

## THE TRANSLOCATION APPARATUS OF THE ENDOPLASMIC RETICULUM

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Eukaryotic proteins destined for the cell surface, extracellular space, or compartments of the secretory pathway are first translocated across or inserted into the endoplasmic reticulum (ER) membrane at sites termed translocons [1-4]. The essential feature of ER translocons is a protein-conducting channel formed by the highly conserved Sec61 complex. Together with several accessory components, the Sec61 complex recognizes translocation substrates, provides a gated conduit for transport across the membrane, and regulates access to the lipid bilayer for membrane protein integration. These combined activities endow translocons with the remarkable capacity to direct the proper biogenesis and topology for a tremendously diverse set of secretory and membrane protein substrates. How is this complex feat accomplished? In this chapter, we subdivide the protein translocation process into a series of decisive mechanistic steps taken by a substrate during its transit across or insertion into the membrane. The translocon components implicated in each step and their proposed mechanisms of action are considered with an eye towards particularly important gaps in our understanding of protein translocation into the ER.

### Translocons receive substrates via two distinct pathways

The first step in translocation is the targeting of a substrate to the translocon. Depending on the substrate and the organism, targeting is achieved in two qualitatively different ways (Fig. 1). In the mammalian system, almost all secretory and membrane proteins are recognized and targeted to the membrane co-translationally (i.e., while they are being synthesized by cytosolic ribosomes). By contrast, a substantial proportion of proteins in yeast can be targeted post-translationally, after their complete synthesis and release into the cytosol.

In the co-translational targeting pathway (see Chapter X), substrates are recognized when the first hydrophobic domain, either a signal sequence or a transmembrane domain (TMD), emerges from the translating ribosome. The signal recognition particle (SRP) binds to the exposed hydrophobic domain, slows translation via further contacts with the ribosome, and targets the entire ribosome-nascent chain complex (RNC) to the ER-localized SRP receptor [5]. The RNC is subsequently transferred to the translocon (in a very poorly understood step), ensuring the delivery of nascent chains for translocation at an early stage in substrate synthesis.

In yeast, SRP fails to efficiently recognize signal sequences whose hydrophobicity falls below a certain threshold [6, 7]. These polypeptides are therefore not targeted to the ER co-translationally, but instead are bound by cytosolic chaperones (see Chapter X) including Hsp70, its cofactor Ydj1p and the Tric/CCT chaperonin complex [8-10]. These factors maintain signal-containing proteins in an unfolded flexible conformation prior to their transfer to the ER translocon. The mechanisms that coordinate chaperone release with delivery of substrates to the translocon remain poorly understood, but may be dependent on a functional signal sequence. Thus, in both pathways, a signal- or TMD-containing polypeptide is presented to an unengaged ER translocon in a configuration (either unfolded or while still being synthesized) that facilitates subsequent translocation through a channel of limited size.

### Substrate recognition by the ER translocon is a decisive step in protein translocation

Upon delivery to the translocon, substrates are recognized and discriminated a second time using the same element (either a signal sequence or TMD) initially used for targeting. This recognition step serves at least three purposes. First, it allows secretory and membrane proteins to be discriminated from non-secretory proteins that may have inadvertently been targeted to a translocon and therefore serves as a 'proofreading' step to improve fidelity of sorting [11, 12].

Second, the engagement of a translocon by a signal or TMD presumably prepares the translocon for protein translocation by gating the channel from a closed to an open configuration [13]. And finally, the orientation in which the signal or TMD is recognized determines the topology achieved by the substrate [14, 15]. Thus, the translocon must not only recognize and interact productively with a signal or TMD, but must also orient this domain with respect to the lipid bilayer.

The orientation taken by the signal or TMD is a critical step in the final outcome of the polypeptide substrate (Fig. 2; see also Fig. 3A and B). An N-terminal cleavable signal sequence is generally thought to be positioned with the N-terminus facing the cytosol and the C-terminal region oriented toward the ER lumen (Fig. 2A). This orientation exposes the signal cleavage site to the luminal side of the translocon (where the active site of signal peptidase is located [16]) and positions the nascent polypeptide in a 'looped' configuration. When positioned this way, the N-terminus of the mature polypeptide can access the ER lumen and translocate across the membrane through the aqueous protein-conducting channel in the translocon (Fig. 3A); failure to achieve this looped configuration results in a lack of translocation. Thus, a cleavable N-terminal signal sequence is employed by a wide range of secretory and membrane proteins whose N-terminus needs to be translocated to the non-cytosolic side of the membrane (Fig. 2A).

A TMD can be positioned in one of two orientations after its recognition by the translocon (Fig. 2B, C). If the N-terminal side of the TMD faces the cytosol, it is often referred to as a signal anchor (or sometimes a type II signal anchor). This orientation is analogous to a cleaved signal sequence, and the nascent chain similarly acquires a 'looped' configuration at this early step in translocation (Fig. 2B). If the TMD is oriented with the N-terminal side facing the lumen, it is called a reverse (or sometimes a type I) signal anchor (Fig. 2C). In this case, the domain N-terminal to the TMD must be translocated to the luminal side of the membrane to permit such an orientation, while the domain C-terminal to the TMD must remain in the cytosol. Regardless of the orientation, the TMD is eventually integrated into the membrane (i.e., moved from the proteinaceous environment of the translocon to the lipid bilayer). Both types of signal anchors are employed widely in the biogenesis both single- and multi-spanning membrane proteins, examples of which are indicated in Fig. 2B and 2C.

From this discussion, it should be apparent that the initial recognition by a translocon of the targeting element (either a signal sequence or TMD) directly determines the portion of a nascent polypeptide that is translocated across the membrane versus retained on the cytosolic side (i.e., the *topology* of the final polypeptide). In the post-translational translocation pathway, this signal-translocon interaction is also likely to be the sole discriminatory event in segregating secretory from non-secretory proteins [17, 18]. Even in co-translational translocation, signal recognition by the translocon may be the decisive discriminatory event since SRP-mediated targeting may not be obligatory for translocation of substrates whose mRNAs are already docked on ER-bound polysomes [11]. Consistent with this notion, yeast mutants depleted of SRP are initially defective in translocation and grow slowly, but substantially recover their translocation capacity and growth rate over time [19, 20]. A plausible explanation for this observation is that co-translational translocation substrates are initially very slow to engage the translocon in the absence of SRP, but become much more efficient after the first (presumably random, and hence rare) targeting event delivers the mRNA to the ER membrane. Indeed, in vitro experiments have shown that a polypeptide can be successfully translocated in a signal-discriminatory manner in the complete absence of SRP, provided that the ribosome-nascent chain complex (RNC) is first docked at the translocon [11, 21-24]. Thus, recognition of a hydrophobic targeting sequence by

the ER translocon is both an obligatory and decisive step in the initiation of all modes of protein translocation.

#### **The remarkable diversity of sequences recognized by the translocon**

Even though essentially every substrate translocated across (or inserted into) the ER engages the translocon, the domains that are recognized (signal sequences and TMDs) share no sequence motifs or homology whatsoever [25-27]. Not only are signal sequences distinctly different from TMDs, but each motif is itself highly variable. N-terminal signal sequences are usually ~15-45 amino acids in length and are often considered to have a three-domain structure: a non-hydrophobic and often basic n-region, followed by a central hydrophobic core of ~8-12 residues (h region), and ending in a c-region that often contains helix-breaking and small-uncharged residues.

TMDs are generally longer than signal sequences and have a hydrophobic membrane-spanning domain of at least ~16 residues. This hydrophobic domain is often flanked by charged residues, the asymmetric distribution of which often correlates with its final orientation in the membrane (with basic residues favoring the cytosolic side; the so-called 'positive-in' rule [28, 29]). Because the TMDs used for targeting need not be at the beginning of the protein, the domain N-terminal to the TMD can be of highly variable size (Fig. 2B, C). Thus, the only feature that is considered common to all signal sequences and TMDs is a ~8 residue hydrophobic region uninterrupted by charges. For this reason, the hydrophobic region of the signal and TMD is generally considered the principal feature that is recognized by the translocon and engages it for subsequent events in substrate translocation.

What then is the functional role of sequence diversity in signals and TMDs? In the case of TMDs, at least two functions are clear. First, statistical and mutagenesis studies have demonstrated that the length, hydrophobicity, and flanking charge distribution of the TMD all influence its orientation in the translocon and hence final protein topology [14, 15, 30]. Second, any structural and functional roles of the TMD in the final membrane protein inevitably constrain the sequences that are allowable. In the case of signal sequences, role(s) for sequence variabilities among substrates remain poorly understood. Only relatively recently have functional differences among signal sequences begun to emerge from a collection of seemingly disparate studies from multiple experimental systems [31, 32].

The reason for this brief discussion on the sequence features of TMDs and signals is that it highlights several important questions regarding translocon function that are considered in subsequent sections. First, how are the rather vague *shared* features of signals and TMDs (primarily hydrophobicity) recognized by the translocon despite their enormous sequence diversity? Second, how are the *variable* regions of signals and TMDs interpreted by the translocon to impart substrate-specific features such as orientation of a TMD? And how might this sequence diversity be physiologically exploited by the cell for differential regulation of translocation in a substrate-specific manner? In considering these and other questions, it is therefore important to keep in mind that even though most of our current knowledge comes from the study of a very few model examples, translocons are in fact designed to handle remarkable substrate diversity.

#### **The machinery of signal sequence recognition**

What does a signal 'see' when it interacts with the translocon? The answer to this question turns out to depend on the organism, substrate, and mode of translocation. This is because the

translocation apparatus is not exactly the same in all organisms, and multiple translocons of differing compositions are likely to exist even within a single cell [1-4]. Nonetheless, it is generally thought that due to their similar hydrophobic character, signal sequences and TMDs of either orientation are all recognized in approximately the same way and at the same general site in a translocon. Furthermore, the high degree of evolutionary conservation in the essential targeting and translocon machinery (i.e., the SRP system and the Sec61 complex) suggests that the fundamental steps in translocation (such as signal sequence recognition) will vary little across experimental systems [33, 34]. With this in mind, let us now consider what is known about the machinery of signal and TMD recognition.

Two types of substrates have been analyzed over the years. In the mammalian system, RNCs of defined length and substrate composition are assembled with pancreatic ER-derived microsomes. In the yeast system, a post-translational substrate is stalled in its translocation across yeast-derived microsomes. Insight into signal sequence and TMD recognition by the translocon in each system comes primarily from fluorescence, crosslinking, and reconstitution studies. Although no single approach or system provides a complete view, a composite can be deduced by combining the resulting data with a reasonable assumption of high evolutionary conservation of basic mechanisms.

In the fluorescence approach, nascent chains containing a fluorescent amino acid incorporated in the signal sequence were used to probe the local environment at different stages in translocation [35]. These pioneering studies demonstrated that during co-translational translocation, the signal sequence of a model secretory protein is in an aqueous environment continuously during its transit through the ribosomal tunnel and engagement of the ER translocation [13, 36]. Upon translocon engagement, the probe in the signal, although still in an aqueous environment, was no longer accessible to ions from the cytosolic side of the membrane [13, 36]. It was presumed that the signal must therefore occupy a non-hydrophobic space at the membrane shielded from the cytosol, consistent with it interacting directly with the translocon.

