocon if the system is to preserve the permeability barrier of the ER membrane. The departure of the nascent chain from the translocon pore would then allow BiP to bind to the translocon and thereby stimulate ribosome release and translocon contraction, perhaps powered by BiP-mediated ATP hydrolysis (Hamman et al 1998).

Cotranslational Integration

When the TM sequence of a nascent membrane protein reaches the translocon, the protein ceases to move through the membrane. Instead, the TM sequence moves laterally out of the aqueous pore, into the translocon, and ultimately into the lipid bilayer. In addition, those TM sequences that are to be aligned with their N-terminal ends facing the cytoplasm must be rotated 180° to obtain the proper orientation before insertion into the bilayer. Thus, in contrast to its relatively passive role in translocation, the translocon appears to be an active participant in the integration process. In fact, because the translocon serves as the entry point for the integration of TM sequences into the lipid bilayer, the translocon is directly involved in the recognition, orientation, lateral movement, and insertion of TM sequences. However, the nature of this involvement has turned out to be surprisingly complex in each of these partial reactions.

Transmembrane Sequence Recognition A TM sequence in a nascent chain was long thought to be recognized only after it reached the membrane, simply because there was no apparent reason for the ribosome to be involved in identifying TM sequences. Yet it was recently discovered that the TM sequence in a nascent chain with a cleavable signal sequence is first detected by the ribosome when the C-terminal end of the TM sequence is only four amino acids from the peptidyltransferase center and well within the ribosomal nascent chain tunnel (Liao et al 1997). Such recognition is presumably mediated by a nonpolar surface inside the ribosomal tunnel (Liao et al 1997), a speculation that is supported by fluorescence lifetime data showing that a fluorescent probe in the TM sequence is in a relatively nonpolar environment inside the tunnel (S Liao & AE Johnson, unpublished data). This recognition by the ribosome initiates a series of events that converts the functional mode of the translocon from translocation to integration, including the closing of the lumenal end of the pore and the subsequent opening of the tight ribosome-translocon seal (see below) (Liao et al 1997). The early recognition of the TM sequence presumably provides the translocon more time to convert its operational mode.

Even though a TM sequence is detected first by the ribosome, the translocon must still independently recognize the TM sequence to position it properly for its lateral move into the bilayer. It is not known which translocon component(s) selects and orients a TM sequence before its insertion. Once oriented properly

(N-terminal end cytoplasmic or lumenal), the nonpolar TM sequence will minimize its exposure to water by associating with (putative) nonpolar patches on the inner surface of the translocon pore that are formed by translocon proteins (Do et al 1996) and/or by phospholipids (Martoglio et al 1995, Mothes et al 1997) and then moving into the hydrophobic interior of the translocon and ultimately the bilayer.

Recognition of the TM sequence is also more complex than simply detecting a stretch of ~20 nonpolar residues, because algorithms based on polypeptide sequence hydrophobicity do not always accurately predict which sequences are transmembranous. A further complication is that particularly strong TM sequences can even force preceding hydrophilic polypeptide segments to adopt a TM conformation, thereby affecting insertion post-translationally (Ota et al 1998). In addition, efficient integration of two otherwise-secreted hydrophobic domains in the TM form of murine immunoglobulin M (Andrews et al 1992) and in the prion-related protein PrP (Lopez et al 1990, Yost et al 1990) requires a hydrophilic sequence in the nascent chain referred to as a stop-transfer effector or an STE sequence. It is not known what component of the system recognizes and/or interacts with an STE sequence.

For many membrane proteins, the signal sequence is not cleaved and is sufficiently hydrophobic to be directed into the bilayer (a signal-anchor TM sequence) rather than to the SP. SRP interacts with signal-anchor sequences when they first emerge from the ribosome and targets them to the translocon. These sequences are adjacent to phospholipids soon after targeting has concluded (Martoglio et al 1995, Mothes et al 1997), but it is not clear exactly how the system distinguishes signal-anchor sequences from typical signal sequences or where their pathways diverge.

Transmembrane Sequence Orientation The mechanism for ensuring that each TM sequence in a membrane protein is oriented properly is also unknown. It has been proposed that the net charges on the polypeptide sequences that flank the TM sequence dictate its orientation, with the more positive of the two remaining on the cytoplasmic side of the membrane (von Heijne 1994). Whereas the positive-inside rule is consistent with the topography of most proteins integrated into bacterial membranes (von Heijne & Gavel 1988, von Heijne 1994), this generalization has been less successful in predicting the topography of proteins inserted cotranslationally into the ER membrane (Andrews et al 1992, Spiess 1995, Gafvelin et al 1997, Sato et al 1998). For example, some TM segments of multiple-spanning proteins can be inserted in either orientation, completely inverting the topography of the protein—such is the case with ductin. This membrane protein with four TM segments is cotranslationally integrated into the ER membrane in two opposite orientations, apparently to carry out two different functions, either as a subunit of the vacuolar H^+-ATPase or as a component of the connexon (Dunlop et al 1995). The prion protein displays a similar behavior, with two membrane-spanning segments that can adopt two different orientations at the membrane in

addition to being completely secreted (Lopez et al 1990, Yost et al 1990, Hegde et al 1998a).

