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# Protein Translocons: Multifunctional Mediators of Protein Translocation across Membranes

**Review**

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**Protein translocation systems consist of complex molecular machines whose activities are not limited to unidirectional protein targeting. Protein translocons and their associated receptor systems can be viewed as dynamic modular units whose interactions, and therefore functions, are regulated in response to specific signals. This flexibility allows translocons to interact with multiple signal receptor systems to manage the targeting of topologically distinct classes of proteins, to mediate targeting to different suborganellar compartments, and to respond to stress and developmental cues. Furthermore, the activities of translocons are tightly coordinated with downstream events, thereby providing a direct link between targeting and protein maturation.**

Cells are defined by membrane boundaries that regulate the selective exchange of matter and information between aqueous environments. The plasma membrane, a universal feature of all cells, contains a multitude of complex transport systems that mediate directional movement of solutes and signals between the cytoplasm and external milieu. In eukaryotes, membrane-bound organelles segregate and organize the vast array of intracellular biochemical processes, providing the framework for cellular development and differentiation. The biogenesis and maintenance of cellular membranes and their corresponding compartments rely on an elaborate system of specific intracellular trafficking pathways that transport proteins to their proper compartment.

The exploitation of in vitro and genetic assays for protein targeting to eukaryotic organelles and the bacterial periplasmic membrane has revealed molecular details of the majority of protein targeting systems. Although the components of the systems vary for each pathway, unifying principles first proposed in the signal hypothesis two decades ago still hold (Blobel, 1980). Nascent or newly synthesized proteins contain intrinsic, organelle-specific targeting signals that are recognized by selective targeting receptors; the receptors are coupled to oligomeric membrane complexes, termed translocons (Walter and Lingappa, 1986) that mediate protein translocation across or integration into the membrane.

In recent years, this basic view of protein translocation systems has expanded tremendously. It now is clear that translocation systems consist of complex molecular machines whose activities are not limited to unidirectional protein targeting. It is more appropriate to view protein translocons and their associated receptor systems as dynamic modular units whose interactions, and therefore functions, are regulated in response to specific signals. This flexibility allows translocons to interact with multiple signal receptor systems to manage the targeting of topologically distinct classes of proteins (i.e., membrane versus soluble proteins), to mediate targeting to different suborganellar compartments, and to respond to stress and developmental cues. Furthermore, the activities of translocons are tightly coordinated with downstream events of protein folding, modification, and assembly, thereby providing a direct link between targeting and protein maturation. In some cases, the translocons can act in reverse to transport misfolded proteins back to the cytoplasm for degradation or provide a means of entry for cellular pathogens into the cytoplasm following endocytosis. This review will highlight the most recent developments in the traditional and expanded view of protein translocation systems. We will limit the scope of the review to protein targeting systems that operate across membrane bilayers. This excludes nucleocytoplasmic transport and specialized transport systems with limited substrate specificities. Our goal is to illustrate the modular activities of these multifunctional mediators of protein trafficking and organelle biogenesis.

## An Overview of Translocon Organization

All translocons possess several essential features (Figures 1 and 2) (Schatz and Dobberstein, 1996). First, they contain intrinsic signal recognition sites that act as the docking sites for the targeting signals of translocation substrates. The docking sites may act as the primary receptors for polypeptides, or they may act downstream of primary signal receptor systems that target polypeptides from their site of synthesis (*cis* compartment) to the translocon. Second, translocons form selectively permeable protein-conducting channels that mediate transport from the *cis* compartment to their destination (*trans* compartment). Finally, translocons must be coupled to a translocation driving force. In most cases, the association of molecular chaperones with the polypeptide in the *trans* compartment provides the energy for translocation.

Viewed from the perspective of classical membrane transporters, translocons represent remarkably flexible transport complexes. The translocation channels of translocons can accommodate hundreds or thousands of distinct protein substrates while maintaining the permeability barrier of the membrane. In addition to providing a conduit for complete translocation of polypeptides across membranes, many translocons sense stop-transfer signals within integral membrane proteins and gate laterally to allow the diffusion of transmembrane segments into the bilayer. Thus, the channels are not passive players in the translocation process.

Two classes of translocons have evolved in response

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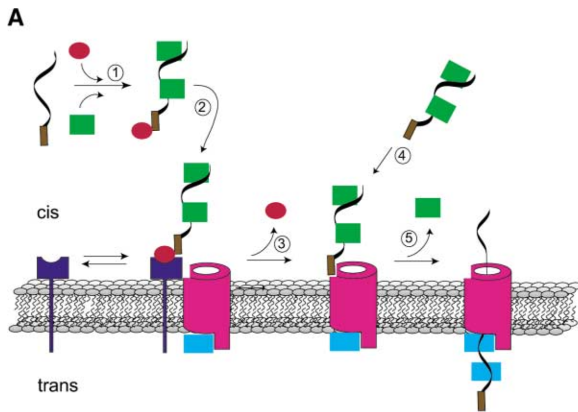


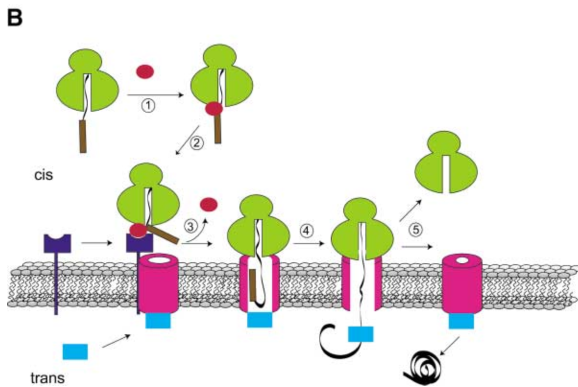


Figure 1. Signal-Gated Translocation

(A) Model for posttranslational protein translocation. (1) Binding of soluble signal receptor (red ball) to targeting signal (brown bar) of nascent polypeptide. (2) Docking of the soluble targeting complex to a membrane receptor (dark blue membrane protein) associated with a translocon (pink membrane channel). (3) Transfer of the targeting signal to a signal docking site on the translocon. (4) Alternatively, the targeting signal docks to the translocon without the aid of a soluble receptor. (5) Targeting signal binding gates open the channel and translocation proceeds with the assistance of molecular chaperones (light blue rectangle) in the *trans* compartment. Proteins are translocated in a largely unfolded state.

(B) Cotranslational translocation model. (1) A soluble signal receptor binds to the targeting signal as it emerges from the ribosome (green double ball). (2) The ribosome nascent chain complex (RNC) is targeted to the membrane by the binding of the soluble signal receptor to a cognate membrane receptor. (3) The RNC is transferred to the translocon and the signal receptor is released to the *cis* compartment. Binding of the RNC gates open the translocon and provides a seal to maintain the membrane permeability barrier. (4) Translocation proceeds cotranslationally until protein synthesis terminates. (5) The ribosome is released from the translocon and the translocon reverts to its closed conformation.

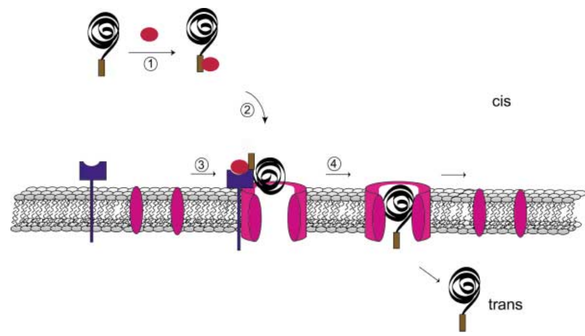


Figure 2. Signal-Assembled Translocation

(1) Folded and oligomeric proteins are bound by a soluble receptor (red ball). (2) The soluble targeting complex docks at the membrane receptor (blue membrane protein). (3) Docking at the membrane receptor triggers assembly of the translocon. (4) The diameter of the translocon channel is determined by the size of the translocation substrate.

to these extraordinary demands. The most common class of translocons is envisioned to function as derivatives of gated ion channels (Figure 1) (Blobel and Dobberstein, 1975). In this class, the nascent or newly synthesized polypeptide is threaded vectorially through a gated protein-conducting channel in a largely unfolded conformation with the aid of molecular chaperones. By maintaining the polypeptide in an unfolded conformation, a single translocon of defined dimensions can accommodate a vast array of substrates. As such, the translocation reactions can be modeled as the transport of polyions through modified channels in a manner analogous to ion or metabolite transport. We refer to these systems as signal-gated translocons.