Coincident with these studies, crosslinking approaches had identified several integral membrane proteins in close proximity to signal-containing nascent chains [37, 38, 39, Thrift, 1991 #189, 40, 41]. The eventual purification and identification of these proteins in the mammalian co-translational system led to the discovery of TRAM (for translocating chain-associated membrane protein [42]), the heterotetrameric translocon-associated protein (TRAP) complex (composed of  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$  subunits [43]), and the heterotrimeric Sec61 complex (composed of  $\alpha$ ,  $\beta$  and  $\gamma$  subunits [44]). In addition to being near (i.e. within crosslinking distance) to a signal sequence, each of these translocon components were also found to be functionally involved in signal sequence-mediated translocation [11, 42, 44-46].

To delineate the signal sequence interaction with the translocon with higher resolution, a site-specific crosslinking approach was combined with biochemical and genetic manipulation of the translocon components. These studies revealed several important observations. In the mammalian co-translational system, nearly every residue in the hydrophobic core of a signal sequence was found to be adjacent to Sec61 $\alpha$  and phospholipids [24, 47-49]. By contrast, TRAM was found to make its predominant contacts to the regions flanking the h-domain [24, 48]. For the signal sequence of prolactin, the n-domain was close to TRAM, while the  $\alpha$ -factor signal sequence (when presented as an RNC) showed the strongest crosslinks to TRAM to regions downstream of the h-domain. Although less precisely mapped, TRAP $\alpha$  seems to crosslink with only longer nascent chains, potentially through residues in the mature domain [42].

In analogous crosslinking experiments using the yeast post-translational system, translocating nascent chains were found in the vicinity of Sec62p and Kar2p (the yeast homolog of BiP) in addition to Sec61p [50, 51]. These early studies together suggested that signal sequence recognition involved a complex protein-based interaction in both the mammalian and yeast systems, but additional resolution was difficult to discern. To address this problem, the genetic manipulability of yeast was combined with site-specific crosslinking to identify the specific regions of Sec61p close to the signal sequence of a stalled translocation intermediate [18]. This systematic study found that the signal sequence of  $\alpha$ -factor occupies a binding site surrounded predominantly by transmembrane helices 2 and 7 of Sec61p (a multi-spanning membrane protein containing 10 TMDs), with additional contacts to Sec62p, Sec71p, and lipid.

Because an approximately similar pattern of crosslinks was observed with mammalian Sec61 $\alpha$  for RNCs of  $\alpha$ -factor [18], it is presumed that the principal signal binding site formed by helices 2 and 7 is conserved across all species and modes of translocation. This is logical since homologs of the Sec61 complex form the central translocation channel in all ER and bacterial translocons, while the other accessory components (such as TRAM, the TRAP complex, Sec62, and others) are neither universally conserved nor present in every translocon. Using the co-translational system, it has further been shown that the Sec61 complex is both necessary and sufficient for recognition of at least some model signal sequences [11]. Although definitive evidence is still lacking, crosslinking analyses suggest that TMDs interact (via their hydrophobic domain) with the same or similar site as signal sequences [52, 53]. Indeed, as for signal sequences, the Sec61 complex alone can suffice for recognition and proper orientation of at least some model TMDs of either topology [44]. Thus, signal recognition involves an essential and major interaction with a specific site within the Sec61 channel. Additional (presumably weaker, but stabilizing) interactions are specific to the translocation system: TRAM and potentially the TRAP complex are involved in the mammalian co-translational system, while Sec62p and Sec71p participate in the yeast post-translational system.

#### **A combined framework for signal and TMD recognition**

Considered together with the available information on protein structure, topology, and functional data, we propose the following working model for co- and post-translational signal recognition (Fig. 3C). In both modes, the hydrophobic core of the signal sequence would interact with roughly the same area of Sec61 $\alpha$  between helices 2 and 7 [18]. Indeed, even in the bacterial translocation system, these same two helices of SecY (the homolog of Sec61 $\alpha$ ) were also observed to interact with a synthetic signal peptide in detergent solution [54]. This binding site is presumably flexible (i.e., helices 2 and 7 can be moved to varying degrees) to accommodate the tremendous sequence diversity of signals (and TMDs). Furthermore, the space near helices 2 and 7 should be capable of providing a route for the signal sequence (or TMD) to access the hydrophobic core of the lipid bilayer [18, 24, 49, 52, 53, 55, 56]. Each of these conclusions derived from the mammalian, yeast, and bacterial systems are consistent with the crystal structure of an archaeal SecY complex [57]. This structure revealed that helices 2 and 7 are indeed adjacent to each other and provide a lateral exit site from the proposed pore within SecY to the lipid bilayer. Thus, it seems reasonable to conclude that in all systems, the helix 2/7 interface represents a generic hydrophobic domain recognition site that is accessed by the core region of all signals and TMDs regardless of the mode of translocation.

In the mammalian co-translational system (Fig. 3C, left diagram), this interaction would be stabilized by additional contacts between the n-domain and TRAM on the cytosolic face of the

translocon [58]. Such contacts, although made with most or all signal sequences, would not be absolutely essential for a stable signal-Sec61 interaction for all signal sequences (e.g., [11, 42, 44]). This would explain why TRAM is stimulatory for the translocation of some, but not other proteins in a signal sequence dependent manner [45] even though it interacts with substrates that don't necessarily require it for translocation. Similarly, the TRAP complex is envisioned to act similarly, but on the luminal face of the translocon [46]. This model is consistent with the interaction between only longer nascent chains and the TRAP complex [59] and the observation that TRAP contains a large luminal domain that appears in cryo-electron microscopy reconstructions to protrude over the luminal aperture of the translocon [60].

Such a stabilizing role for both TRAM and TRAP would explain why both factors seem to be more important for substrates whose signal sequences are potentially weaker as judged by a non-canonical n-domain and/or a shorter and less hydrophobic h-domain [45, 46]. Furthermore, TRAM and TRAP may allow the core Sec61 complex to recognize a much broader range of signal sequences than would otherwise be possible due to constraints on the flexibility of the helix 2/7 binding site. This breadth of recognition may be particularly important in systems (such as mammalian cells) where essentially all substrates are translocated in the same mode (obligately co-translational) instead of being sub-divided between co- and post-translational systems that involve different translocation machineries (as in yeast [6]).

A similar model of primary and stabilizing interactions may apply for post-translational translocation in yeast (Fig. 3C, middle diagram). Here, the translocon is composed of Sec61p, Sec62p, Sec63p, Sec71p and Sec72p [61]. It is plausible to view the role of Sec62p and Sec71p as being designed, at least partially, for the recognition of modestly hydrophobic signal sequences that are selectively targeted to this translocon [6]. In this view, a translocon lacking these components (composed only of the Sec61 complex) could only handle signals and TMDs whose interaction with the helix 2/7 binding site is sufficiently stable to obviate additional stabilizing factors. Hence, the co-translational translocon in yeast (which lacks homologs of TRAM and the TRAP complex) would accommodate only the limited subset of signals that could be accommodated into its principal helix 2/7 binding site (Fig. 3C, right diagrams). This constraint may explain why in yeast, there exist two different co-translational translocons composed of either Sec61p or its homolog Ssh1p [62]: each may be capable of recognizing different subsets of signal sequences and operate in parallel to together accommodate a wider range of substrates [Wittke, 2002 #133]: each may be capable of recognizing different subsets of signal sequences and operate in parallel to together accommodate a wider range of substrates [63].

Thus, in yeast, the problem of accommodating the remarkable diversity of signal sequences seems to have been solved by the use of multiple parallel translocation machineries that each have distinct (but perhaps overlapping) substrate-specificity. By contrast, higher eukaryotes (such as the mammalian system) may have overcome this same problem by evolving translocon-associated factors such as TRAM and TRAP to stabilize weakly interacting signal sequences that otherwise could not be efficiently recognized by the Sec61 complex alone. Such a qualitatively different solution (of multiple weak interactions colluding to accomplish the final outcome) to the signal recognition problem may have significant implications for how translocation could be regulated by the cell (see the final section of this chapter).

The same basic mode of signal-translocon interaction is likely to be applicable for the initial recognition of TMDs during translocon engagement (Fig. 3B; compare to Fig. 3A). Because the hydrophobic domain is usually both longer and more hydrophobic than signal sequences,

stabilizing interactions with TRAM and TRAP may be less critical for initial TMD recognition and engagement of the translocon (although this remains to be tested experimentally). Even if initial recognition is less dependent on accessory factors, the problem of correct orientation could very well involve interactions with accessory components of the translocon. This seems entirely plausible in the case of TRAM and TRAP because these components seem to interact with regions flanking the hydrophobic domain of a signal sequence [24, 42, 47, 58] and may similarly associate with flanking domains of a TMD. As with signal sequences, features intrinsic to Sec61 may simply be insufficient to fully accommodate the diversity of TMDs (especially in the mammalian system) with which it must interact. Thus, accessory components (while dispensable for the few model TMDs thus far studied; [44]) may be particularly important for assimilating the various parameters of the substrate (including hydrophobicity, charge of flanking domains, length, and folding of flanking domains) into a final unique topologic orientation.

The sequence features of a signal or TMD that determine the need for components in addition to the Sec61 complex are not well studied in either the mammalian or yeast systems. Furthermore, the mechanisms by which such accessory components like TRAM, the TRAP complex, or Sec62p facilitate signal recognition remain unknown. And finally, whether yet other components are also involved in substrate-specific aspects of signal recognition is also not known. Numerous proteins, particularly in the mammalian system, have been identified to be at or near the site of translocation (reviewed in [3, 64]). These include proteins with known functions (such as the multi-protein oligosaccharyl transferase complex or five protein signal peptidase complex), as well as many others whose functions are not known (including for example p180, Mtj1, RAMP4, and p34). While none of these are absolutely essential for translocation of at least the simplest model substrates [44], it is not clear whether they play essential or stimulatory roles in translocation of select substrates. As was exemplified by the TRAP complex [46], the functional role(s) of such accessory factors in translocation may elude detection until the proper substrate(s) are examined.

#### **Gating of the protein conducting channel of the translocon**

In addition to discrimination from non-secretory proteins, signal sequence recognition is an important event in preparing the translocon for subsequent protein translocation. Hence, the signal recognition step is thought to be coincident with a change in the translocon that leads to its opening towards the ER lumen [11, 13], a step termed translocon gating. Although it is clear that this is an essential step in initiating translocation, the mechanism by which it occurs remains unknown. Presumably, the initial binding of a signal sequence (or TMD) to the Sec61 complex (likely at the helix 2/7 interface) triggers a conformation change in either the Sec61 complex itself and/or associated components to convert the translocon from a 'closed' to 'open' configuration. Understanding the mechanism by which this occurs is intricately tied to the general issue of membrane permeability: the translocon should normally remain closed when it is inactive yet open during substrate translocation such that in neither situation can small molecules easily pass across the membrane.