In early work with model proteins containing duplications of the same TM sequence, the ultimate orientations of the TM sequences in the membrane alternated, and hence the topography of the protein was defined by the decision that established the orientation of the first TM sequence (Wessels & Spiess 1988, Lipp et al 1989, see also High & Laird 1997). Yet the story is not always so simple and straightforward. Some multispanning membrane proteins are inserted with multiple topographies and thus deviate from a strictly alternating orientation of predicted TM sequences, probably because some predicted TM sequences are not always recognized as such (Skach et al 1993; Zhang et al 1993, 1996; for a review, see Levy 1996). Cystic fibrosis transmembrane regulator integration is further complicated by the fact that its first TM sequence is inserted into the bilayer post-translationally, not cotranslationally (Lu et al 1998).

These and other instances of nonstraightforward threading of the polypeptide into the bilayer demonstrate that no single, simple property of the nascent chain controls the recognition and processing of TM sequences. Instead, it seems likely that the balance of several nascent chain sequence and flanking region properties [e.g. hydrophobicity, charge, conformation (Denzer et al 1995), magnitude of the α-helical dipole] dictates what happens to the polypeptide at the translocon (Johnson 1997, Wahlberg & Spiess 1997, Harley et al 1998, Moss et al 1998). A system that is complex enough to simultaneously assess multiple properties of the nascent chain near the TM sequence would provide flexibility and the opportunity to achieve multiple structural and functional outcomes (e.g. ductin), thereby accommodating the structural requirements of each of the many different membrane proteins that are integrated at the translocon.

Currently, the mechanisms that result in proper TM sequence orientation are largely unknown, including the extent to which this process is mediated by proteins either in the translocon or transiently associated with it. Membrane proteins with uncleaved N-terminal signal-anchor sequences are correctly oriented in proteoliposomes containing only the Sec61 complex and TRAM (Görlich & Rapoport 1993, Oliver et al 1995), which suggests that the core translocon proteins are sufficient to orient signal-anchor TM sequences accurately. However, the final topography of some integral membrane proteins may also be influenced by unknown soluble cytoplasmic factors (Zhang & Ling 1995, Falk & Gilula 1998, Hegde et al 1998b).

Lateral Movement and Insertion of the Transmembrane Sequence How does a TM sequence move laterally through the translocon and into the bilayer? Different stages of integration were examined by creating integration intermediates with nascent single-spanning membrane proteins of increasing length and incorporating a single photoreactive probe in the middle of the TM sequence of each to determine its environment (Do et al 1996). After entering the translocon, the TM

sequence was initially adjacent to both TRAM and Sec61α, but it was then moved sequentially to two different sites within the translocon from which only TRAM was photocross-linked. It is surprising that the TM sequence remained adjacent to TRAM until translation of the single-spanning membrane protein was terminated. These data suggest that the TM sequence moves through the translocon via a multistep pathway, by use of protein-protein interactions to regulate the location of the TM sequence in the translocon until the TM sequence is released into the bilayer after translation terminates (Figure 4*A*) (Do et al 1996).

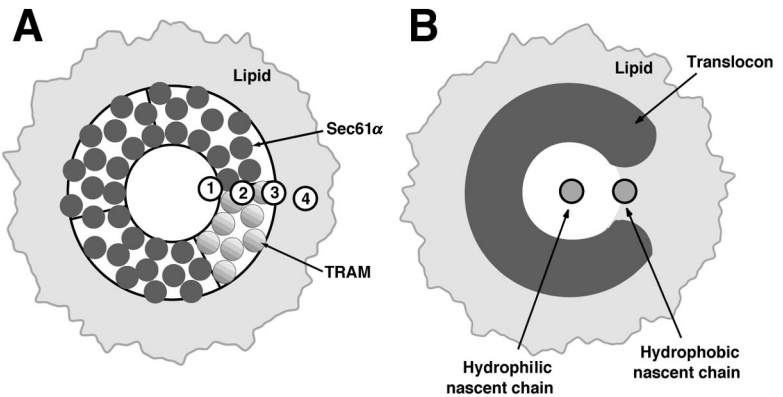


Figure 4 Models of transmembrane segment integration into the membrane. (*A*) Protein-regulated integration. The pore is viewed in cross section as in Figure 1*C*. During its integration, the TM segment moves sequentially through three different translocon protein environments that are distinguishable by photocross-linking (Do et al 1996). Upon entering the pore, the TM segment is adjacent (is cross-linked) to both TRAM and Sec61α (1). After the nascent chain increases in length by $\sim$20 residues, the TM segment is then found adjacent only to TRAM (2) and is actively held in this position, because releasing the nascent chain from the ribosome by using puromycin before photolysis has no effect on the photocross-linking. The TM segment then moves to a site (3) that is adjacent to TRAM but is distinct from site (2) because the TM segment can diffuse away from TRAM if the nascent chain is released by puromycin treatment. The TM segment of a single-spanning signal-cleaved membrane protein remains adjacent to TRAM until translation terminates, after which the TM segment diffuses laterally into the bilayer (4). (*B*) Phospholipid-mediated integration. The pore is opened laterally to the lipid phase, either transiently or constantly, and the positioning of the nascent chain within the pore is determined by the hydrophobicity of the polypeptide occupying the translocon. Hence, a hydrophilic nascent chain will remain in an aqueous milieu, whereas a nonpolar portion of the nascent chain—such as a TM or signal anchor sequence—will diffuse laterally and partition spontaneously into the bilayer (Martoglio et al 1995).