The second class of translocons is distinguished by their ability to transport fully folded and/or oligomeric proteins of large dimensions while maintaining the membrane permeability barrier (Berks et al. 2000; Cline and Mori 2001; Gould and Collins 2002) (Figure 2). Stable translocon channels have not been detected in these systems, leading to the proposal that translocons of variable apertures are assembled in response to the size of the translocation substrate. We refer to this class as the signal-assembled translocons.

## The Signal-Gated Translocons

### *The Sec Translocons Represent a Conserved Group of Translocation Systems*

The SecYEG complex of the bacterial periplasmic membrane (Manting and Driessen, 2000) and the homologous Sec61 complex of the ER membrane (Johnson and van Waes, 1999) are the prototypes of the signal-gated, protein-conducting channels (Table 1). A Sec-related translocon also operates at the chloroplast thylakoid membrane (Mori and Cline 2001). These translocons contain an oligomeric membrane protein complex at their core. The trimeric core of the SecYEG complex reversibly associates with SecA, a cytoplasmic ATPase. SecA has a dual function as both a component of the cytoplasmic signal receptor system and as the major component of the membrane translocation motor (see below). The mammalian core contains the SecYEG-homologous Sec61$\alpha$, Sec61$\beta$, and Sec61$\gamma$ subunits and a fourth unique subunit, TRAM, that is required for membrane protein integration.

High-resolution cryoelectron microscopic examination of the Sec-related translocons indicates that they consist of multiple membrane-spanning $\alpha$ helices that

Table 1. Protein Translocons and Their Associated Factors

| | | Signal-Gated Translocons | | | | | | | | | Signal-Assembled Translocons | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Mitochondria | | | | Chloroplast | | | | |
| | | *E. coli* SecYEG System | ER Sec61 System | Thylakoid cpSec System | TOM Systems | TIM22 system | TIM23 System | Oxa1p System | TOC System | TIC System | Peroxisome PEX System | *E. coli* TAT System | Thylakoid TAT/ΔpH System |
| Targeting signals | | Leader (signal) sequence | Signal sequence | Thylakoid targeting domain (signal sequence) | Presequence | Modules of multiple transmembrane segments and their intervening sequences | Presequence | Internal signal encompassing a single transmembrane segment | Transit peptide | Transit peptide | PTS1 and PTS2 | Twin arginine signal sequence | Twin arginine signal sequence (lumenal targeting domain) |
| *Cis* components | Chaperones | SecB | Hsp70 | Hsp70 | Hsp70 | Tim9p-10p, Tim8p-13p, Tim12p | | | | | Hsp70 | | |
| | Soluble targeting-signal receptors | SecA; Ffh | SRP | cpSecA; cpSRP54, cpSRP43 | MSF;RAC | | | | Toc159; guidance complex | | Pex5p; Pex7p | | |
| | Membrane receptors | FtsY | SR | cpFtsY | | | Tim23p | | Toc33/34 | | Pex13p, 14p, 17p | | |
| Translocon components | Targeting signal receptors | SecY; YidC | Sec62p,63p, 71p,72p; TRAM | CpSecE; Albino3 | Tom20p, 22p,5p; Tom70p, 37p | Tim22p | Tim23p | | Toc33/34, 159 | Tic22 | | TatB,C | cpTatB/Hcf106, cpTatC |
| | Channels | SecY,E,G | Sec61$\alpha,\beta,\gamma$ | CpSecY,E | Tom40p | Tim22p | Tim17p, 23p | Oxa1p | Toc75, 159 | Tic20; Tic40 | (Pex10p, 12p, 8p, 2p) | TatA,B,C,E | cpTatA/Tha4, cpTatB/Hcf106, cpTatC |
| | Trans chaperone binding sites | | Sec63p | | | | Tim44p | | | Tic110 | | | |
| | Accessory factors | SecD, F, YajCp | | | Tom6p,7p | Tim54p; Tim18p | Tim50 | | Toc64 | Tic55 | 15 additional PEX proteins | TatD | |
| *Trans* components | Chaperones | | BiP; calnexin; calreticulin | | | | MtHsp70-Mge1 | | Hsp70-IAP | Hsp93; cpn60 | | | |
| | Processing peptidases | Leader peptidase | Signal peptidase | Leader peptidase | | | Mitochondrial processing peptidase; Imp1/Imp2 peptidase | | | Stromal processing peptidase | | Leader peptidase | Leader peptidase |

Protein translocons from the bacterial periplasmic membrane and eukaryotic organelles are categorized as signal-gated or signal-assembled translocons. The names of the targeting signals that direct proteins to the corresponding translocons, factors that assist in targeting polypeptides from their site of synthesis (*cis* compartment) to the translocon and factors that assist with protein translocation and maturation at the opposite side of the membrane (*trans* compartment), are listed for each known translocon.

form a central cavity (Beckmann et al., 2001; Breyton et al., 2002). The central cavity appears to correspond to the aqueous translocation channels originally identified by biochemical and electrophysiological measurements. Cross-linking studies with trapped translocation intermediates in conjunction with the structural studies identify Sec61$\alpha$ and SecY as the major constituents of the protein-conducting channels (Johnson and van Waes, 1999). Biochemical and microscopic measurements of the Sec61 translocon suggest that the protein-conducting channel exists in two states: a constricted closed channel with dimensions ranging from 9–25 Å (Beckmann et al., 2001) and a translocation-active open channel with dimensions of 40–60 Å (Hamman et al., 1997). Inactive SecYEG channels possess a channel of $\sim$16 Å (Breyton et al., 2002).

The dimensions of the Sec-related channels are exceptionally large compared to known ion or metabolite channels, raising the question of how they are gated while maintaining the membrane permeability barrier in the presence or absence of a translocation substrate. Channel dimensions likely are dictated by the need to accommodate two or more polypeptide strands at various stages of translocation. The Sec61 complex appears to be gated by peripheral components of the translocation system. Translocation occurs in two modes: the posttranslational mode in which proteins are translocated after synthesis is complete (Figure 1A), and the cotranslational mode in which protein synthesis is coincident with membrane translocation (Figure 1B) (Johnson and van Waes, 1999). The primary pathway for Sec61-mediated translocation in mammalian cells is cotranslational, and it appears that a bound ribosome at the translocon forms a tight seal to prevent exchange between the cytoplasm and ER lumen. Images of the ribosome-translocon complex suggest that the exit channel for nascent polypeptides on the ribosome and the Sec61 translocon channel are aligned to provide a direct conduit from the peptidyl transferase to the translocon (Beckmann et al., 2001). In this mode, translocation is coupled directly to translation.

During posttranslational translocation, the polypeptide is threaded through the translocon vectorially after the completion of protein synthesis, and a bound ribosome is not present to seal the channel. In the case of the Sec61 translocon, the lumenal molecular chaperone BiP associates with the *trans* face of the translocon and appears to prevent ion movement across the membrane in the absence of bound ribosome (Hamman et al., 1998). BiP is a member of the hsp70/DnaK family of chaperones. The extraction of BiP from ER microsomes reveals an open channel of dimensions consistent with those of the Sec61 translocon. The mechanics of the gating activity of BiP remain a mystery. It is not clear whether BiP itself acts as a plug for the channel or acts as a regulator of an undefined gating mechanism. The binding of BiP to translocation substrates also provides the unidirectional driving force for translocation in the absence of the bound ribosome (see below). These dual activities suggest that chaperone binding and channel gating are highly coordinated activities.