At present, the question of how the membrane permeability barrier is maintained during different stages of protein translocation remains a matter of considerable debate. It is clear, however, that resolving this issue will require information about both the architecture of the translocon, the structure of its individual constituents, and how they are assembled and changed during the functional translocation cycle. This will provide critical information about the nature of the translocation pore, its size, how it might be opened and closed, and how its permeability to

small molecules can be controlled both during and in the absence of substrate translocation. At present, such structural and organizational information about the translocon and the pore are only beginning to emerge, leaving the mechanism of membrane permeability maintenance unresolved.

The first experimental studies to begin addressing the issues of pore size and membrane permeability were in the mammalian co-translational system. Translocation intermediates containing a fluorescently labeled amino acid were used as a probe of both the environment surrounding the nascent chain [36] and the accessibility of this environment to exogenously added fluorescence quenchers [13, 65-67]. The ability to control substrate length (and hence, the stage of translocation), the position of the probe, and the size and location of the fluorescence quenchers allowed various parameters of the translocon to be deduced. From these studies [3], the pore sizes of inactive versus engaged translocons were measured to be ~8-10 Å and ~40-60 Å, respectively [65]. Preventing the passage of small molecules through this pore depended on alternately sealing the channel with either a ribosome on the cytosolic side or BiP on the luminal side [65-67]. Sequences in the nascent polypeptide are proposed to choreograph the dynamics of channel gating by the ribosome and BiP to allow substrate transport without small molecule leakage [67]. Recently, an electrophysiological approach also suggested that purified Sec61 complex in lipid bilayers may contain pores as large as 60 Å that can be blocked by BiP [68].

Although the model derived from the fluorescent probe approach is internally consistent and compatible with many other biochemical experiments in the mammalian co-translational system, several arguments against it have been raised. In one experiment, the inability to detect folding of even a small domain while it is inside the translocon [69] seemed at odds with the proposed 40-60 Å pore size [65, 68]. However, it is not clear how generalizable the results from either approach are since in each case, a single (and different) substrate has been examined to measure pore size. In other experiments, structural studies using cryo-electron microscopy (EM) of RNCs bound to the translocon failed to see a tight seal between the ribosome and translocation channel that was expected from the fluorescence quenching studies [70-73]. However, an *inability* to see density by cryo-EM can be difficult to interpret since it could be due to increased flexibility in those regions of the structure, loss of ancillary translocon components upon solubilization and sample preparation, or sample heterogeneity. Thus, cytosolic or membrane components in addition to the ones visualized by cryo-EM may form the putative seal between the ribosome and membrane. Indeed, several abundant membrane components have been identified associated with the translocon (some with large cytosolic domains such as p180) [74] whose functions remain unclear. Thus, there are some potentially plausible ways to reconcile much of the seemingly conflicting data gathered on membrane permeability and translocon architecture of the mammalian co-translational system.

More problematic however is the argument that the proposed mechanism involving the ribosome and BiP during mammalian co-translational translocation does not shed light on how the permeability problem is solved in other modes of translocation or in bacterial systems. In the post-translational pathway, the ribosome is not involved in translocation, precluding a role for it in maintaining the permeability barrier. In bacteria, it is unclear what would serve the function of the luminal gate proposed for BiP in the mammalian system. Because of these difficulties, a more generally applicable and evolutionarily conserved solution to the permeability barrier problem has been sought. The most insight into such a putatively conserved mechanism comes from interpretation of the recent high resolution crystal structure of an archaeal SecY complex [57, 75].

In this structure, a single SecY complex was found to form a channel-like structure with a very small pore flanked on the luminal and cytosolic sides by funnels. The narrow constriction between these two funnels is only ~5-8 Å in diameter and lined by several hydrophobic residues that together form the 'pore ring.' If the channel formed by a *single* SecY complex is the functional pore through which the substrate is transported, the small size and flexibility of the 'pore ring' side chains would then form a relatively snug fit around a translocating polypeptide. This mechanism of translocation would solve the permeability problem because the nascent chain itself can occlude the channel during translocation. Furthermore, another small segment of the SecY protein (termed the 'plug' domain) appears to occlude the pore in its inactive state [57]. Thus, no additional components would be required to maintain permeability except the Sec61/SecY complex, which forms the channel in all modes of translocation.

Mutagenesis studies have demonstrated movement of the plug domain upon initiation of translocation, supporting its proposed role in gating [76]. However, other studies question an essential role for the plug domain [77] since even its complete deletion was nonetheless compatible with viability in yeast (although various translocation defects were noted). In addition, crosslinking between nascent chains and residues near the pore-ring (but not elsewhere) supports the polypeptide transit path proposed on the basis of the SecY structure [78]. Based on these and other considerations, it has been argued that a hydrophilic pore is not formed at the interface of multiple SecY complexes, but within a single SecY whose permeability is maintained by its plug domain (reviewed by [4, 75]). Whether this model proves to be true in all modes and systems of translocation, and hence universally explains the permeability problem, remains to be investigated by high resolution structures of translocons engaged in active translocation. At present, computational fitting of the archaeal X-ray structure into a cryo-EM structure of a RNC-engaged *E. coli* translocon suggests that two SecY molecules may 'face' each other with communicating pores [79]. If validated, this may suggest that the basic unit of translocation can be re-organized significantly by associating components (like the ribosome) when changing between inactive and active states.

Hence, in eukaryotic systems, the basic unit of translocation may have evolved into a more malleable oligomeric structure in which the pores of multiple Sec61 complexes can indeed be combined to form a larger translocon that changes to meet the demands of the substrate. This explanation would necessitate additional protein complexes that facilitate this reorganization and new mechanisms to solve the permeability problem. This view could help to reconcile the fluorescence data in the mammalian system with alternate models of gating derived from other systems. While this might seem unnecessarily complicated, it is not unreasonable given the existence of numerous eukaryotic-specific translocation components whose functions remain largely unknown (such as Sec62, Sec63, TRAM, or TRAP, among many others).

At present, the choice among the different views depends largely on where a philosophical line is drawn. On the one hand is the tremendous degree of evolutionary conservation of the most fundamental features of protein translocation that has allowed information across multiple kingdoms to be combined into explanations applicable to all systems. On the other hand is the equally powerful feature of evolution to forge new biological principles using the same basic constituents. Clearly, the former is justified when one considers examples such as the SRP pathway, while the latter is strikingly exemplified by the evolution in eukaryotes of mechanisms to 'pull' nascent chains across the membrane from a system initially designed to 'push' such chains from the cytosolic side. Ultimately, experimental results will be needed to resolve these

issues and determine the degree to which evolution has been conservative versus inventive in shaping eukaryotic protein translocation across the ER.

### **The energetics of protein translocation**

Regardless of the structural features of the translocon pore or the precise mechanism of translocon gating, it is clear that after this event has occurred, the nascent polypeptide spans the membrane bilayer through the aqueous channel formed by the Sec61 complex [13, 47]. Hence, a portion of the nascent chain can access the ER luminal environment, while the remainder of the chain has yet to be translocated. The channel within which the nascent chain resides does not appear to interact with the chain; it is instead thought to be a relatively inert passive conduit. Indeed, early studies artificially releasing a nascent chain stalled in its co-translational translocation showed it to be capable of bidirectional movement to either the luminal or cytosolic side of the membrane [80]. This suggested that unidirectional vectorial movement of the nascent chain must be imparted by accessory factors and presumably requires the input of energy.

In the co-translational pathway, the substrate is thought to be 'pushed' across the membrane by the ribosome (Fig. 4A). In this model, the pushing is critically dependent on the architecture of a ribosome-translocon complex that provides a continuous path from the peptidyl transferase site within the ribosome to the luminal aperture of the translocon. When configured this way, the nascent chain is thought to essentially have only one path of transit as it is elongated by continued translation. Thus, the energy of protein synthesis is simultaneously used to support translocation. This view is supported by the demonstration of a contiguous path through the ribosome-translocon complex [13], the apparent alignment of conduits through both structures [70], the shielding of translocating nascent chains from the cytosol [11, 13], and the reconstitution of successful translocation with the Sec61 complex as the only translocon component in the membrane [11, 44]. Thus, for at least some substrates, co-translational translocation can proceed solely on the basis of energy expended for protein synthesis.

However, several observations suggest that this model of co-translational translocation is insufficient to explain the vectorial transport of all co-translationally translocated substrates. First, the putative tight seal between the ribosome and translocon that precludes nascent chain slipping during translocation may not be uniformly maintained for all substrates. In some instances, nascent chains have been shown to be accessible to the cytosol during translocation [81, 82]. In other cases, the nascent polypeptide did not become shielded from the cytosol shortly after its docking at the translocon, instead being cytosolically accessible for prolonged periods early in translocation [83]. Cytosolic accessibility is also predicted on the basis of a relatively large gap observed between the ribosome and translocon in cryo-EM structures [60, 70-73, 84], and the fact that cytosolic loops in multi-spanning membrane proteins must have some means of escaping the ribosome-translocon tunnel during translocation. Thus, it is reasonable to conclude that many nascent chains have access to the cytosol at one or another point during their translocation, and therefore at least have the potential to slip into the cytosol rather than being translocated.

This potential to slip may explain why some studies have found that nascent chain binding proteins on the luminal side of the membrane can stimulate co-translational translocation [85-89]. It is plausible that such additional factors are required to bias transport only in some circumstances or for some substrates, perhaps explaining why this luminal factor requirement has not been uniformly observed by all investigators. Additional studies systematically and

quantitatively examining the requirements for co-translational translocation of many different types of substrates will be needed to fully identify all of the factors that impart unidirectionality to the transport process.

In the yeast post-translational pathway (Fig. 4B), the ribosome and energy of protein synthesis cannot be exploited for translocation. Thus, other factors, such as the luminal chaperone Kar2 (the yeast homolog of BiP), are obligately required for vectorial translocation [61, 89-91]. Kar2 is recruited to the luminal side of the translocation site via its interaction with Sec63, a J-domain protein that regulates the ATPase activity of BiP [90, 92]. At the site of translocation, Kar2 binds to the substrate and prevent its back sliding to the cytosol [61, 90, 91]. Repeated rounds of ATP-dependent binding and release, coupled with brownian motion of the nascent chain, are thought to drive substrate translocation into the ER lumen by a 'molecular ratchet' mechanism [93]. This role of BiP/Kar2 in the translocation of proteins across the ER membrane is discussed in more detail in the next chapter of this book.

A yet different translocation mechanism is involved in post-translational translocation in bacteria (Fig. 4C). SecA, a dissociable subunit of the translocon, appears to harness its ATPase activity to 'push' substrates in a step-wise manner through the SecY pore. In addition, the proton motive force can drive translocation after the initiation by SecA and can be continued and completed even after depletion of ATP [4, 94]. This translocation system (described in detail in chapter X) further underscores the basic idea that components of the membrane-embedded translocon or translocation channel do not directly provide any intrinsic directionality to transport; instead, the energetics and mechanisms for directionality are imparted by reversibly associated accessory factors. The reason for this 'division of labor' is not clear, but may allow translocons to be adapted for multiple purposes (such as forward translocation and retrotranslocation) simply by changing the spectrum of associated components.