A different view of TM sequence insertion was obtained when a photoreactive probe was positioned in the middle of an uncleaved signal-anchor sequence, the first topogenic sequence in the nascent chain. In contrast to the above case, where the TM sequence was the second topogenic sequence in the nascent chain (a cleavable signal sequence was located at the N terminus of the protein), most signal-anchor probes reacted covalently with phospholipid molecules, not translocon proteins, after entering the translocon (Martoglio et al 1995, Mothes et al 1997). These data suggest that the TM sequence moves into the bilayer immediately or soon after reaching the translocon, possibly by passing directly from the aqueous pore into the nonpolar core of the bilayer (Figure 4*B*). In this model, a TM sequence is recognized and transferred to the interior of the bilayer in a concerted, one-step movement at the translocon via a direct interaction between the TM sequence and the bilayer, although one cannot rule out transient protein involvement in this process.

The above studies of TM sequence movement at the translocon involved different types of membrane proteins and also focused on different photocross-linking targets (proteins vs phospholipids). The different conclusions of these studies could therefore result from these experimental differences. One suggestion (Mothes et al 1997), that the extended photocross-linking to TRAM observed by Do et al resulted from the use of a nitrene-reactive group rather than a shorter-lived carbene, was shown not to be the case because the same photocross-linking results were obtained in parallel experiments with carbene and nitrene photoreagents (H Do & AE Johnson, unpublished data). Thus, the differences are not probe dependent. It remains to be determined whether the insertion process actually differs for signal-anchor and other TM sequences.

When does the TM sequence leave the translocon? The above two possibilities represent the extremes for a single-spanning membrane protein, one in which the TM sequence immediately enters the nonpolar core of the bilayer (Martoglio et al 1995, Mothes et al 1997) and one in which the TM sequence is not released until translation is terminated (Do et al 1996). If the latter model is correct, at least for some proteins, then what happens with the TM sequences in a multispanning membrane protein? Does the entry of a second TM sequence into the translocon cause the first to leave? *P*-glycoprotein nascent chains containing as many as five TM sequences were extracted from microsomes by urea while they were still covalently attached to the tRNA, but not after they were released from the tRNA by use of puromycin (Borel & Simon 1996). These urea extraction data suggested that the TM sequences had not been integrated into the bilayer but were instead all located in the aqueous pore of the translocon until they were integrated into the bilayer at the same time (Borel & Simon 1996). A different study of *P*-glycoprotein integration concluded that its TM sequences integrated into the bilayer in pairs (Skach & Lingappa 1993), perhaps as helical hairpins (Engelman & Steitz 1981). Pairwise integration of TM sequences was also indicated in studies of the insertion of *Neurospora* H^+-ATPase (Lin & Addison 1995). It is clear from the wide spectrum of models derived from the work cited in this paragraph that we

still have much to learn about how nascent chains and TM sequences interact with the translocon and about whether there are multiple modes of translocon-mediated integration.

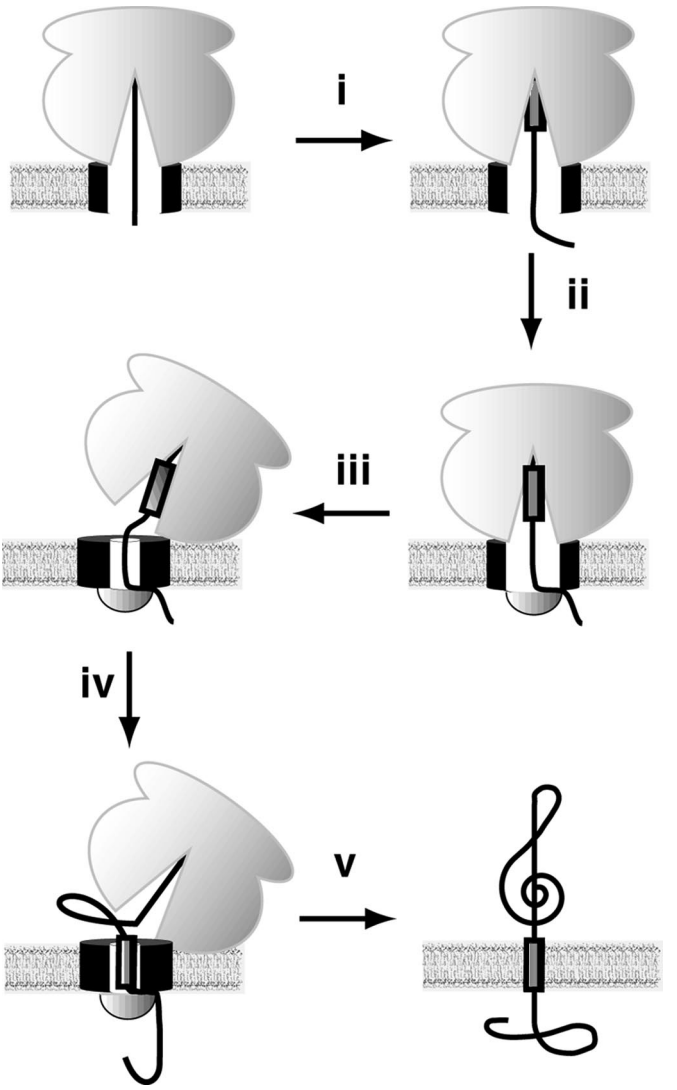
Maintenance of the Permeability Barrier An integral membrane protein has one or more domains or loops on the cytoplasmic side of the membrane. Because the permeability barrier of the ER membrane is maintained during translocation by the binding of the ribosome to the cytoplasmic end of the aqueous translocon pore (Crowley et al 1993, 1994), how are domains of a nascent membrane protein introduced into the cytoplasm without disrupting the permeability barrier? To assess nascent chain accessibility to one side of the membrane or the other, fluorescent probes were incorporated into nascent membrane proteins of different lengths, and their exposure to both cytosolic and lumenal iodide ions was then determined. The resulting collisional quenching data (Liao et al 1997) revealed that (i) the ribosome-translocon junction is opened to allow the cytoplasmic domain of the nascent chain to move into the cytoplasm while translation proceeds; (ii) before the ribosomal seal is broken, the other end of the aqueous translocon pore is first sealed by an unknown mechanism; and (iii) these major structural changes on both sides of the translocon occur while the TM sequence is still inside the ribosome (Figure 5). The series of events depicted in Figure 5 is both structurally complex and also highly precise: Step (ii) is completed (100% open to 100% closed), while the nascent chain is extended in length by only two residues, as is true for step (iii) (Liao et al 1997). Because the purpose of these structural changes is to prevent the movement of small ions and molecules from one side of the membrane to the other, the system has evolved to ensure that the translocon pore is sealed at all times. Specifically, the lumenal end of the pore is closed before the cytoplasmic end is opened during integration (Figure 5), just as BiP is not released from the lumenal end of the pore until the ribosome is firmly seated on the other end of the pore during targeting (Figure 3).