Two-dimensional membrane crystals of the SecYEG complex contain 15 transmembrane helices that associate into dimers (Breyton et al. 2002). Two membrane helices of SecE tilt inward and form contacts between the two monomers on the periplasmic face of the translocon. SecE is known to be a dynamic component of the translocon, and these helices might act as the gate to the central channel that opens in response to translocation (Veenendaal et al., 2001). This activity would serve an analogous function to BiP in the ER lumen. The bacterial periplasm lacks nucleoside triphosphates precluding the participation of chaperones on the *trans* side of the translocon.

The translocation of preproteins at SecYEG is driven directly by the cytoplasmic ATPase SecA. SecA is a homodimer that binds to both the signal sequences and mature portions of preproteins as well as to the SecYEG complex via SecY (Manting and Driessen 2000). The formation of the SecA-preprotein-SecYEG complex stimulates SecA ATPase activity, resulting in a dramatic conformational shift in which a domain of SecA inserts across the membrane through the SecYEG channel (Economou and Wickner 1994). This has led to a model in which SecA acts as a two-stroke piston, pushing $\sim$2–3 kDa segments of preproteins through the channel with each ATP-dependent insertion reaction. The membrane potential also is required for translocation and may act in channel gating (i.e., via SecE) for SecA insertion (Nouwen et al. 1996). The SecA translocation cycle compensates for the lack of a periplasmic energy source such as ATP that could fuel molecular chaperones in the periplasmic space.

***The Sec Translocons Interact with Multiple Signal Targeting Systems***. The Sec-related translocons act as common convergence sites for multiple targeting pathways by interacting with multiple receptor systems to target topologically distinct classes of proteins into or across membranes. The primary targeting signals for the Sec pathways, termed signal sequences (Zheng and Gierasch 1996), consist of a short ($\sim$20 amino acids) peptide with a hydrophobic core and short polar flanking regions. Signal sequences of soluble preproteins are typically located at their N termini and are proteolytically cleaved during or after translocation by specific peptidases at the *trans* side of the membrane. The signal sequences of integral membrane proteins can be located internally and are not cleaved.

Two targeting pathways to the Sec translocons in eukaryotes and prokaryotes are recognized: the cotranslational or signal recognition particle (SRP)-dependent pathway (Figure 1B) and the posttranslational or SRP-independent pathway (Figure 1A) (Johnson and van Waes, 1999). The SRP-dependent pathway is universally conserved in all cells (Keenan et al., 2001). In eubacteria, the pathway functions primarily in the targeting of nascent integral membrane proteins of the periplasmic membrane to the SecYEG translocon, thereby avoiding exposure of these hydrophobic substrates to the cytoplasm. In chloroplasts, the SRP homolog participates in both the co- and posttranslational targeting of hydrophobic components of the photosynthetic light harvesting complexes of the thylakoid membrane. In mammalian cells, the SRP-dependent pathway is the major pathway for the targeting of both nascent secretory and membrane proteins to the Sec61 translocon. The central player in the targeting process is the soluble GTPase SRP (Keenan et al., 2001). SRP binds to

the hydrophobic signal sequences of nascent secretory and membrane proteins as they emerge from the ribosome in the cytoplasm and targets the ribosome-nascent chain complex (RNC) to the translocon. The SRP acts in concert with a cognate membrane GTPase, the SRP receptor (SR), to perform unidirectional targeting via a GTP regulated targeting cycle. SRP thereby coordinates the coupling of translation with membrane translocation to prevent premature folding or misfolding of the translocation substrate prior to membrane targeting.

The GTPase cycle regulating SRP-dependent targeting has been an area of intense investigation, yet the complexity of unraveling the roles of multiple GTPases working in concert with large molecular machines, such as the ribosome and translocon, leave many questions unanswered. As a consequence, a consensus model for the GTP-dependent targeting cycle has not been reached. Nevertheless, elements of the cycle are coming to light (Keenan et al., 2001). The emerging signal sequence triggers the binding of SRP to the RNC, inducing elongation arrest in some cases. The docking of SRP-RNC at the membrane occurs via a homotypic interaction between homologous domains of SRP and SR at a site adjacent to the translocon. Docking is stabilized by reciprocal GTP-loading at SRP and SR resulting in unidirectional targeting of the RNC to the rough ER. Current models propose that the ribosome and signal sequence play complementary roles in the initial stages of the GTPase cycle by promoting GTP loading and inhibiting GTP hydrolysis by SRP, respectively (Miller et al., 1993; Bacher et al., 1996). In the next step, the RNC is transferred to the translocon and the signal sequence inserts into the channel. The mechanistic details of these transfer reactions remain a mystery.

In the final reaction, GTP hydrolysis by the SRP-SR complex results in dissociation of SRP from SR (Connolly et al., 1991). The translocon appears to stimulate the GTPase activities of SRP-SR (Bacher et al., 1996; Song et al., 2000). As such, the multiple sequential steps regulating GTP binding and hydrolysis at SRP and the SRP-SR complex function as a complex molecular switch, regulating the proper assembly of translocon components with the RNC to ensure the fidelity of protein translocation.

Recent studies suggest that SRP-dependent targeting may not be required each time the synthesis of a secretory protein is initiated (Nicchitta, 2002). These studies presented data suggesting that membrane-bound ribosomes can initiate protein synthesis. The dissociation of ribosomes from the ER appeared to be triggered only if the mRNA encoded a cytosolic rather than a secretory protein. As such, the SRP-dependent targeting cycle would not be required for each round of preprotein translocation but would represent a scavenging pathway that maintains the proper segregation of membrane-bound and soluble ribosomes in relation to the prevalence of mRNAs for secretory/membrane versus non-secretory proteins. Although controversial, this model would provide a means of maintaining a dynamic equilibrium between ER bound and soluble ribosomes in response to the demand for secretory/membrane protein synthesis.

The posttranslational modes of targeting to the Sec translocons differ markedly in prokaryotes and eukaryotes. The eukaryotic pathway has been studied primarily in yeast, where it represents a significant proportion of targeting to the Sec61 translocon. In this pathway, the Sec61 translocon associates with a second oligomeric membrane protein complex, the Sec62/63 complex (Rapoport et al. 1999). The Sec62/63 complex contains a cytoplasmic signal sequence receptor site that binds newly synthesized secretory proteins. The substrates are maintained in an unfolded, translocation-competent conformation with the aid of cytoplasmic chaperones (Chirico et al., 1988). Subsequent to binding, the signal sequence is transferred from Sec62/63 to the signal sequence receptor of the Sec61 translocon, and translocation occurs via the Sec61p channel. As previously mentioned, BiP plays direct roles in channel gating and polypeptide transport. These activities are coordinated by the Sec63 subunit of the Sec62/63 complex (Brodsky and Schekman, 1993). Sec63 contains a lumenal J domain that is homologous to a region of the DnaJ cochaperone of the *E. coli* Hsp70, DnaK. The J domain acts as a docking site for BiP, localizing the chaperone to the lumenal exit site of the translocon.

The posttranslational mode of targeting to the SecYEG translocon predominates in prokaryotes. In addition to its role as the translocation motor, SecA also selectively binds to the signal peptides of exported proteins in the cytoplasm (Lill et al., 1990). It is assisted by SecB, a molecular chaperone, that binds both to SecA and to the mature portions of preproteins (Hartl et al., 1990). SecB is released from the targeting complex immediately upon docking at the SecYEG translocon and appears to function in maintaining the import competence of newly synthesized proteins.

### *Mitochondria and Chloroplasts Contain Multiple, Coupled Translocons*

Mitochondria and chloroplasts are complex organelles containing multiple membranes. As a consequence, they possess multiple translocons that work in sequence to target proteins to the outer membrane, intermembrane space, inner membrane, and internal soluble compartments. The TOM and TOC translocons of mitochondrial and chloroplast outer membranes, respectively, associate reversibly with translocons of their corresponding inner membranes (TIMs in mitochondria and TICs in chloroplasts). These are highly regulated events that are dictated by multiple topogenic targeting signals of the translocation substrate.