### **The biogenesis of membrane proteins**

For secretory and many single-spanning proteins, the issues thus far considered (recognition, engagement of the translocon, and vectorial transport) largely suffice to explain their translocation. However, multi-spanning membrane proteins have several TMDs that must each be recognized, oriented appropriately, laterally inserted into the membrane bilayer, and assembled with other TMDs to create the final product. All of these events are thought to occur co-translationally in all systems ranging from bacteria to mammals. The reason may have to do with the relatively intractable problem of even transiently maintaining a highly hydrophobic protein containing several TMDs in a configuration capable of subsequent translocation. Instead, cells appear to deal mostly with TMDs sequentially, as they emerge from a ribosome docked at an already engaged translocon. The mechanisms involved in the handling of internal TMDs is very poorly studied, and remains largely in the realm of speculative working models.

It is reasonable to assume (albeit with little experimental data) that recognition and orientation of internal TMDs operates by principles similar in many ways to the initial recognition event of signal sequences and signal anchor sequences (Fig. 5). The one obvious exception is that the orientation of preceding TMDs already embedded in the membrane is likely to impose rather strict constraints on the topology that can be achieved by subsequent TMDs. Unless previously synthesized TMDs are re-oriented after their initial handling (an event for which some precedent exists), the incoming TMD must acquire a topology opposite to the preceding TMD (Fig. 5A,B). In the context of this constraint, how then are co-translationally synthesized internal TMDs recognized, oriented, integrated, and assembled?

Quite surprisingly, fluorescence probes incorporated into TMDs suggest that they may first be recognized while still inside the ribosome [67]. Not only does the TMD begin to acquire secondary structure inside the ribosomal tunnel, but this recognition seems to induce changes in the translocon machinery to which the ribosome is bound [95, 96]. In particular, the translocon becomes sealed (i.e., a closed configuration) on the luminal side, while the ribosome-translocon interaction changes in a way that allows nascent chain exposure to the cytosol [97, 98]. This translocon configuration (sealed from the lumen and open to the cytosol) is similar in many ways to the configuration encountered by a signal sequence or TMD when it is first targeted to a vacant translocon. It is therefore appealing to consider a model in which every TMD, regardless of its position in a multi-spanning membrane protein, encounters the translocation apparatus in essentially the same way. In this view, the translocon is effectively 'reset' to a baseline configuration before the emergence of every TMD.

From this baseline configuration, we would imagine that the translocon recognizes and orients each successive TMD by using similar parameters as those used for signals and signal anchors. This conclusion is consistent with the observation that the charge distribution flanking internal TMDs is similar (although not quite as obvious) to that of first TMDs [28]. These same recognition parameters, together with the additional constraint of previous TMDs, stabilize the intended orientation of most TMDs. Because many TMDs may not be as robustly oriented on their own as others, the constraint of previous TMDs may be more important in some instances than others. Indeed, experiments intentionally altering the flanking charges of internal TMDs have shown that TMDs can be 'forced' into specific orientations solely on the basis of surrounding TMDs (Fig. 5C). For example, a TMD that is essentially incapable of engaging the translocon on its own could potentially be forced to span the membrane by two very strongly oriented flanking TMDs [99, 100]. Such unusual insertion mechanisms may be especially important for the biosynthesis of many membrane proteins (such as ion channels) whose internal TMDs may need to be relatively non-hydrophobic for correct function of the final product.

Considering the tremendous range of TMDs that are handled by the translocon, it seems unlikely that a single mechanism is used for their uniform recognition and orientation. Instead, a combination of self-contained sequence elements (e.g., hydrophobicity, flanking charges), constraints imposed by other TMDs, cooperation between adjacent TMDs [56, 101], and even re-orientation mechanisms [102] all contribute to complex membrane protein biogenesis. This multi-factorial aspect of TMD integration may help to understand why prediction of topology is non-trivial, and how many regions of limited hydrophobicity can nonetheless serve as TMDs.

#### **Lateral exit of TMDs from the translocon**

After recognition and orientation of a TMD by the translocon, it must be moved from the aqueous translocation channel to the hydrophobic lipid bilayer. Several non-mutually exclusive mechanisms have been proposed to explain how this lateral exit occurs (reviewed in [3, 103, 104]). It is likely that each of these mechanisms operates under different circumstances, depending on the features of the TMD and other parts of the substrate (Fig. 6)

Prior to integration, crosslinking studies have shown that the TMD is adjacent to Sec61 $\alpha$ , lipids, and, in some cases, TRAM [52, 53, 55]. Because crosslinking probes positioned in adjacent sites within a TMD show different efficiencies of crosslinking to Sec61 $\alpha$  and TRAM, it is thought that the TMD is bound to a specific site in the translocon [96]. This site may be the same as the signal sequence binding site involving helix 2/7 of Sec61 $\alpha$ . Binding to the

translocon is also presumably important to prevent further translocation into the lumen during continued translation. From this site, the TMD then moves into the lipid.

The simplest mechanism of lateral TMD movement involves its partitioning via its hydrophobic core into the lipid bilayer [52]. Because the TMD, even when in the translocon, can be crosslinked to lipids, it is presumably already positioned at the lateral exit site. The model is that upon further translation, the tether keeping the TMD in close proximity to the translocon is lengthened, allowing the TMD free reign to access its energetically favored environment. Hence, the TMD moves from the translocon to the lipid bilayer before protein synthesis had terminated (Fig. 6A). Consistent with this partitioning model, introduction of charges into the TMD slowed its movement into the lipid bilayer [52]. Furthermore, this mechanism (at a minimum) appears to involve only the Sec61 complex since integration by partitioning has been reconstituted using purified components [52].

In another set of studies with different substrates, the TMD was observed to remain within crosslinking distance to Sec61 $\alpha$  and/or TRAM for a prolonged time, up until the terminal codon was reached by the ribosome [55, 96]. This has been interpreted as a mode of integration being distinct from the partitioning model. It is possible that the conformational change in the ribosome-translocon complex upon termination is the critical event in driving TMD integration (Fig. 6B). The mechanistic difference between integration during synthesis versus integration upon termination is not clear, but could have to do with different substrates or experimental conditions. Given that most detailed studies of TMD integration are performed using artificially designed substrates, it is not yet obvious how commonly each mechanism is used in naturally occurring membrane proteins.

The third mechanism of integration involves 'displacement' of a TMD from its binding site in the translocon by a subsequent TMD (Fig. 6C). In one version of this view, only one principal binding site for a TMD (or signal sequence) exists within the translocon. Hence, each subsequent TMD would displace the previous TMD from this site, forcing its integration into the lipid bilayer [105-107]. A variation of this view is that some TMDs are not sufficiently stable on their own in the lipid bilayer, and are thus held at a protein-lipid interface until it can co-integrate with another TMD (with which it interacts). Indeed, examples of membrane protein integration where two TMDs cooperate to facilitate each other's integration have been described [56, 101]. Extrapolation of this line of thought leads to a model where the translocon (perhaps in combination with associated factors) can retain multiple TMDs (or retrieve them to the translocon ever after integration) to facilitate the assembly of three or more TMDs co-translationally before their en bloc release into the lipid bilayer [106, 108].

And finally, although poorly studied, complex membrane protein assembly events such as multiple TMD assembly may require chaperones specialized for handling TMDs (Fig. 6D). TRAM, an importin-like ER protein termed importin $\alpha$ 16, and a yet unidentified factor termed PAT10 have each been suggested to serve in this role on the basis of crosslinking studies [52, 55, 105, 109]. At present, experiments examining the consequences of removing any of these components for membrane protein integration remain to be performed. Thus, their functional roles in the integration process remain largely a matter of speculation. In fact, aside from the Sec61 complex, no other component has been functionally shown to directly influence integration (defined as the lateral movement of a TMD from the aqueous translocon to the lipid bilayer). Setting up robust and well-defined assays for TMD orientation, integration, and assembly in a system readily amenable to selective depletion of individual translocon

components will be an important goal in defining the mechanisms of membrane protein biogenesis.

### Regulation of protein translocation

The evolution of a complex endomembrane system in eukaryotes provides several advantages to the cell, some of which are more obvious than others. These advantages include increased capacity, quality control, quantity control, and regulation. Upon translocation into the ER, a protein is still available to a eukaryotic cell before its secretion or exposure at the cell surface; by contrast, translocation is largely synonymous with exit from the cell in prokaryotes. This intracellular availability prior to secretion or presentation on the cell surface has been thoroughly exploited to confer several important advantages to eukaryotes.

The most important advantage is the opportunity for quality and quantity control: since a translocated protein in eukaryotes is not lost to the extracellular space, there is time to impose a 'recall' in instances where the protein is not desired. Hence, if a protein is not matured or assembled properly, it is re-routed for degradation (i.e., quality control) [110, 111], thereby avoiding the potentially detrimental consequences of misfolded or incomplete secretory and membrane proteins. This has almost certainly facilitated the evolution of very complex secretory proteins (such as apolipoprotein B) or multi-component membrane protein complexes (such as the T-cell receptor). Similarly, regulated degradation during or shortly after translocation allows the abundance of secretory or membrane proteins to be modulated in response to need (i.e., quantity control, exemplified by HMG-coA reductase [112] or apolipoprotein B [113, 114]).

Furthermore, the intracellular compartmentalization of secretion allows secretory and membrane proteins to be stored until they are needed [115], at which point they can be rapidly delivered to selected regions of the cell surface by exocytosis. Thus, secretion of extracellular proteins or surface expression of membrane proteins can be rapid, quantal, and temporally and spatially regulated. These examples illustrate an important general principle: the disadvantages of increased cost and lower efficiency of a more complex, multi-step process (e.g., the secretory pathway) can be offset by the benefits of a greater degree of regulatory control. Thus, potentially regulatory aspects of the secretory pathway are likely to be most thoroughly developed in systems where control, and not just energetic cost, is of the utmost importance.

In which organisms is the highest premium placed on precise control of secretory and membrane protein biogenesis? The answer is multicellular organisms, whose fitness depends not only on the health of individual cells, but equally (or perhaps even more) on the ways those cells interact, communicate, and function as complex units. Such communication and interactions are intimately dependent on secreted and cell surface proteins whose amounts at the right time and place must be carefully regulated. Thus, completely healthy *individual* cells in a complex organism can nonetheless lead to failure of the organism if they do not function coordinately in extremely precise ways. Countless examples of this idea can be found in human physiology and disease, including the regulation of blood pressure, reproductive cycles, stress, appetite, and weight regulation. It should therefore come as no surprise that *each and every step* in the secretory pathway that has been examined was discovered to be regulated to tightly control the levels of secretory and membrane proteins in response to cellular and organismal needs. Will protein translocation prove to be any different once more complex (and subtle) aspects of this process have received experimental attention? Almost certainly not.

How then might one conceptualize a framework for translocational regulation that can guide future investigation? At the outset, it is instructive to consider analogies to other regulatory

systems for common themes that can be applied to translocation. In this vein, a grossly simplified discussion of transcriptional promoters and their regulation is useful [116-119] (although similar arguments can be made equally well with any other regulatory process). In transcription, sequence features that are common to all promoters are accompanied by sequence elements that are unique to each individual promoter [120]. Thus, each promoter is unique, but contains at least some common elements that allow it to be recognized as a promoter *per se*. The common elements allow a core (or 'general') machinery to mediate transcription [117, 118], while the unique elements impose requirements for additional machinery that regulate the recruitment or activity of the core components [116, 119]. The combinatorial expression or modification of the unique machinery can dramatically influence the activity of any given promoter. By regulating individual components of the unique machinery in a temporal or cell type-specific manner, transcriptional regulation of individual promoters can be achieved independently of each other. Thus, sequence diversity of promoters combined with diversity in the components that recognize them allows selective regulation of genes that all nonetheless use a commonly shared core machinery for transcription.