Also, protease protection experiments with a type-I signal-anchor membrane protein showed that in integration intermediates nascent chain domains destined to end up in the cytoplasm were accessible to proteases added to the outside of the vesicle (Mothes et al 1997).

Nascent Chain Processing and Folding

Some enzymes act on nascent proteins while they are still in the translocon. Signal peptidase cleaves off signal sequences when the nascent chain length totals ~150 residues (e.g. Mothes et al 1994, Nicchitta et al 1995). OST glycosylates the nascent chain whenever an *N*-glycosylation sequence (Asn-X-Thr/Ser) extends more than 12–14 residues from the lumenal end of the translocon (Nilsson & von Heijne 1993, Popov et al 1997). In addition to covalent modifications of the nascent chain, some of its folding also occurs cotranslationally. The clearest example of the latter is provided by studies of nascent hemagglutinin, whose interactions

with calnexin, calreticulin, protein disulfide isomerase, BiP, and other lumenal proteins control the folding rate to prevent misfolding or aggregation (Haynes et al 1997, Tatu & Helenius 1997, Zhang et al 1997; see also Helenius et al 1997). Assembly of multicomponent complexes can also occur cotranslationally. For example, the association of triglycerides and cholesteryl esters with nascent apoB occurs cotranslationally during the formation of lipoprotein particles (Boren et al 1992). Similarly, heterodimers of the inositol trisphosphate receptor (Joseph et al 1997) and polypeptides of photosystem II (Zhang et al 1999) may assemble



cotranslationally, although little is known about the details of either process. In view of the large number of proteins that may interact with nascent chains in the ER lumen, one might expect that access to the nascent chain is regulated, at least to some extent, to ensure that each interaction can occur at the proper time. It is therefore reasonable to wonder whether the nascent chain is substrate channeled by the translocon, that is, directed along a specific path through the translocon to the functional sites of OST, SP, and then perhaps to other proteins such as calnexin. Such channeling would have the added advantage that it would (presumably) restrict folding of the nascent chain, during the time when its primary sequence needs to be surveyed by the OST and/or SP (Whitley et al 1996).

Retrotranslocation

So far, we have considered only the vectorial transfer of proteins from the cytoplasm to the ER lumen. However, a growing body of work indicates that the translocon may be involved in the transport of proteins from the ER lumen to the cytoplasm. At least some translocation components seem to be involved in the removal of misfolded or misassembled proteins from the ER lumen for degradation by the proteasome (Brodsky & McCracken 1997, Kopito 1997, Sommer & Wolf 1997).

One of the first indications that the Sec61 complex may be involved in a quality control process at the ER (Hammond & Helenius 1995) was a mutation in the yeast Ubc6p (a protein involved in the ubiquitination of substrates for degradation by the proteasome) that suppressed a known Sec61p mutation (Sommer & Jentsch 1993). The involvement of the mammalian Sec61 complex in proteolytic processing at the ER membrane was later shown in a study of cytomegalovirus-mediated degradation in which a newly deglycosylated immunoglobulin heavy chain was coimmunoprecipitated with the Sec61 complex when degradation by the proteasome was inhibited (Wiertz et al 1996). Using an in vitro system, Pilon

Figure 5 Nascent chain exposure during membrane protein integration. The conversion of the translocon function from translocation to integration begins soon after the TM sequence is synthesized by the ribosome. Immediately after the TM segment is synthesized (i), the system is in its translocation mode, with the lumenal end of the pore open and the ribosome seal intact. When the C-terminal end of the TM segment is ~4 residues from the peptidyl transferase center (and still inside the ribosome), the lumenal end of the pore is closed by an unknown mechanism (ii). After five more residues are added to the nascent chain, the ribosome-translocon junction is opened (iii), thereby allowing the growing cytoplasmic domain of the nascent chain to move into the cytoplasm (iv). For a signal-cleaved single-spanning membrane protein, the ribosome-translocon junction remains open until translation terminates (v), and the permeability barrier is maintained by closing the lumenal end of the pore (Liao et al 1997).