*TOM and TOC Translocons Mediate the Initial Stages of Protein Import into Mitochondria and Chloroplasts*. Although there is no apparent sequence similarity between the components of the TOM and TOC translocons, both are distinguished by the presence of core subunits that form the major constituent of their respective translocation channels. The core subunits, Tom40p (Vestweber et al., 1989; Hill et al, 1998) and Toc75 (Kouranov and Schnell, 1997; Hinnah et al., 2002), both possess cation-selective channel activity when reconstituted in proteoliposomes, and covalent cross-linking studies demonstrate that they interact with proteins during membrane translocation. The Tom40p and Toc75 channels are distinguished from the Sec-type translocons in that they form β-barrel channels containing mul-

tiple transmembrane sheets similar to those of many bacterial outer membrane pores.

The TOM translocon of yeast corresponds to a 400 kDa membrane complex containing oligomers of Tom40p and associated subunits (Table 1) (Pfanner and Chacinska, 2002). Biochemical and electron micrographic analysis of the TOM complex indicates that it contains channels of ~20 Å diameter in the open state (Hill et al., 1998; Kunkele et al., 1998). This size is larger than that required to transport a fully unfolded polypeptide chain. In fact, polytopic membrane proteins destined for the mitochondrial inner membrane contain internal targeting signals and appear to translocate as partially folded hairpin loops with two segments of the polypeptide passing through the channel at one time. Therefore, the TOM translocon is flexible enough to accommodate both unfolded substrates and those with limited secondary structure. Translocons isolated from yeast mutants lacking the Tom22p subunit are in the constitutively open state, suggesting that this subunit acts as the gate for the translocon (van Wilpe et al., 1999). Tom20p appears to be the initial preprotein receptor subunit of the complex, but other Tom subunits, including Tom40p, also posses binding sites. These sites are located on both the *cis* and *trans* faces of the TOM translocon, and it has been proposed that they function as a series of presequence binding sites of increasing affinity to mediate preprotein recognition and sequential translocation through the TOM channel (Pfanner and Chacinska, 2002). An additional energy source is not required for TOM translocation.

At least two distinct targeting signals direct proteins to the TOM translocon (Table 1)(Pfanner and Chacinska, 2002). Most matrix, inner membrane and intermembrane space proteins contain cleavable N-terminal presequences that direct proteins, at least partially, across both TOM and TIM translocons. The classical presequence is an oligopeptide of varying length that forms an amphiphilic helix with a positively charged face. A class of polytopic carrier proteins of the mitochondrial inner membrane is targeted via multiple internal signals whose initial receptor is formed by an accessory complex, the Tom70 dimer (Steger et al., 1990). The carriers are subsequently transferred to the core TOM translocon for translocation. These proteins are unusually hydrophobic and likely require a specialized receptor system to prevent misfolding and aggregation in the cytoplasm.

The TOC translocon of the chloroplast outer membrane consists of a trimeric core containing Toc75 in association with two membrane GTPases, Toc34 and Toc159 (Table 1) (Bauer et al., 2001). These units also associate into larger membrane assemblies. Electrophysiological measurements of reconstituted Toc75 indicate a potential channel with a diameter of ~14 Å; a pore large enough to accommodate an unfolded polypeptide chain (Hinnah et al., 2002).

All chloroplast preproteins identified to date contain an N-terminal cleavable targeting signal, the transit peptide. Transit peptides are typically longer than mitochondrial presequences (30 to 70 amino acids), are enriched in hydroxylated amino acids, and lack acidic amino acids (Bauer et al., 2001). The homologous Toc34 and Toc159 GTPases mediate recognition of preproteins and the GTPase activity of one or both are required for preprotein translocation across the outer membrane, leading to the proposal that they gate the TOC channel. Toc159 exists in both cytoplasmic and membrane bound forms and appears to act as a soluble transit peptide receptor that delivers preproteins to the TOC translocon via a GTP-regulated targeting cycle with its cognate receptor, Toc34, in a model analogous to the SRP cycle that operates at the ER membrane (Hiltbrunner et al., 2001; Smith et al., 2003).

***Multiple Translocons Mediate the Translocation of Proteins across the Inner Membranes of Chloroplasts and Mitochondria***. Two independent TIM translocons exist in mitochondria (Table 1) (Jensen and Dunn, 2002). The TIM23 complex mediates the translocation of proteins containing N-terminal presequences. This includes mitochondrial matrix proteins and a subset of integral inner membrane proteins mostly with single transmembrane domains. The TIM22 complex is required for the integration of polytopic membrane proteins into the inner membrane. The distinct topogenic signals of these proteins trigger the association of the TOM translocon with one of the two alternative TIM translocons, thereby defining the pathway of targeting.

The structurally related Tim23p and Tim17p subunits form the core of the TIM23 complex (Jensen and Dunn 2002). Tim23p has been shown to form a channel with a 13 Å diameter when reconstituted in proteoliposomes; a diameter similar to that of native TIM23 channels (Truscott et al., 2001). This corresponds to a relatively narrow channel that would accommodate a single, unfolded polypeptide chain, while excluding the simultaneous movement of small ions. Tim23p also appears to serve as the gate to the TIM23 translocon by sensing the presequence as is emerges from the TOM translocon into the intermembrane space. Upon triggering channel opening, the presequence has been proposed to insert across the channel via an electrophoretic push from the membrane potential (Jensen and Dunn, 2002). Complete translocation of the preprotein is driven by an ATP-regulated cycle of mtHsp70 binding in the matrix. This is facilitated by the Tim44p subunit of the translocon that acts as a *trans* docking site for the mtHsp70 at the translocon (see below).

Polytopic membrane proteins engage a second inner membrane translocon, the TIM22 complex, upon translocation across the outer membrane (Jensen and Dunn, 2002). Tim22p exhibits significant similarity to Tim23p and Tim17p of the TIM23 translocon and forms the core of the TIM22 translocon. Recombinant Tim22p forms a voltage-activated, peptide-sensitive ion channel with a diameter of 11–18 Å (Kovermann et al., 2002). The targeting information for directing proteins to the TIM22 translocon consists of both transmembrane segments and intervening sequences, but a consensus sequence has not been defined. Insertion into the translocon appears to proceed in modules of transmembrane segments pairs (Davis et al., 1998). The proteins are proposed to integrate into the membrane sequentially as each subsequent module engages the translocon. In contrast to the TIM23 translocon, translocation at the TIM22 complex requires only the membrane potential. The matrix-localized loops between transmembrane segments of TIM22 substrates are enriched in basic

residues. These charges are required for proper integration, suggesting that the membrane potential may exert an electrophoretic force on these regions to drive them across the membrane in a mechanism similar to that proposed for the initial stages of translocation at the TIM23 translocon.

In chloroplasts, only a single TIC translocon has been identified to date (Bauer et al., 2001). The exact constituents of the TIC translocon channel are not known, although both Tic20 and Tic110 have been directly implicated in translocation (van den Wijngaard et al., 2000; Chen et al., 2002). Tic20 shares distant homology to the Tim17p/22p/23p family of mitochondrial inner membrane translocation channels. Tic110 contains a large stromal domain that binds to stromal chaperones, leading to the proposal that it serves a function similar to Tim44p and Sec63p as a component of the ATP-dependent translocation motor (Bauer et al., 2001).

Translocon coupling facilitates protein import into mitochondria and chloroplasts. Protein import into mitochondria and chloroplasts occurs simultaneously across both the outer and inner membranes, thereby preventing protein accumulation in the intermembrane space between the two membranes. This is facilitated by direct communication between outer and inner membrane translocons at membrane contact sites. In chloroplasts, this is accomplished by a direct, transient interaction between TOC and TIC translocons. In mitochondria, the situation is more complex because proteins emerging from a single TOM translocon must be sorted to one of two TIM translocons.