Applying this general idea to translocation allows at least one mechanism of regulation to be conceptualized. Here, signal sequences are viewed as loosely analogous to promoters, and the evolutionarily conserved components of the translocation machinery (i.e., SRP, SR, and Sec61 complexes) are analogous to the core transcriptional machinery. Signal sequences are indeed extremely diverse, with each substrate containing an effectively unique signal, while nonetheless sharing certain common, recognizable features [25, 121]. The common features of the signal appear to be the elements that are recognized by the core machinery, such as SRP54 and the Sec61 complex. The unique features of the signal appear to impose additional constraints on signal function by requiring the presence of additional factors at the translocation site such as TRAM or the TRAP complex [42, 45, 46]. These additional components can be modified (e.g., by phosphorylation [122-124]), which potentially may selectively modulate their activity (although this has yet to be examined). Thus, even using only the limited information that is currently known, one can easily envision the basic elements of a substrate-specific system of translocational regulation (Fig. 7): (a) diversity in structure and function of signal sequences that share a bare minimum of common features, (b) diversity in 'accessory' components that influence recognition by a core translocation machinery of some, but not other signals, and (c) selective changes in expression or modification of the 'accessory' components that could affect the outcome of translocation for some, but not other substrates.

This view of regulating translocation by the combinatorial functions of accessory components can be readily expanded to incorporate the many other factors at or near the site of translocation whose functions remain elusive. In the mammalian system, these include Sec62, Sec63, p180, p34, Mtj1, RAMP, a TRAM homolog, and yet unidentified proteins observed by cross-linking studies. Each of these components could potentially play stimulatory (or inhibitory) roles in the translocation of selected substrates, with the specificity encoded in the sequence diversity of the signal. Such accessory components can not only be modified, but themselves regulated at steps such as alternative splicing [125] or differential expression [126] to influence their function. Thus, there exist more than enough sources for modulatory activities to theoretically provide exquisite specificity in the regulation of signal sequence function, and hence translocation. Proof-of-principle that differences in signal sequences among substrates can indeed be exploited to selectively modulate translocation comes from the recent discovery of translocational inhibitors [127, 128]. These molecules appear to work by selectively inhibiting

the interaction between Sec61 and some, but not other signal sequences [127]. Thus, signal sequence function can be selectively, potently, and reversibly modulated in trans.

Initial evidence that protein translocation can indeed be physiologically modulated in a substrate-selective, cell-type specific way has recently been provided by quantitatively examining the efficiency of signal sequence function in vivo [129]. Not only were different signal sequences found to have different efficiencies within a given cell type, but they also varied independently in a cell type-specific manner. For example, one signal sequence was observed to be significantly more efficient than another signal in a particular type of cell; however, in a different cell type, the two signals were found to be equally inefficient. Similarly, the relative efficiencies of different signal sequences appeared to change even within a population of cells when assessed at different stages of growth.

In another series of experiments, it has recently been shown that translocation efficiencies of selected substrates can be responsive to environmental stimuli. For example, during acute ER stress, the translocation of some but not other proteins was shown to be attenuated to varying degrees in a signal sequence-selective manner [130]. These aborted translocation products were routed for degradation by the cytosolic proteasome system. One purpose for this re-routing from a translocated to degradative fate (a process termed 'pre-emptive quality control' or pQC) may be to protect the ER lumen from excessive protein misfolding during ER stress. Indeed, forcing the prion protein (PrP) to be constitutively translocated under these conditions by use of a highly efficient signal sequence caused increased sensitivity to ER stress and increased PrP aggregation in the ER lumen. Thus, the entry of proteins into the ER is not necessarily a constitutive process pre-destined by the sequences of the substrate. Rather, it is dependent on and potentially regulated by a translocation machinery that may be responsive to changes in cellular conditions.

Modulating translocation not only provides a mechanism for quantity control (i.e., the ability to change the abundance of the protein in the secretory pathway), but may also be a means to generate alternative forms of certain proteins that reside in another compartment (where it could potentially serve a second function). Examples of proteins that may have such alternative functions in different compartments have been suggested (summarized in [32, 129]). At present, at least one example of the physiological relevance of such an alternatively localized population has been provided by analyses of the ER-luminal chaperone Calreticulin (Crt). Here, the signal sequence of Crt was shown to permit a small but detectable percent of the total to fail in its translocation and reside in the cytosol [131]. Remarkably, forcing efficient translocation by use of another signal sequence influenced gene expression mediated by the glucocorticoid receptor, a function previously ascribed to cytosolic Crt.

The degree to which translocational regulation is beneficially utilized for the generation of functional diversity or quantity control of secretory pathway proteins remain to be investigated and represents a largely unexplored area of protein translocation. It is clear that in addition to developing a working framework for the plausible ways that translocation might be regulated (e.g., as in Fig. 7), it will be important to identify additional tractable model systems. Whereas the study of essential and constitutive facets of translocation has required simple and highly robust model systems, the study of regulation will probably necessitate more complex substrates and potentially new experimental methods. Furthermore, as in other fields, the consequences of mis-regulation may be more nuanced than defects in basic translocation. For example, mice disrupted for the translocon accessory component RAMP4 display an ER stress-related phenotype [132]. In addition, humans containing a mutant Sec63 develop polycystic liver disease [133, 134]. Hence, the study of translocational regulation may require analyses in more complex organisms

and systems (such as *C. elegans*, *Drosophila*, or mouse models) than have yet to be employed in this field. These areas of study represent challenging but physiologically important directions for the future.

### **Figure Legends**

**Figure 1. Two modes of substrate delivery to translocons at the ER.** (A) Co-translational targeting. The signal recognition particle (SRP) binds the hydrophobic regions of N-terminal signal sequences and transmembrane domains (TMDs) as they emerge from the ribosome (bottom). The ribosome-nascent chain-SRP complex is delivered to a translocon at the ER (top) via a GTP-dependent interaction between SRP and its receptor (SR). The principal components of the mammalian co-translational translocon are indicated, with the highly conserved Sec61 complex forming the central protein-conducting channel. (B) Post-translational targeting. Polypeptides with N-terminal signal sequences of lower hydrophobicity fail to be recognized efficiently by SRP in some organisms (such as *S. cerevisiae*). In this case, the nascent chain is bound by various cytosolic chaperones that keep it in a loosely folded configuration until signal sequence recognition by an ER translocon initiates chaperone disengagement and protein translocation. The principal components of the post-translational translocon from *S. cerevisiae* are indicated, with the Sec61 complex again forming the central protein-conducting channel.

**Figure 2. The initiation step of protein translocation influences protein topology.** (A) An N-terminal signal sequence is generally recognized by the translocon in a 'looped' orientation with the N-terminus facing the cytosol (lower-left diagram). Upon cleavage of the signal sequence by signal peptidase (indicated by the scissors), the new N-terminus of the mature polypeptide (N') is committed to translocation into the ER lumen. Hence, nearly all proteins with an N-terminal signal sequence have the N-terminus of the mature protein in a non-cytosolic location. Examples include secretory proteins like the hormone prolactin, simple membrane proteins like the EGF-receptor (EGFR) and complex membrane proteins like the G-protein coupled receptor for the corticotropin releasing factor (CRFR). Although rare, some proteins (like the 2a isoform of the CRFR) may retain the N-terminal signal sequence in their final structure [135]. (B, C) Proteins whose targeting is achieved by a TMD are made into membrane proteins. Depending on features of the TMD and its flanking sequences, the orientation it acquires in the translocon is either with the N-terminus facing the cytosol (panel B) or translocated into the lumen (panel C). When the targeting TMD acquires the topology depicted in panel B, it is often known as a 'signal anchor' or type I signal anchor sequence. Panel C depicts a 'reverse signal anchor' or type II signal anchor. Examples of membrane proteins that utilize these targeting mechanisms are shown. Note that unlike the N-terminal signal sequence, the targeting element is part of the final protein structure.

**Figure 3. The signal sequence-translocon interaction.** In each of the diagrams, the lumen is on top, and the cytosol on the bottom. For simplicity, the ribosome (which would be bound to the bottom of each translocon) is not shown in any of these pictures. (A) Shown in the brackets is the initial interaction between a signal sequence and a translocon (depicted as a cylinder). This interaction is thought to be dynamic (indicated by the double arrows), and occurs during ongoing protein translocation. If excessive protein is synthesized and folding initiates on the cytosolic side before a 'looped' orientation is achieved (left diagram), the polypeptide fails to be translocated.

Otherwise, the polypeptide can commit to being successfully translocated. Signal sequences from different proteins seem to achieve the looped orientation and hence successfully gate the translocon with differing efficiencies [136]. The membrane bilayer is not shown for simplicity. (B) As with the signal sequence in panel A, the interaction between a TMD and the translocon is thought to be similarly dynamic [137]. The possible configurations that may be sampled by a TMD are shown in the brackets, with the arrows indicating an ability to interconvert among the different states. Depending on which configuration is stabilized sufficiently long to permit lateral movement into the lipid bilayer, the substrate can be committed to either final orientation. The membrane bilayer is not shown for simplicity. (C) The signal-translocon in different systems is shown in molecular detail. In each case, the view is essentially from 'within' the channel of the translocon, looking out laterally toward the lipid bilayer. Thus, the only portion of the translocation channel that is displayed is the putative lateral exit site formed by helices 2 and 7 of Sec61 $\alpha$  (white bars). In the mammalian co-translational system, the signal sequence (black bar) is positioned in the helix 2/7 interface such that portions flanking its hydrophobic region may contact portions of TRAM and the TRAP complex for additional stabilization. The analogous interaction in the yeast post-translational system also involves the helix 2/7 interface of Sec61p. Here, additional stabilizing interactions are provided by Sec62p, Sec63p, Sec71p, Sec72p, and/or the luminal chaperone BiP (also called Kar2p). Co-translational translocation in yeast may rely solely on the helix 2/7 interaction since homologs of TRAM and TRAP do not appear to exist. This may explain why only a subset of signal sequences (presumably those that do not need stabilizing factors) are directed into the co-translational pathway in yeast [6, 7]. Furthermore, it could also explain why a second co-translational translocon formed by a Sec61 homolog (Ssh1p), which has a somewhat different helix 2/7 site (indicated by shaded bars), displays different signal sequence specificity [63].

**Figure 4. Energetics of protein translocation.** Energy from several sources are harnessed for vectorial transport of substrates in the different translocation systems. In co-translational translocation (panel A), the architecture of the ribosome-translocon complex allows translocation to occur concurrently with polypeptide elongation, thereby harnessing the energy of protein synthesis for transport. In post-translational translocation (panel B), the luminal chaperone BiP utilizes ATP hydrolysis for repeated rounds of binding and release from translocating polypeptides to effect transport by a 'molecular ratchet' mechanism. The recruitment of BiP to the translocon and regulation of its ATPase cycle are provided by Sec63. BiP may also play a role in co-translational translocation, although this remains to be fully investigated. In bacterial systems (panel C), a cytosolic ATPase termed SecA seems to act as a motor that 'pushes' nascent chains through the translocon. A comparable system in eukaryotes has not been described.