et al (1997) demonstrated that a mutated form of prepro-α-factor that serves as a proteasome substrate (McCracken & Brodsky 1996) remained intact after translocation into ER microsomes prepared from strains carrying Sec61p mutations. The same study also showed that this defective protein was associated with Sec61p on the lumenal side of the membrane when retrotranslocation was inhibited (Pilon et al 1997). In another study, the degradation substrate was co-immunoprecipitated with antibodies against Sec61β (Bebök et al 1998). These results, together with other mutation analyses (Plemper et al 1997, 1998; Pilon et al 1998), confirm the involvement of the translocation machinery in quality control. However, it is not clear whether the movement toward the cytoplasm occurs before the release of the nascent chain from the translocon or whether there is a targeting mechanism that delivers the degradation substrate to the lumenal end of a translocon pore or laterally into the pore.

Numerous ER lumenal and membrane proteins have been implicated in the process of retrotranslocation: Kar2/BiP (e.g. Plemper et al 1997, Brodsky et al 1999), calnexin (e.g. McCracken & Brodsky 1996, Chen et al 1998, Brodsky et al 1999, Liu et al 1999), Sec63p (Plemper et al 1997), protein disulfide isomerase (Wang et al 1997), Der1p (Knop et al 1996), Hrd3p and Der3p/Hrd1p (Hampton et al 1996, Bordallo et al 1998), and Cue1p (Biederer et al 1997). However, it is not yet clear to what extent these proteins interact directly with the translocon to influence polypeptide movement. As is true for most aspects of this process, many questions remain unresolved, and much work still needs to be done to elucidate the role of the translocon in ER protein degradation.

REGULATION AND DYNAMICS OF TRANSLOCON STRUCTURE AND FUNCTION

Multiple lines of evidence demonstrate that the translocon is anything but a static pore. The large difference in pore diameter for ribosome-free and functioning translocons (Hamman et al 1998) demonstrates that the translocon undergoes major structural rearrangements during its functional cycle (Figures 1*B*,1*C*; Figure 3). The translocon also changes conformation as TM sequences pass laterally through the translocon during integration (Do et al 1996). Conformational changes also accompany the sequential sealing of the lumenal end and the opening of the cytoplasmic end of the translocon pore during integration (Liao et al 1997). Pause-transfer sequences in the apoB nascent chain cause the ribosome-translocon junction to open, reversibly, during the cotranslational processing of apoB (Hegde & Lingappa 1996). In addition, the composition of the translocon changes as its functional state changes, as evidenced by the transient association of the translocon with BiP (Brodsky & Schekman 1993, Hamman et al 1998) and the SRP receptor (Walter & Johnson 1994). Thus, translocon structure is time dependent, and the figures in this review depict some of the dynamic changes in translocon structure during its functional cycle.

Translocon Conversion from Functional to Nonfunctional

The most dramatic change is the large alteration in pore diameter, from 9–15 Å in a ribosome-free translocon (Hamman et al 1998) to 40–60 Å in a functioning translocon (Hamman et al 1997). This difference in pore size is controlled by the ribosome because large pores become small pores when ribosomes are released from the microsomes in high salt (Hamman et al 1998). It therefore seems likely that the arrangement of translocon components and hence the diameter of the pore are determined by the specific binding interactions that create the tight junction between the ribosome and the translocon components. We speculate, with little supporting experimental data, that the contraction of the translocon is driven by BiP-dependent ATP hydrolysis after translation terminates.

Changes in Nascent Chain Accessibility to Cytoplasm and ER Lumen

Immediately after targeting has been completed, the nascent chain is not exposed to either the cytoplasm or the ER lumen (Crowley et al 1993) (Figure 3). After the nascent chain reaches a length of ~70 residues, BiP is released from the lumenal end of the pore, and the nascent chain is then exposed to the lumen (Crowley et al 1994, Hamman et al 1998). During the translocation of an ordinary secretory protein, the tight seal between the translocon and the ribosome is then maintained and the nascent chain need never be exposed to the cytoplasm (Crowley et al 1994).

In at least one case, however, this simple model does not hold. When apoB association with lipid material is irreversibly disrupted during the normal cotranslational assembly of an apoB lipoprotein, the apoB nascent chain is degraded by proteasomes located in the cytoplasm to regulate apoB translocation and hence lipoprotein production, as discussed above. This degradation requires the breakage of the ribosome-translocon seal to allow the apoB nascent chain access to the cytoplasm where it is ubiquitinated and then proteolyzed. The mechanisms for initiating the cytoplasmic exposure and the proteolytic degradation of apoB are still obscure, but the former appears to require an interaction between a pause-transfer sequence in the nascent chain (Chuck & Lingappa 1992, Hegde & Lingappa 1996) and TRAM in the translocon (Hegde et al 1998c).