For the mitochondrial TIM23 translocon, the coordinated activities of Tim23 and the newly discovered Tim50 subunit are key players. In addition to binding presequences, the N-terminal domain of Tim23 has been shown to reversibly insert into the outer membrane and associate with the TOM translocon in response to a translocation substrate (Donzeau et al., 2000). Tim50 appears to coordinate this coupling by directing preproteins from the TOM translocon to the TIM23 translocon and facilitating Tim23 insertion (Geissler et al., 2002; Yamamoto et al., 2002). Tim50 also may participate in discriminating between matrix and integral inner membrane proteins, thereby contributing to the molecular switch that determines whether the translocon will form a stable channel to transport matrix proteins or gate laterally to facilitate membrane protein integration.

Coupling of translocation across the TOM and TIM22 translocons is facilitated by a set of small proteins of the intermembrane space, Tim8p, Tim9p, Tim10p, Tim12p, and Tim13p (Koehler et al., 1999). These proteins share a common "twin $CX_3C$" motif and appear to act as specific molecular chaperones to insure safe passage of the hydrophobic carrier proteins through the intermembrane space.

### *Molecular Chaperones Perform Multiple Roles during Translocation*

Molecular chaperones play critical roles in protein translocation in both the *cis* and *trans* compartments (Figure 1). On the *cis* side, chaperones assist in maintaining preproteins as unfolded or loosely folded monomers that can be threaded through the membrane channel posttranslationally. These can be specialized chaperones that function solely in protein translocation, such as SecB in the bacterial cytoplasm or the small Tim proteins of the mitochondrial intermembrane space. Alternatively, general chaperones, such as those of the cytoplasmic Hsp70 family, act to maintain translocation competence. Targeting to the mitochondrial TOM complex and posttranslational targeting to the ER both require cytoplasmic Hsp70s for translocation in vivo.

Chaperone binding provides the driving force for translocation at the *trans* side of the membrane in most organelles. The roles of members of the Hsp70/DnaK family of chaperones in mitochondrial and ER translocation have been studied extensively. The functions of hsp70s are supported by their ability to reversibly bind short hydrophobic segments (Bukau and Horwich, 1998). Hsp70 can bind a wide variety of non-native proteins at multiple sites on a given protein. The status of the peptide binding pocket is determined by adenine nucleotide binding. The ADP bound state is the high affinity state. In this state, peptide exchange is slow because the binding pocket has closed on to its substrate. The displacement of ADP by ATP initiates the release of substrate placing the chaperone in the unbound or low-affinity state. Restoration of the high-affinity site can then be reinitiated by the hydrolysis of ATP to ADP. This hydrolysis can be stimulated by J domain-containing proteins that interacts with hsp70s (Misselwitz et al., 1998). Membrane anchored J proteins also function to recruit hsp70s to the *trans* face of a translocon (e.g., Sec63p in the ER and Tim44p for the mitochondrial inner membrane).

Two models have been proposed to explain the mechanism of action of hsp70s during protein translocation (Pilon and Schekman, 1999). The root of the differences in the models is in the magnitude of the force that is instilled upon the polypeptide chain during translocation. The "Brownian ratchet model" proposes that hsp70 binding traps the chain on the *trans* side of the membrane. Polypeptide movement is powered by spontaneous diffusion or Brownian oscillation through the membrane. Binding on the *trans* side hinders backward sliding biasing the movement toward the *trans* space. The alternative "molecular motor model" proposes that hsp70 acts as a motor by generating a pulling force. Here, hsp70 uses the membrane anchored J domain protein as a fulcrum and the conformation change initiated by ATP hydrolysis to pull the polypeptide into the organelle. Vigorous debate for the past decade has centered on the validity of these two models, and there is experimental evidence to support elements of both mechanisms.

In addition to acting as a gatekeeper and determining the directionality of transport, hsp70s also act as chaperones to assist in the maturation of proteins both co- and posttranslocationally. The exposed hydrophobic sequences that hsp70s bind are also hallmarks of a non-native protein in need of protection. Therefore, the interaction of hsp70s on the *trans* side of the membrane with the translocating substrate serves bipartite roles. They help to control the directionality of the translocation process as well as act as the first chaperone in the production line to ensure proper folding and assembly of the customer protein upon emergence into the maturation compartment.

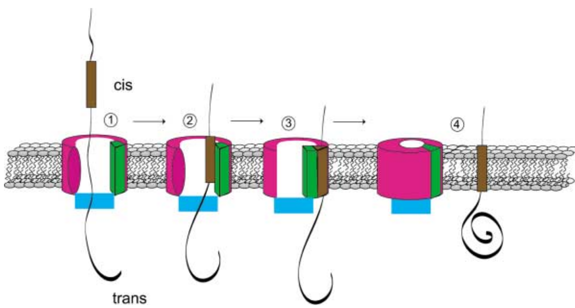


Figure 3. Membrane Protein Integration

(1) Translocation proceeds co- or posttranslationally until a stop transfer signal (brown bar), encompassing a transmembrane segment, enters the channel. (2) Binding of the stop-transfer signal to a site on the translocon (green bar) induces a pause in translocation. (3) The translocon undergoes a conformational shift that allows lateral diffusion of the transmembrane segment into the lipid bilayer. (4) The translocon closes and the membrane protein is free to diffuse through the membrane.

### *Membrane Protein Integration Requires a Dynamic Translocon Channel*

The vectorial transport of polypeptides across a membrane is conceptually well defined if viewed in the context of the precedence of ion channel function. However, the mechanism by which translocons mediate membrane protein integration is considerably more complex and presents numerous experimental hurdles to our understanding of translocon function. Nonetheless, several common events are proposed to occur during integration at all translocons (Figure 3) (Johnson and van Waes, 1999). The translocation of integral membrane proteins pauses upon the entry of a transmembrane domain into the translocon. The transmembrane domain acts as a topogenic signal (stop-transfer signal or signal-anchor sequence) that is recognized by a component of the translocon. This initiates a series of events that result in the lateral opening of the translocation channel, thereby exposing the transmembrane domain to the lipid bilayer. The hydrophobic region partitions into the bilayer core and the translocon channel closes.

Integration by the Sec61 translocon has been best studied, but similar mechanisms are proposed to operate at the SecYEG, TIM22, TIM23 and TIC translocons. During cotranslational translocation, a stop-transfer signal encompassing a transmembrane segment initially enters the translocon. The transmembrane segment may remain in close association with Sec61$\alpha$ and TRAM until the end of protein synthesis, at which time the translocon gates laterally, allowing diffusion of the protein into the lipid bilayer (Do et al., 1996). Alternatively, the stop transfer signal may trigger the immediate transfer of the transmembrane segment to the lipid bilayer upon entering the translocon (Martoglio et al., 1995; Do et al., 1996). Major questions remain as to how multi-spanning membrane proteins are sequentially threaded into the membrane. It now appears that two or more transmembrane domains may accumulate in the translocon prior to release into the lipid bilayer. This is another factor that could account for the relatively large dimensions of translocon channels. The accumulation of multiple membrane-spanning domains within the translocon also may be necessary to ensure proper transmembrane topology. Alternating transmembrane domains of multi-spanning membrane proteins must be inverted 180° as they enter the translocon, requiring at least two strands of the polypeptide to accumulate in the channel. Complex topogenic signals within and adjacent to the transmembrane segments including charge distribution also appear to play a role in determining topology.

BiP also may play an important role during the integration of membrane proteins during cotranslational translocation. The ribosome-translocon seal must be broken to allow the cytoplasmic domains between adjacent transmembrane domains to escape at the *cis* side of the membrane. The membrane permeability barrier is strictly maintained during the translocation process, leading to the proposal that a breach in the ribosome-translocon junction triggers the binding of BiP at the lumenal face of the translocon (Liao et al., 1997). This coordinated gating mechanism would insure that the translocon maintains a tight seal during and after protein translocation.