**Figure 5. Recognition and membrane integration of internal TMDs.** When a TMD within the ribosomal tunnel approaches the translocon (left diagram in each panel)), it appears to acquire secondary structure [95] and may induce conformational changes in the translation and translocation machinery such that the channel is closed to further translocation (not depicted). The TMD is subsequently recognized by the translocon (second diagram in each panel) in a step that is poorly understood by may be similar to the initial signal/TMD recognition step (see Fig. 3). Depending on constraints such as the topology of preceding TMDs, the charge distribution flanking the TMD, length, and hydrophobicity, the TMD is oriented within the translocon (third

diagram in each panel). In some cases, these constraints may force a moderately hydrophobic or non-hydrophobic segment of the polypeptide (white segment in panel C) to acquire a membrane-spanning configuration. In the final step, the TMD is laterally moved into the lipid bilayer, either on its own or perhaps in conjunction with preceding TMDs with which it assembles.

**Figure 6. Lateral exit of TMDs from the translocon into the lipid bilayer.** After recognition and orientation of a TMD in the translocon (see Fig.2 and Fig. 5), it is thought to reside at a lateral exit site at the interface between the channel and lipid bilayer (possibly between helix 2 and helix 7 of Sec61 $\alpha$ ; see Fig. 3C). Each of the several mechanisms of TMD movement into the lipid bilayer that have been suggested is indicated in panels A thru D. See text for additional details.

**Figure 7. Potential mechanisms for selective translocational regulation.