The requirement for a tight ribosome-translocon seal also creates a problem during cotranslational integration at the ER membrane because the cytoplasmic domain(s) of a nascent membrane protein must, at some point, move past the ribosomal seal and into the cytoplasm. As noted above, recognition of the TM sequence by the ribosome causes the lumenal end of the pore to close, and shortly thereafter the cytoplasmic end of the pore is opened (Figure 5) (Liao et al 1997). The disruption of the ribosome-translocon seal therefore allows the cytoplasmic domain of the nascent membrane protein to enter the cytoplasm. For a single-spanning membrane protein, nascent chain access to the cytoplasm then continues until protein synthesis terminates (Liao et al 1997).

For multispanning membrane proteins, the above results suggest that the ribosomal seal would be established (and the lumenal end of the pore would be open) whenever the ribosome was synthesizing a lumenal domain of the nascent chain and that the ribosomal seal would be open (and the lumenal seal established) whenever the ribosome was synthesizing a cytoplasmic domain of the nascent chain. However, this putative cyclic alternation of translocon structure and nascent chain exposure during the cotranslational integration of multispanning membrane proteins has not yet been demonstrated experimentally.

Conversion of Translocon Operational Mode from Translocation to Integration

For nascent chains with cleavable signal sequences, the default functional mode of the translocon is translocation. The system converts to integration only after a TM sequence is synthesized and recognized by the ribosome (Liao et al 1997). The conversion in functionality is therefore initiated before the TM sequence reaches the translocon and hence appears to be effected allosterically through ribosome-mediated changes at the translocon.

In contrast, for uncleaved signal-anchor sequences, the shift into the integration mode apparently occurs soon after the signal-anchor sequence is released from the SRP and interacts with the translocon (Martoglio et al 1995, Mothes et al 1997). In this case, the conversion in translocon function from translocation to integration may be elicited by a direct interaction between the signal-anchor sequence and the translocon. The extent of ribosome involvement in this process is unclear. Whereas the signal-anchor sequence may have a unique means of converting the functional state of the translocon, once the translocon is in the integration mode it seems very likely that the integration process will be the same for all of the other TM sequences in a multispanning membrane protein, regardless of whether they have a signal-anchor or a cleavable signal sequence.

Coordination Between Translocon and Ribosome

Whereas it was once possible to consider the ribosome and translocon as independent units at the ER membrane, one synthesizing a protein and the other transporting it, it has become exceedingly clear that the ribosome and the translocon are tightly coupled, both structurally and functionally. In fact, these two multicomponent complexes are more accurately thought of as a single structural and functional unit during cotranslational sorting, and one could reasonably argue that the ribosome is just one component of the complete functioning translocon.

Structurally, the ribosome and translocon associate to form a tight seal that creates the permeability barrier during translocation. This seal is tight in terms of not permitting small ions to pass through the ribosome-membrane junction (Crowley et al 1993) and also in terms of binding affinity to the Sec61 complex (Kalies et al 1994). The binding surfaces on the ribosome and translocon therefore must complement each other very well. This complementarity is particularly impressive given

the size of the binding surfaces, sufficient to form an impermeable junction around an aqueous pore with a diameter of 40–60 Å (Hamman et al 1997) (Figure 1).

Together, the ribosome and translocon direct and control nascent chain movement at the ER membrane. This coupling is especially evident during the integration process, when the detection of a TM sequence inside the ribosomal nascent chain tunnel leads to major structural changes at each end of the translocon pore, first closing the lumenal end of the pore and then opening the ribosomal seal at the cytoplasmic end (Liao et al 1997). This coordination of ribosome and translocon is remarkable for several reasons. First, the TM sequence is detected inside the ribosome on the cytoplasmic side of the ER membrane, but the initial ramification of this recognition is the closing of the pore on the other side of the membrane. Second, this communication from one side of the membrane to the other is a very long-range coupling, probably spanning >150 Å (an ∼50-Å-thick bilayer + nearly halfway up the ∼250-Å-diameter ribosome). Third, the structural communication from one side of the membrane to the other includes a variety of molecular species: ribosomal proteins, rRNA, translocon proteins, and perhaps lumenal proteins, as well as both membrane and nonmembrane proteins. Fourth, the coordination is highly precise: both steps (ii) and (iii) in Figure 5 are completed while the nascent chain increases in length by only two residues.

Other examples in which the ribosome and translocon together control nascent chain movement and exposure, often in response to signals or changes in the nascent chain, include the opening of the ribosomal seal at the cytoplasmic end of the pore during the processing of apoB (Hegde & Lingappa 1996); the opening of the lumenal end of the aqueous pore only after the ribosome-synthesized nascent chain reaches a length of ∼70 residues (Crowley et al 1994); and retention of the TM sequence of a signal-cleaved single-spanning membrane protein in the translocon until translation terminates (Do et al 1996).

Thus, the intimate merging of ribosome and translocon creates a multifaceted unit that dynamically alters its structural state to accomplish or accommodate a particular functional state. Extended multicomponent structural linkages must effect the long-range, TM communications that mediate various stages of cotranslational targeting, translocation, and integration. Although no specific components of any of these signal transduction systems have been identified as yet, photocross-linking studies have identified possible ribosomal protein participants in TM sequence detection (Liao et al 1997) and a membrane protein that may be involved in apoB pause-transfer processing (Hegde & Lingappa 1996). Delineation of these signaling pathways represents a major experimental challenge of the future.

SOME UNRESOLVED ISSUES

As is evident from the text thus far, we still have much to learn about translocon structure and function. Despite the often fragmentary information, detailed mechanisms and models are frequently proposed to explain a particular aspect of

translocon function. Such speculation is useful in terms of providing new models to test experimentally, but it is sometimes difficult, especially for those new in the field, to discern exactly what is documented experimentally and what is presumed to be true.