The Oxa1p, YidC, and Albino3 proteins of the mitochondrial inner membrane, bacterial periplasmic membrane, and the chloroplast thylakoid, respectively, are homologous integral membrane proteins that are required for the integration of a subclass of membrane proteins (Table 1) (Luirink et al., 2001). Data suggest that YidC and Albino3 participate directly in SRP-dependent pathways. YidC also has been shown to interact with the Sec machinery, suggesting that it may act as an adaptor for the cotranslational pathway. However, targeting involving Albino3 does not appear to require chloroplast Sec components. Furthermore, mitochondria lack both SRP and Sec machinery. These data suggest that the Oxa1p, YidC, and Albino3 proteins also may represent independent translocons. Confirmation of this assignment must await further investigation of their activities and the identification of additional components that may participate in translocon formation.

### The Signal-Assembled Translocons

Recent studies of the TAT/$\Delta$pH translocation systems of bacteria and thylakoids, and the protein import system of peroxisomes have established a second paradigm for protein translocation systems. Although the TAT/$\Delta$pH and peroxisomal systems are not structurally or evolutionarily related, we group them together based on a common mechanistic theme, the ability to transport large, fully folded, and/or oligomeric proteins (Figure 2). In the case of the TAT/$\Delta$pH translocons, their substrates appear to be limited to a subset of metalloproteins that must fold and acquire complex cofactors in the cytoplasm or stroma prior to transport across the periplasmic or thylakoid membrane (Berks et al., 2000). The peroxisomal translocon can transport substrates as large as 9 nm colloidal gold particles coated with a peroxisomal targeting signal (PTS) (Walton et al., 1995). In some cases, the oligomeric substrates of these translocons employ a piggy-back mechanism in which only one or a subset of subunits contain targeting signals.

How do these translocons function while maintaining the membrane permeability barrier of their respective

organelles? The most popular current hypothesis proposes that the translocons assemble at the site of translocation in response to the docking of a protein substrate at the membrane (Figure 2). In this model, the dimensions of the protein-conducting channels would be determined in response to the size of the transport substrate. Upon translocation, the translocon would immediately disassemble to minimize the free diffusion of molecules across the channel, thereby maintaining the critical permeability barrier.

At least 23 PEX genes have been identified in fungi that are required for peroxisome biogenesis (Table 1). The protein products of these genes, termed peroxins, include a number of soluble proteins and peroxisomal membrane proteins (PMPs). Although genetic analyses have implicated these proteins in protein import, the precise functions of most peroxins remain largely unknown. The most detailed information on the translocation pathways has come from the analysis of the targeting receptors. Two types of peroxisomal targeting signals (PTSs) operate in protein import (Subramani et al., 2000). The predominant PTS1 pathway is defined by a C-terminal tripeptide targeting signal (-SKL or conserved variants) that is recognized by a soluble receptor, Pex5p, via a cluster of six tetratricopeptide repeats at its C terminus. The targeting signal for the minor PTS2 pathway consists of a degenerate nine-residue sequence located internally or near the N terminus. PTS2 is recognized by the Pex7p receptor. Pex5p and Pex7p bind their respective PTSs in the cytoplasm, and the receptor-cargo complexes dock independently to the peroxisome surface at a single membrane-associated complex containing Pex13p, Pex14p, and Pex17p. The two pathways converge at the point of membrane translocation, implicating a single translocon in the translocation of all matrix proteins.

The peroxisomal import field was confounded for some time by the observation that Pex5p is localized to the cytoplasm, peroxisomal membrane, and peroxisomal matrix. A potential explanation for the multiple localizations was presented by data suggesting that Pex5p shuttles between the cytoplasm and peroxisomal matrix (Dammai and Subramani 2001). This has led to the extended shuttle model for peroxisomal protein import. The model predicts that after docking at the membrane surface, Pex5p remains bound to its substrate and is translocated into the matrix along with its cargo. Upon arrival in the matrix, Pex5p is triggered to release its cargo and the receptor is then transported back into the cytosol where it is available to undergo another import-export cycle.

The nature of the translocon remains elusive. However, Pex10p, Pex12p, and Pex2p are peroxisomal membrane proteins that function downstream from the receptor complexes (Holroyd and Erdmann, 2001). They all contain cytoplasmic zinc RING domains, and Pex10p and Pex12p bind Pex5p. These observations implicate them in membrane translocation reactions. ATP hydrolysis is required for the import of peroxisomal matrix proteins, providing the potential for a nucleotide-hydrolysis driven import cycle. Two peroxins, Pex1p and Pex6p, are ATPases, but their direct or indirect association with Pex5p has not been established, and their precise roles in import are unknown. The nucleotide cycle may be required for the assembly of the translocon and/or the dissociation and recycling of the PTS receptors.

The TAT/$\Delta$pH pathways (Table 1) were first distinguished from the Sec translocons because translocation requires only the transmembrane potential (Berks et al., 2000). The N-terminal targeting signals for these pathways are similar to those of the Sec pathways with the exception of a twin arginine motif following the hydrophobic core of the signal peptide. The *E.coli* TAT system consists of five known components, TatA–E. TatA, TatB, TatC, and TatE are membrane proteins. The thylakoid proteins Hcf106, Tha4, and cpTatC are orthologs of TatB, TatA/E, and TatC, respectively. TatB/Hcf106 and TatC form a stable membrane complex that binds the TAT signal. Binding appears to trigger association of the TatA/Tha4 with the receptor complex. TatA/Tha4 exists as an oligomer, suggesting that the association of the two subcomplexes may produce a functional translocon. The dissociation of the TAT translocon recently has been observed in response to translocation in thylakoid membranes, consistent with the reversible assembly of the translocon (Mori and Cline, 2002). Although preprotein binding to the TatB/Hcf106-TatC complex does not require energy, the membrane potential is required for the association of TatB/Hcf106-TatC with TatA/Tha4. These observations have led to the proposal that structural changes induced by preprotein binding at the receptor complex harness the membrane potential to assemble the functional translocon. The membrane potential likely also participates in driving translocation.

***Translocation Is Coupled to Protein Maturation***

In recent years, studies on protein folding and modification events have revealed the tight interconnection between membrane translocation and protein maturation. This relationship is most pronounced for proteins entering the ER because translocation is coupled to the relatively slow rate of translation. An average eukaryotic protein of 500 amino acids takes $\sim$2 min to be translated. This extended duration permits a variety of maturation processes to occur, while the ribosome-attached nascent chain is associated with the translocon. These cotranslational maturation events include signal peptide cleavage, the transfer and trimming of N-linked glycans, disulfide bond formation, transmembrane domain integration, chaperone binding, and protein folding (Daniels et al., 2003).

Translocon-associated processing and modification events involve a variety of large protein complexes that reside in the ER membrane. Cleavage of N-terminal signal sequences is mediated by the signal peptidase complex, containing five proteins, of which two contain the protease active site. The *en bloc* addition of N-linked carbohydrates ($Gluc_3$-$Man_9$-$GlcNAc_2$) in yeast involves nine transmembrane proteins that form the oligosaccharyl transferase complex (30 transmembrane domains total). These bulky, hydrophilic and flexible modifications act as tags within the lumens of the secretory pathway to recruit chaperones and maturation factors (Helenius and Aebi, 2001). After transfer, glycans are immediately trimmed by glucosidases I and II to generate monoglucosylated side chains that are cotranslocational substrates for the lectin chaperones calnexin (type I membrane protein) and calreticulin (a soluble paralog).