** Translocons in the ER of mammalian cells are capable of residing in multiple states. These different states might be distinguished by the presence or absence of various accessory factors (like TRAM, TRAP, Sec62, Sec63, Mtj1, p180, RAMP4, and others), modifications such as phosphorylation [122-124]), or use of alternative splice variants or homologs of key components. Hypothetical translocons in two different states (A and B) are depicted in the top and bottom diagrams. Signal sequence recognition by these two translocons is speculated to be different with respect to the efficiencies with which they position signals of different features. For substrate #1, whose N-domain is highly basic, translocon A efficiently positions the signal in the 'looped' orientation and hence favors translocation (top-left). By contrast, the same substrate is not efficiently recognized by translocon B (bottom-left). For substrate #2, whose signal sequence is different than substrate #1, efficient recognition is mediated by translocon B (bottom-right), but not translocon A (top-right). Thus, the differences among signal sequences combined with the malleability of translocon states would allow substrate-specific regulation of translocation into the ER.

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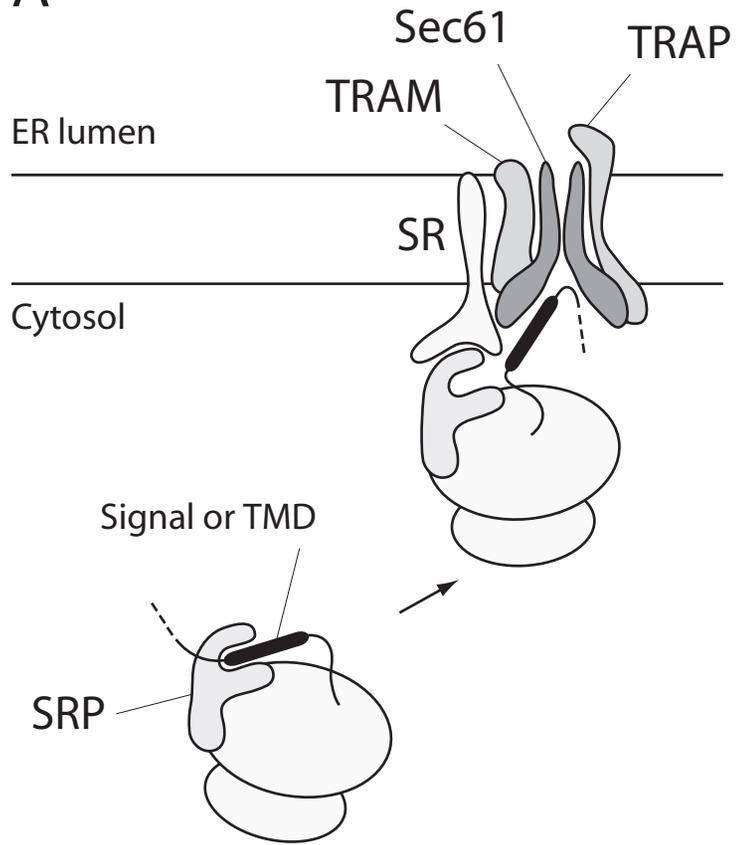
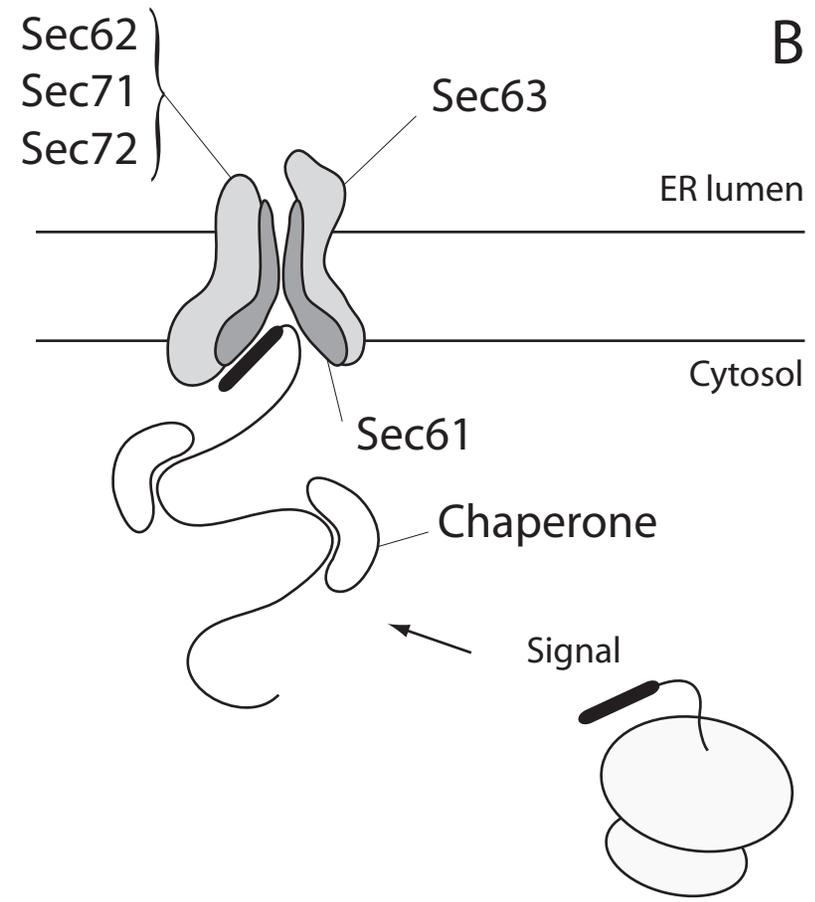
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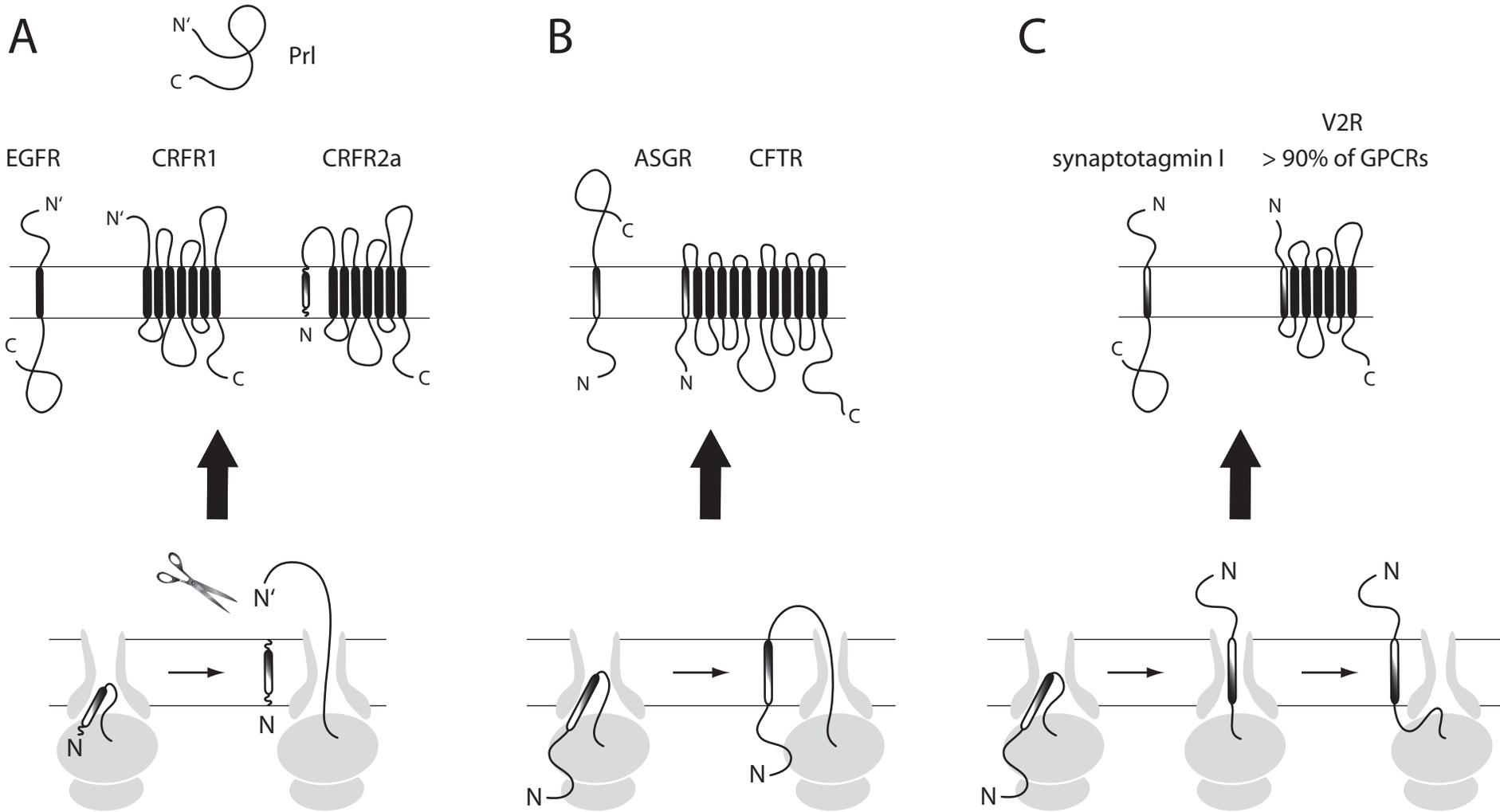
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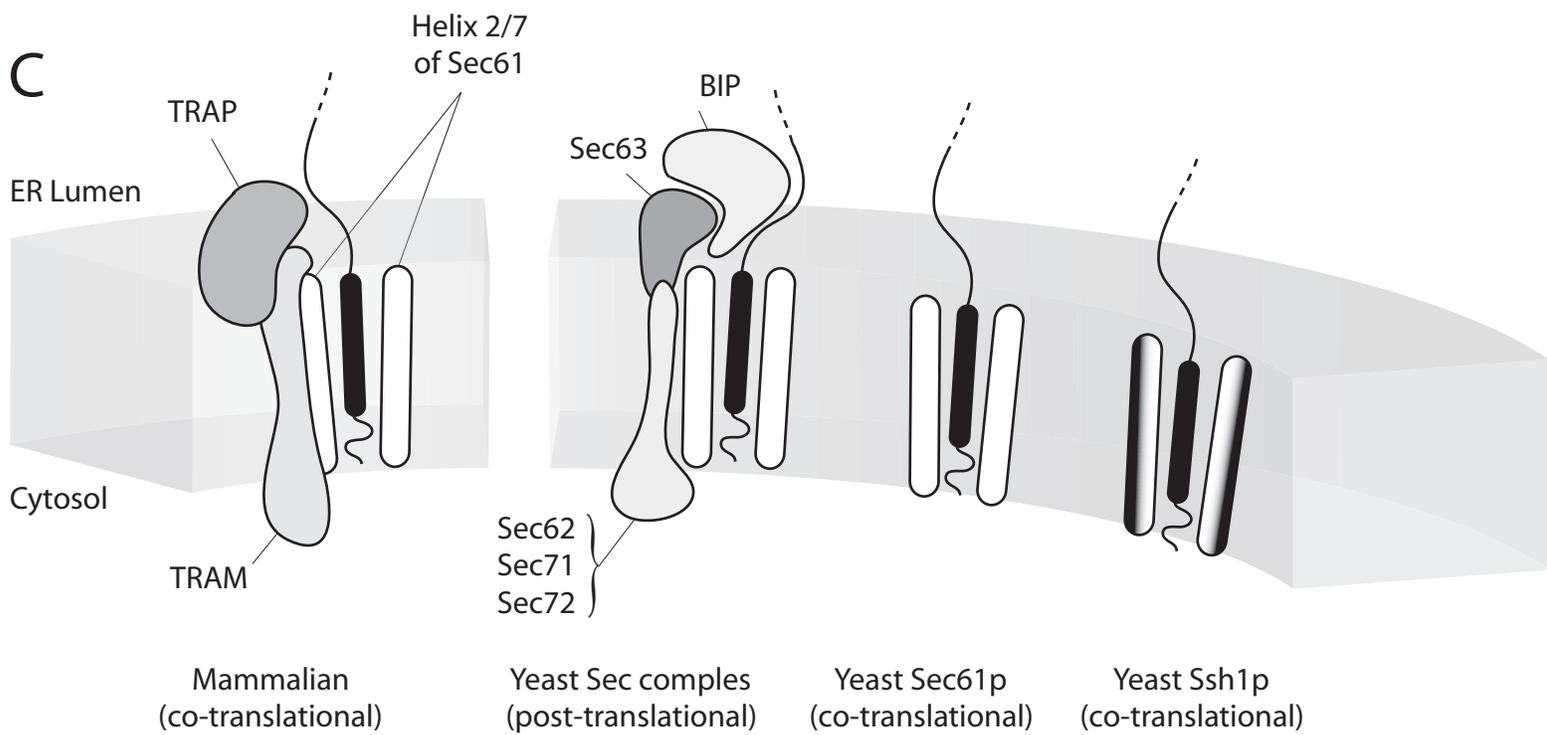
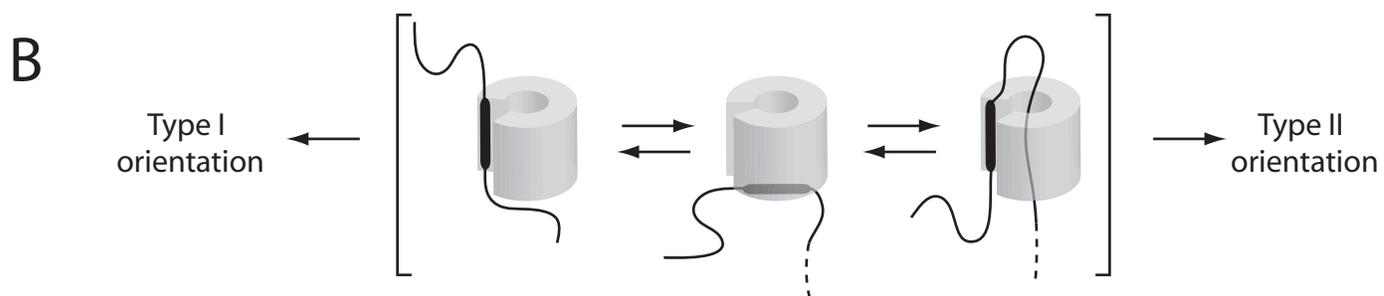
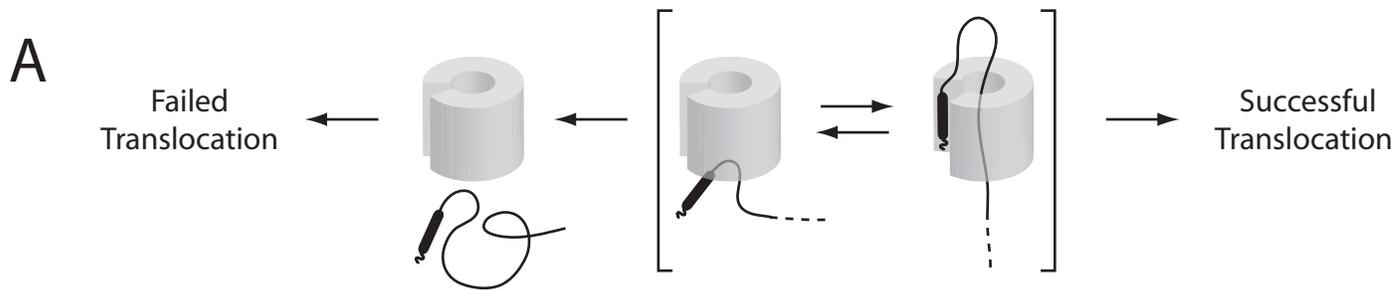
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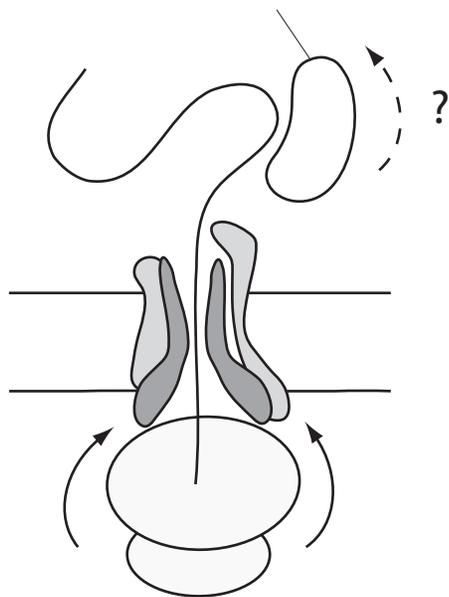
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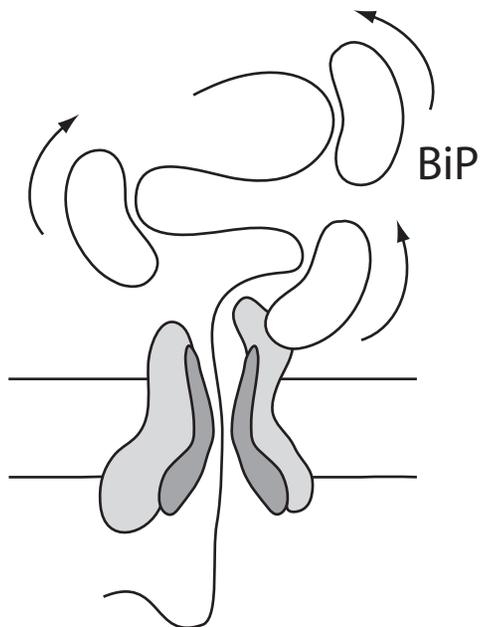
A