Thus, in this last section we address a few major issues that are still unresolved and that involve the translocon to various extents. In doing so, we highlight the alternative or controversial aspects of the process to clarify what remains uncertain. More experiments and likely new experimental approaches will be required to identify which of the mechanisms discussed below are real and which are imagined.

Where Does the Signal Sequence Go After It Leaves the Signal Recognition Particle?

The signal sequence is currently thought to be released from the SRP after a GTP-dependent interaction between the SRP receptor and the SRP-nascent chain-ribosome complex. Also, the signal sequence is cleaved from the nascent chain after it reaches a length of ~150 residues (e.g. Mothes et al 1994, Nicchitta et al 1995), and the signal sequence is then further processed after a second proteolytic cleavage (Lyko et al 1995, Martoglio et al 1997). So where does the signal sequence go after it leaves the SRP and before it reaches the SP?

Based on early photocross-linking results, the signal sequence was proposed to bind to the signal sequence receptor or SSR protein (Wiedmann et al 1987, Prehn et al 1990). Later studies identified TRAM (Görlich et al 1992a, High et al 1993, Mothes et al 1994) and Sec61α (Görlich et al 1992b, High et al 1993, Mothes et al 1994), instead of SSR (now termed TRAPα) (Hartmann et al 1993), as the primary photocross-linking targets of photoreactive probes in the signal sequence. The signal sequence is therefore in proximity to both Sec61α and TRAM after leaving the SRP. Furthermore, signal sequence–dependent binding of ribosomal complexes to translocons in the absence of SRP indicated that the translocon contains a binding site for the signal sequence (Jungnickel & Rapoport 1995). This binding site has been assumed to be formed by Sec61α (Jungnickel & Rapoport 1995), largely because photocross-linking studies suggest that Sec61α is the primary protein exposed on the inner surface of the translocon pore (High et al 1993, Mothes et al 1994). This view is supported by the recent demonstration that the signal sequence photocross-links to TM2 and TM7 of yeast Sec61p in post-translational translocation complexes (Plath et al 1998), as well as by experiments in detergent solution indicating that signal sequence recognition by the translocon is based on protein-protein interactions (Mothes et al 1998). Although it is very tempting to assume that the signal sequence binds to the same TM2-TM7 site in a mammalian translocon, this supposition has not yet been shown experimentally. TRAM has also been proposed to interact with the signal sequence (High et al 1993, Voigt et al 1996). Is this latter interaction direct or indirect? If direct, does the signal sequence bind to TRAM before, after, or instead of binding to Sec61α?

It has also been proposed that the hydrophobic core of the signal sequence enters the nonpolar interior of the bilayer after being released from SRP, based on the greater extent of signal sequence photocross-linking to phospholipids than to TRAM or Sec61α (Martoglio et al 1995). Does this result indicate that the signal sequence is embedded in the bilayer and that the observed protein cross-linking results simply from random encounters between signal sequence and translocon proteins? Alternatively, does the signal sequence bind specifically to Sec61α or TRAM, as suggested in the previous paragraph, whereas photoreactive probes in the signal sequence react primarily with nearby phospholipid molecules interspersed between protein α-helices in the bilayer?

The current dogma has also been questioned in two studies that examined the protease sensitivity of nascent chains in membrane-bound ribosomes (Murphy et al 1997, Nicchitta & Zheng 1997). The signal sequence in each complex was cleaved by exogenous proteases, thereby indicating that a nascent chain long enough to target, but too short for SP cleavage, was exposed to the cytoplasmic surface of these membrane-bound complexes. These data elicited a model in which the signal sequence dynamically moves back and forth between being buried in the lipid bilayer and being exposed to the cytoplasm (Nicchitta & Zheng 1997). Does the nascent chain therefore move through the bilayer into the translocon or to the SP at the appropriate time, or does the ribosome have to dissociate from the membrane and then reinitiate SRP-dependent targeting to the translocon?

If the signal sequence does in fact bind specifically to Sec61α, TRAM, and/or phospholipids after leaving the SRP, what initiates the ultimate release of the signal sequence from this binding site(s) and the movement of the nascent chain to the SP for cleavage?

Thus, the path followed by the signal sequence between the SRP and the SP is anything but clear; almost every possibility has some experimental support among the contradictory models that have been published. A methodical, high-resolution examination of all possibilities, including kinetics experiments, will be necessary to eliminate the current confusion and rigorously establish signal sequence interactions and their ordering.

Is Transmembrane Sequence Integration Mediated by Translocon Proteins or by Phospholipids?

As discussed above, different conclusions have been reached in photocross-linking studies involving membrane proteins with a cleavable signal sequence (Do et al 1996) or a signal-anchor sequence (Martoglio et al 1995, Mothes et al 1997). Whereas the former study concluded that TM sequence insertion was a regulated multistep movement through the proteinaceous translocon, the latter two studies concluded that the TM sequence is inserted directly into the hydrophobic interior of the bilayer with little or no translocon protein involvement. Is the mechanism of insertion actually different for signal-anchor and other TM sequences or do the different conclusions reflect differences in experimental perspective? Both

translocon proteins and phospholipids are undoubtedly involved at some stage in integration, but the timing and extent of their involvement have not yet been defined experimentally.