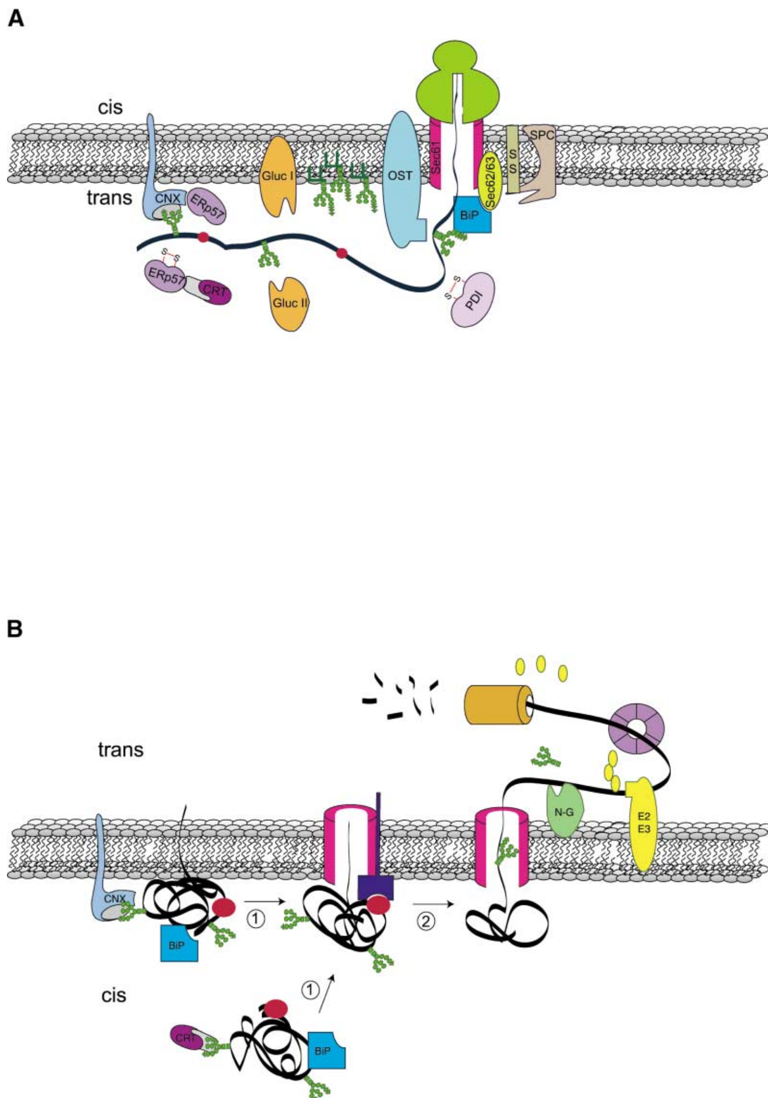


Figure 4. ER Translocons and Their Associated Proteins

(A) The cotranslational translocon. A nascent chain can interact with a large number of protein modifiers and chaperones while attached to the ribosome. These proteins include the signal peptidase complex (SPC) that cleaves the signal sequence (SS), BiP, protein disulfide isomerase (PDI), the oligosaccharyl transferase (OST), glucosidases I and II (Gluc I and II), calnexin (CNX), calreticulin (CRT), and ERp57.

(B) ERAD model. (1) Malfolded proteins are recognized by a quality control receptor (red ball) and targeted to its cognate membrane receptor (dark blue membrane protein) for retranslocation. (2) Retranslocation of the ERAD substrate proceeds through the translocon in a relatively unfolded state where it is deglycosylated by an N-glycanase activity (N-G) on the *trans* side of the membrane in the cytosol. In addition, ubiquitin (yellow ovals) is added to the polypeptide chain by ubiquitinating enzymes E2 and/or E3. The force of retranslocation is generated by an AAA-ATPase (purple wheel) with the eventual degradation of the ERAD substrate by the 26S proteasome (orange can).

This binding helps to increase the fidelity of the folding process by minimizing protein aggregation and slowing the folding reaction (Hebert et al., 1996).

While the tight confines of the ribosome polypeptide tunnel and the translocon channel provide little opportunity for folding to take place, the folding process can immediately commence cotranslationally and cotranslocationally upon emergence into the ER lumen (Kowarik et al., 2002). A vectorial folding process where folding occurs from the N- to C terminus permits the separation of domain acquisition, allowing each domain to potentially fold independently for a protein that contains sequential folding domains (Hardesty et al., 1999). This greatly limits the total number of conformations that can be sampled, helping to increase the overall efficiency in the acquisition of native structure in the cell. It also provides a mechanism to control the environment of the nascent chain when it is most susceptible to being diverted to a non-productive folding pathway. By organizing a controlled environment or assembly line for nascent chains at the *trans* face of the translocon, the vulnerable polypeptides can have optimal opportunity to fold properly. It can also ensure a concerted order of events; for instance, modification sites can be recognized prior to being hidden within the core of a folded domain. This environment appears to involve some 30 components that can interact with the ribosomal-attached nascent chain (Figure 4A). While these proteins assist in the modification and folding of the protein, they also provide a barrier to separate nascent chains in the crowded confines of the cell minimizing aggregation (Chen and Helenius, 2000). Together, the translocon and its associated proteins create a privileged environment that can permit maturation levels to approach 100% for some proteins traversing the eukaryotic secretory pathway, a feat unattainable in a test tube for even the simplest proteins. Elucidating the organization of these translocon-associated proteins and their mechanisms of assistance in protein maturation will be an area of vigorous investigation in the coming years.

### *Retrograde Translocation Is Used for Degradation of ER Proteins*

While the ER is optimized for efficient protein maturation, not all proteins mature correctly. An estimated one

third of all proteins synthesized are degraded immediately after translation (Schubert et al., 2000). To monitor the integrity of the maturation process and prevent terminally misfolded or unassembled non-functional proteins from being deployed throughout the cell, the cell possesses a quality control system that sorts aberrant proteins for destruction (Ellgaard et al., 1999). However, the ER appears to be devoid of non-specific proteases, presumably because the placement of promiscuous proteases within the maturing compartment of the ER could present an hostile environment for an emerging nascent chain. The cell is able to reconcile these demands by segregating, at least in part, the degradation process to the cytosol (Figure 4B). The 26S cytosolic ubiquitin-dependent proteasome is involved in the degradation of malfolded proteins that traverse the secretory pathway through a process termed ERAD (ER-associated protein degradation) (McCracken and Brodsky, 1996). Therefore, a retrograde protein translocation process is required for presentation of the misfolded substrates to the cytosolic protease. This process is referred to as retranslocation, retro-translocation, or dislocation. It requires a signal/receptor, targeting to membrane channel, a translocon, and extraction method as previously outlined for a protein translocation process, in addition to the degradation by a protease within the *trans* compartment.

A small number of proteins appear to be dedicated to monitoring the fidelity of the maturation process for a large number of substrates that pass through the secretory pathway. Therefore, the quality control test must generally involve a fundamental structural signal rather than a complex test of functionality (Ellgaard et al., 1999). Malfolded or unassembled proteins often possess exposed hydrophobic sequences that appear to act as signals for degradation. Therefore, the protein machinery involved in facilitating the correct maturation of a protein also possesses the binding properties required to recognize the signals of aberrant proteins and potentially act as receptors to sort malfolded proteins for degradation. Genetic studies with yeast have demonstrated that BiP is required for ERAD of a variety of soluble proteins (Plemper et al., 1997; Fewell et al., 2001). The precise role for BiP in the ERAD process is not known; however, potential functions include: (1) maintaining a substrate in a retranslocation-competent state by holding it in a loosely folded conformation or inhibiting aggregation in a similar manner to Hsp70s assistance in the delivery of substrates to translocons for anterograde translocation, and (2) acting as a receptor that is involved in the trafficking of proteins to the translocon.

The quality control system of the ER employs carbohydrates as tags to mediate chaperone interactions involved in ER-retention and ERAD of glycoproteins (Helenius and Aebi, 2001). The soluble protein UDP-glucose: glycoprotein glucosyltransferase serves as a folding sensor in the ER by reglucosylating aberrantly folded proteins with exposed hydrophobic regions. The regenerated monoglucosylated protein is then retained in the ER in the calnexin and calreticulin binding cycle, suggesting a role of the lectin chaperones in ERAD. The trimming of mannose residues is also involved in sorting substrates for ERAD. When trimming by ER mannosidase I is inhibited ERAD substrates can be stabilized (Jakob et al., 1998), implicating a mannose-trimmed structure as a potential ERAD sorting tag. The putative ER lectin Html1p/EDEM of yeast or mammals, respectively, has been proposed to be the lectin that recognizes mannose trimmed glycans; however, further experimentation will be required to solidify the role of Html1p/EDEM in ERAD (Hosokawa et al., 2001; Jakob et al., 2001).