Luminal chaperone



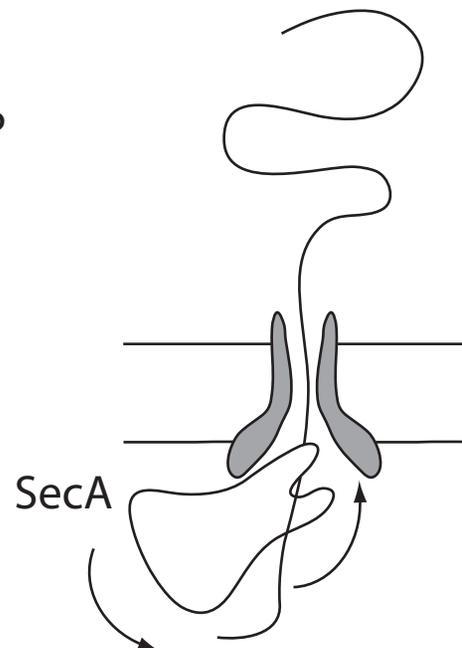
Ribosome

B



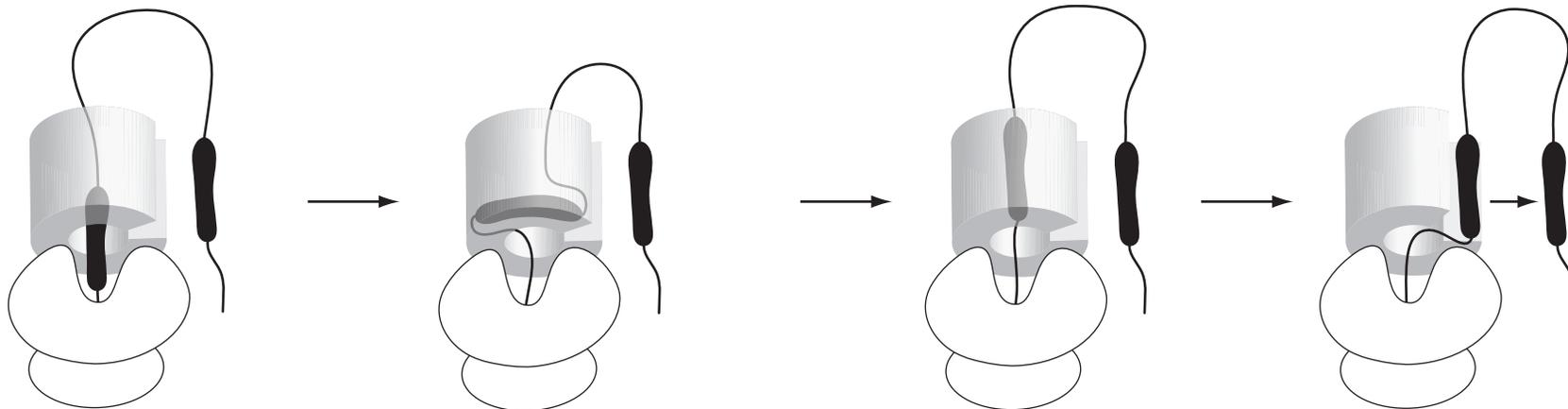
BiP

C

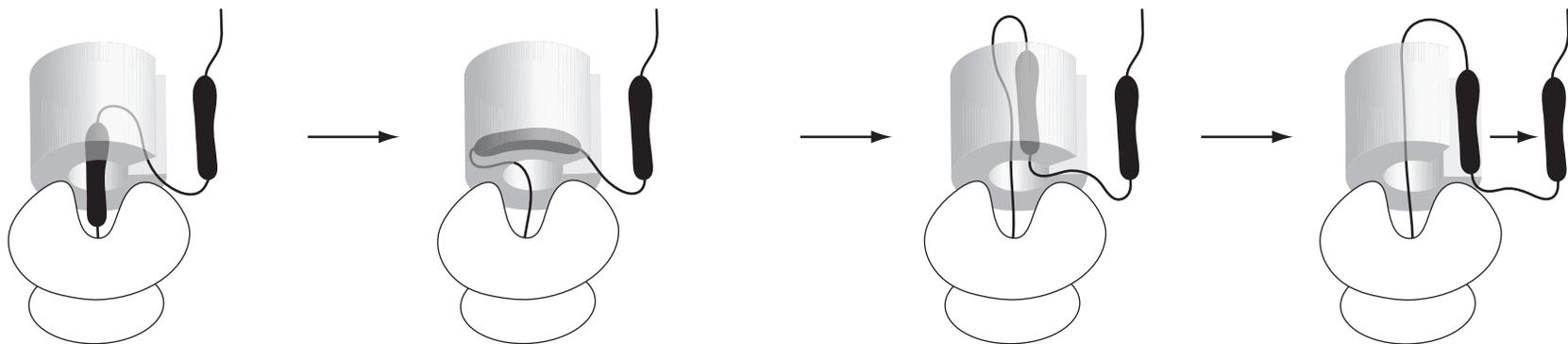


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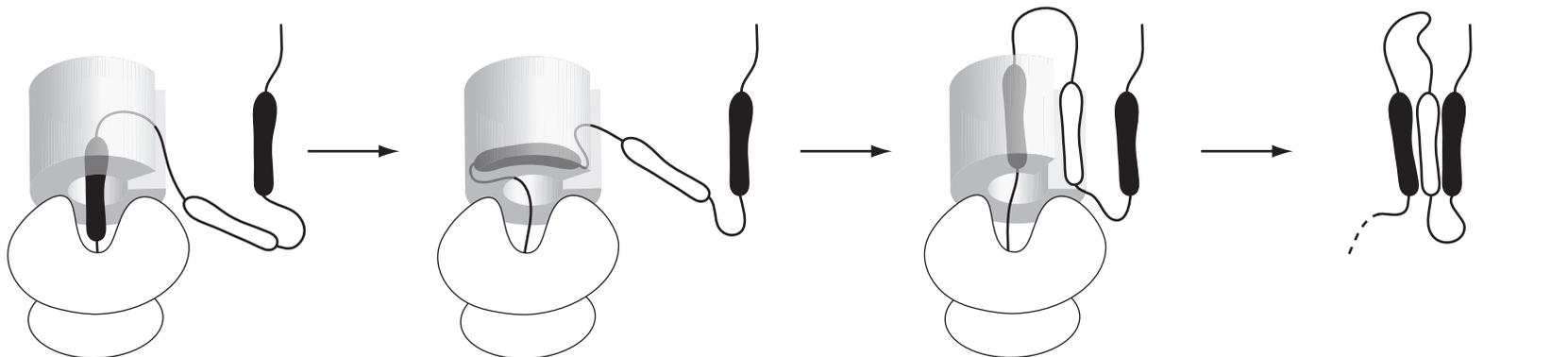
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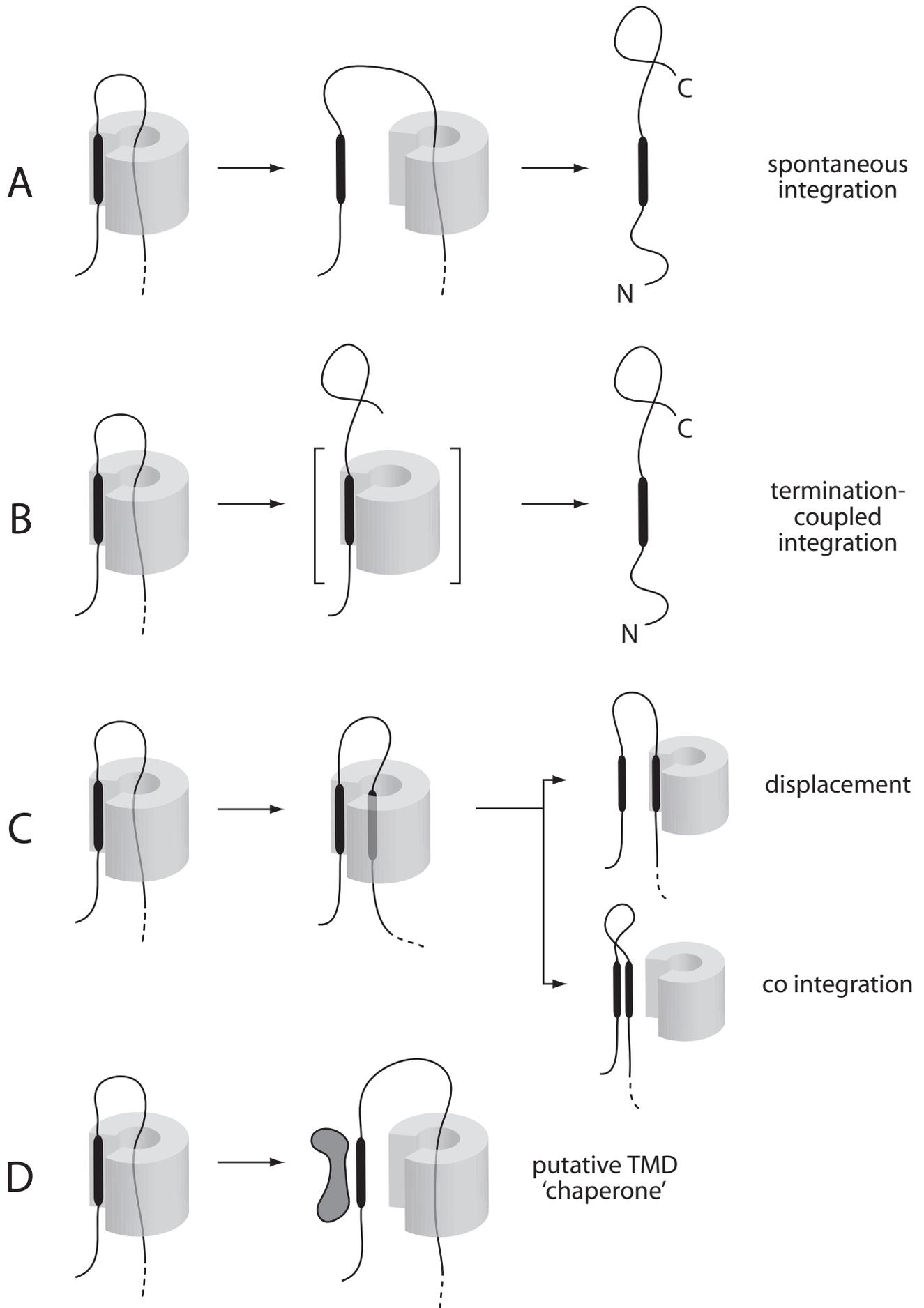


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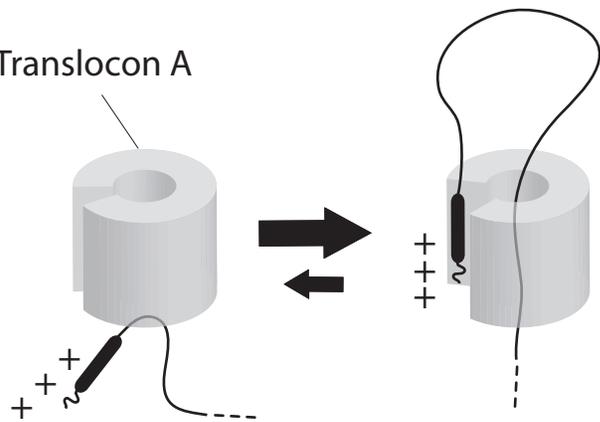


C

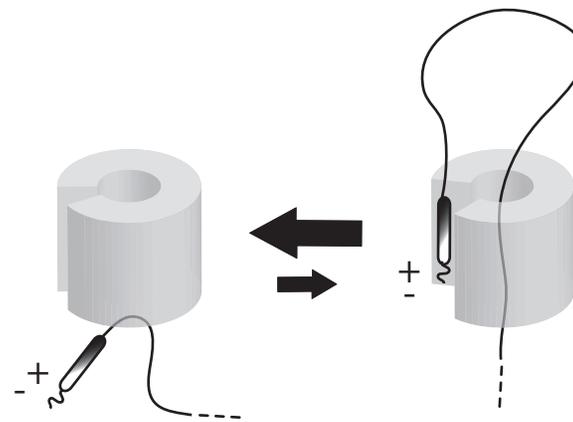




Translocon A

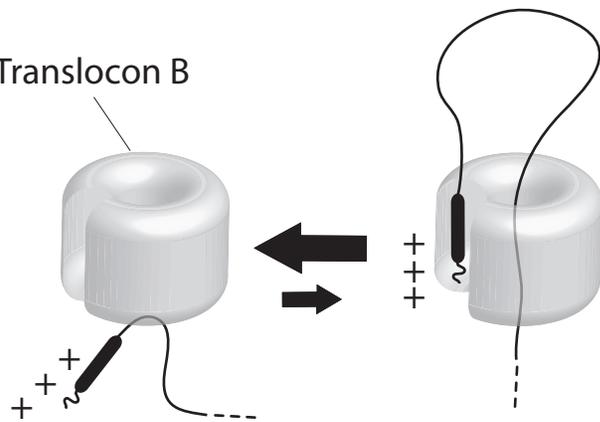


Substrate 1

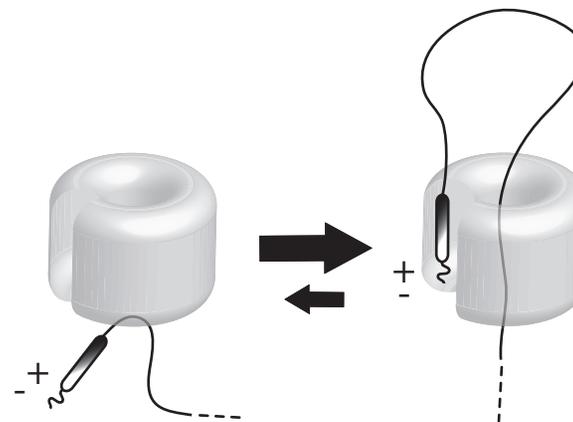


Substrate 2

Translocon B



Substrate 1



Substrate 2