How Do Phospholipid Molecules Contribute to Translocon Structure?

Is the nonpolar core of the bilayer exposed to the aqueous pore in the translocon, as has been suggested (Figure 4*B*) (Martoglio et al 1995)? If so, is such exposure constant or transient? Alternatively, do individual (or groups of) phospholipid molecules intercalate between the TM helices of TRAM, Sec61α, and other translocon components to provide an environment that is not completely proteinaceous? Distinguishing between these possibilities experimentally may prove difficult, especially because translocon structure is dynamic and the bilayer may be transiently exposed to the aqueous pore in vivo.

What Orients Transmembrane Sequences Before Their Insertion into the Bilayer?

The molecular basis of how TM sequence orientation is determined during the integration process in mammals is still a complete mystery. Some believe that specific proteins actively rotate the necessary TM sequences at the ER membrane, but no clear experimental evidence for such proteins has been reported despite efforts to detect such proteins by photocross-linking and other techniques. This negative evidence may indicate that no such proteins exist. However, even if the orientation of a TM sequence is dictated solely by the positive-inside rule at the translocon, something at the translocon must assess whether the TM sequence in a nascent chain is oriented correctly (i.e. is the positive end of the TM helix actually at the cytoplasmic surface?). Because single-spanning signal-anchor membrane proteins are correctly oriented in proteoliposomes containing only TRAM and the heterotrimeric Sec61 (Görlich & Rapoport 1993, Oliver et al 1995), it is possible that only the core translocon proteins are required for this function.

Why Is the Diameter of the Aqueous Pore in a Functioning Translocon So Large?

One obvious explanation for why the diameter of the acqueous pore in a functioning translocon is so large is that the sites on the ribosome that bind to the translocon components to form the tight seal are widely separated, and hence the binding of the translocon proteins to the ribosomal sites leaves a large space or pore in the middle. But the advantage of evolving such an arrangement is not so obvious. The large pore and ribosome nascent chain tunnel would allow some protein folding to occur, although premature folding would appear to interfere with OST recognition of nascent chain glycosylation sites. A large pore could also accommodate multiple TM sequences from a multispanning membrane protein if, as has been proposed,

such TM sequences accumulate inside the translocon and then enter the bilayer all at once (Borel & Simon 1996). Another possibility that we favor (Hamman et al 1997), but for which there is no direct experimental evidence, is that the large pore is important for integration, not for translocation. Every second TM α-helix sequence in a multispanning membrane protein must rotate 180° before insertion at the translocon, and each TM segment has a length of at least 30 Å. If such a TM sequence were to rotate without losing its helical conformation, then the pore would have to be >40 Å in diameter to accommodate this rotation. Clearly, the reason for the large translocon pore is still very much an open question.

A related question is why does the pore contract upon ribosome release? Because there are no ribosome-sized proteins in the ER lumen, it seems likely that the translocon pore must contract to allow a smaller protein, such as BiP, to bind to the lumenal end of the translocon and seal off the pore.

Are Nascent Secretory Proteins Other Than apoB Ever Exposed to the Cytosol?

In principle, a nascent secretory protein need never be exposed to the cytoplasm during translocation. But because nascent apoB is exposed to the cytoplasm without compromising the permeability barrier (as are nascent membrane proteins), it is conceivable that under certain conditions other nascent secretory proteins are also exposed to the cytoplasm. Consistent with this possibility, long preprolactin and other nascent chains in membrane-bound ribosomes have been degraded by proteases added to the cytoplasmic side of the membrane in some studies (Connolly et al 1989, Jungnickel et al 1995, Murphy et al 1997, Nicchitta & Zheng 1997) but not all (Mothes et al 1997). In contrast, short nascent chains were protected from proteases in all of the studies. We have also observed that long fluorescently labeled preprolactin chains are more susceptible to quenching agents and proteases than are shorter nascent chains (Hamman et al 1997), but we were unable to ascertain whether the increased exposure of the longer nascent chains represents a normal physiological occurrence or is a result of the experimental conditions (e.g. ribosome-released nascent chains adsorbed to the surface of the microsomes and/or instability of translocation intermediates with longer nascent chains). Further experiments are necessary to clarify whether and why nascent chains are exposed to the cytoplasm at later stages of translocation.

CONCLUSION

The focus of translocation research has changed over the past few years from demonstrating the existence and composition of the translocon to identifying the molecular mechanisms in which it participates. These studies have revealed a translocon that is far more intricate and sophisticated than is generally appreciated. In the near future, research will continue to focus on mechanistic aspects

of the translocon, both to fill in the gaps in our current understanding and to resolve existing discrepancies. X-ray crystallography has recently provided three-dimensional structures for the GTP- and signal sequence–binding domains of Ffh (Freymann et al 1997, Keenan et al 1998) and for FtsY (Montoya et al 1997), the bacterial homologs of the signal sequence-binding protein component of SRP and the SRP receptor, and such structures are illuminating as well as useful in designing new experiments. Structures of individual translocon components may also be available before long, but obtaining atomic structures of the entire membrane-bound translocon, or even the core translocon, will be a challenging long-term goal, particularly in view of the documented dynamics of translocon conformation and composition. Thus, maintaining the recent rapid progress in understanding the structure, function, assembly, and regulation of the translocon will require continued efforts with established techniques and the application of new approaches to examine this complex molecular machine.

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