The Sec61p channel that assists in the co- and post-translational translocation of proteins into the ER also appears to support the post-translational retranslocation of ERAD substrates to the cytosol. This role was initially suggested by the co-immunoprecipitation of the viral induced ERAD substrate MHC class I heavy chain with antibodies raised against Sec61β under conditions where anterograde translocation was minimized (Wiertz et al. 1996). The identification of mutations of Sec61p with genetic screens that are defective in retranslocation but proficient for translocation into the ER supports these findings (Plemper et al. 1997; Gillece et al. 2000). A role in retranslocation for the Sec61p yeast homolog Ssh1p has also been identified suggesting that multiple ER translocons may be involved in the retranslocation of malfolded substrates (Wilkinson et al. 2001). While these results implicate Sec61p and Ssh1p in the retranslocation of ERAD substrates, a direct demonstration using a reconstituted ERAD system is missing.

The need to target an ERAD substrate to the translocon can be averted by making the quality control decision cotranslationally while the substrate is still associated with Sec61p. In this case, the direction of the translocation process has to be reversed. This phenomenon has been observed for the degradation of truncated ApoB translation products (Fisher and Ginsberg, 2002). The basis for the interplay between the ribosome and the quality control machinery is an area that will require further exploration to elucidate how a quality control cue, evidently based in the ER lumen, is communicated to the ribosome.

The retranslocation of ERAD substrates requires a pushing or pulling driving force to ensure unidirectional translocation into the cytosol. Emerging evidence implicates cytosolic machinery in dictating the directionality of transport. First, polyubiquitination at the cytosolic face of the ER membrane assists in the retranslocation process (Shamu et al., 2001). Genetic screens have identified several of the enzymes involved in the this process in yeast and the discovery of mammalian orthologs has begun to follow (Fewell et al., 2001). The attachment of multiple ubiquitin moieties could provide a molecular ratchet that ensures movement toward the cytosol. In addition, ubiquitin may serve as a tag to recruit protein machines involved in the pulling of ERAD substrates to the cytosol. The cytosolic AAA-ATPase p97 and its partner proteins (Ufd1-Npl4) are localized to the ER membrane and have been demonstrated to release ERAD substrates from ER membranes (Ye et al., 2001). The p97-Ufd1-Npl4 complex binds both ubiquitin and the proteasome providing a potential link between retranslocation and the degradation machinery. AAA-ATPase family members are also responsible for the extraction and degradation of membrane proteins in bacteria, mitochondria and chloroplasts; however, here

the AAA-proteases appear to work in a translocon-independent manner (Langer, 2000). Alternatively, a large proteasomal fraction colocalizes with ER membranes. The 19S cap of the proteasome contains multiple ATPases in addition to ubiqutin binding subunits. These ATPases are capable of unfolding proteins to thread the substrate into the proteasomal tunnel. A similar force could also help to unfold a malfolded protein across the ER membrane and drive the retranslocation process. The wide spectrum of ERAD substrates likely employ multiple methods for ER retranslocation.

The use of the Sec61p channel in both the anterograde and retrograde direction could potentially create a logjam if the need for ERAD is increased by chemical treatment or mutations that produces an increase in ERAD load. This potential problem can be alleviated since the accumulation of non-native protein in the ER creates a stress that initiates the unfolded protein response (UPR) (Travers et al. 2000). This response produces an SOS signal to the cell to assist in the clearing of the secretory pathway by shutting down the synthesis of general secretory pathway substrates (anterograde Sec61p substrates) and turning on the expression of protein machinery that helps with the proper maturation or disposal of accumulated proteins. These genes are transcriptionally induced and correspond to many of the proteins discussed above that are involved in protein translocation, including proteins involved in glycosylation and chaperone activities, as well as ERAD. Their induction helps to create an optimal folding environment within the ER. That Sec61p itself is induced by the UPR underscores its central role in protein maturation and degradation within the ER. Similar to the anterograde translocation of proteins into the ER, an assembly line of proteins also appear to await the arrival of the ERAD substrate at the cytosolic face of the translocon (Figure 4B). These activities include ubiquitination, deglycosylation by an N-glycanase, extraction/unfolding machinery, and a protease.

***Alternative Uses of the ERAD Pathway***

The ERAD pathway also plays a role in the regulation of native proteins involved in cholesterol synthesis and lipoprotein metabolism (Hampton, 2002). This pathway is most thoroughly understood for the yeast Hydroxymethylglutaryl-coenzyme A reductase proteins referred to as Hmg1p and Hmg2p. Here, the mevalonate pathway signals the regulated destruction of native Hmg proteins by the ERAD pathway. The level of ApoB is also controlled by the ERAD process with the presence of cholesterol permitting the completion of the translation and maturation process, whereas in its absence, translation and translocation of ApoB is aborted and ERAD of the partially synthesized product is initiated (Fisher and Ginsberg, 2002).

Plant and bacterial toxins and viruses appear to have evolved mechanisms to co-opt the ERAD process. Toxins such as cholera, shigera, pertussis, and ricin enter the cell by endocytosis eventually reaching the ER lumen. In the case of cholera toxin, a toxic peptide fragment is liberated by protein disulfide isomerase (PDI) and then retranslocated to the cytosol where its toxic effects are exhibited (Tsai et al., 2001). Viral proteins can also exploit the ERAD pathway to subvert the immune response. The human cytomegalovirus (HCMV) encodes two proteins US2 and US11 that bind and target normal MHC class I molecules for destruction, allowing the virus to evade class I detection (Wiertz et al., 1996).

## Future Directions

Significant progress has been made in the past two decades on understanding the mechanism of protein translocation across membranes. What are the major questions yet to be addressed? The events that initiate the translocation reaction remain a mystery in all systems. Although the details of targeting signal recognition by signal receptors are at hand, the mechanisms by which signal-receptor complexes trigger translocon gating and/or assembly, resulting in transfer of the polypeptide into the translocation channel, remain elusive. Furthermore, our current models do not explain how the translocon and its associated components maintain the membrane permeability during the dynamics of the translocation reaction. The remarkable discovery that translocons can work in reverse to mediate protein efflux raises a number of confounding questions. While recent studies have shed light on the process of retranslocation in ERAD, the identification of the targeting signals, their receptors, and the details of membrane translocation have yet to be defined.

In the case of the signal-gated translocons, recent advances in the reconstitution of translocons into chemically pure systems and the definition of minimal requirements for translocation activity have aided the development of models of translocon function. In addition, low-resolution structures of isolated translocons are providing glimpses into the structural organization of these complex molecular machines. A major goal for future studies will be to combine the biochemical analyses with high-resolution structural approaches to capture snapshots of translocation intermediates. This will lead to the visualization of molecular events at each stage in the translocation reaction. The ability to capture translocation intermediates will be of paramount importance in defining the nature of the signal-assembled translocons because the apparent lack of stable translocons in the absence of membrane transport precludes their purification and analysis.

Another exciting prospect for future investigation is the regulatory mechanisms associated with translocon function. It has been argued that the ER translocon directly regulates protein topology and targeting, thereby determining the fate and function of proteins in response to cellular signals (Hegde and Lingappa, 1999). Furthermore, genome sequencing has revealed the existence of families of related translocon components. This raises the possibility that assembling these homologs in different combinations can generate translocons with functions tailored to the gene expression profile of a particular developmental state or cell type (Bauer et al., 2001). Finally, it is clear that the coordination of translocation and retranslocation with protein maturation and degradation requires exquisite regulation of a multitude of simultaneous molecular interactions. Revealing these mechanisms will lead to the integration of protein translocation into the broader set of events in the overall biogenesis of membrane bound compartments.

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