Multiple Mechanisms of Protein Insertion Into and Across Membranes

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Summary. Protein localization in cells is initiated by the binding of characteristic leader (signal) peptides to specific receptors on the membranes of mitochondria or endoplasmic reticulum or, in bacteria, to the plasma membrane. There are differences in the timing of protein synthesis and translocation into or across the bilayer and in the requirement for a transmembrane electrochemical potential. Comparisons of protein localization in these different membranes suggest underlying common mechanisms.

Each of the 20 membrane-limited compartments of a mammalian cell contains a particular set of proteins that enables it to carry out its specific functions. The accurate and swift delivery of each protein to its correct compartment is an important step in gene expression. Except for the few proteins made within mitochondria and chloroplasts, protein synthesis begins with the formation of polysomes in the cytoplasm. An important concept that has guided recent work is that protein localization is initiated by binding to a specific receptor on an intracellular membrane. For different organelles, binding occurs either while the protein is still growing on the ribosome or after it is completed. Subsequent translocation of the protein into or across the membrane requires an input of energy. In different cases this is provided either by a transmembrane electrochemical potential or by the folding of the protein during or after its translocation through the membrane. In many cases there is one or more "maturation" steps involving covalent modifications or folding on the opposite membrane surface.

Different integral membrane proteins—those bound to the phospholipid bilayer by hydrophobic interactions—can have distinct asymmetric structures (Fig. 1). A protein may span the membrane once or several times, with the NH_2- and COOH-terminus on either side of the bilayer. Our ideas of membrane assembly necessarily rest on our knowledge of the complexities of the structures of these proteins. Surprisingly different answers have emerged for various membrane proteins, organelles, and organisms, confounding early global hypotheses (*1–3*) and raising the need for better definition of fundamental mechanisms. In this article we focus on protein assembly into the endoplasmic reticulum, mitochondria, and the bacterial cell surface (Fig. 2). We do not discuss other assembly-competent organelles such as chloroplasts, peroxisomes, and the nuclei, nor will we include organelles (such as Golgi or lysosomes) that are derived by membrane fission and fusion.

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Secretory Proteins and the Endoplasmic Reticulum

Classic studies by Palade and his colleagues (*4*) showed that secretory proteins are first found within the lumen of the rough endoplasmic reticulum (RER). Sealed fragments of the isolated organelle (rough microsomes) carry polysomes, which are highly enriched for secretory proteins and capable of chain completion in vitro (*5–7*). Nascent chains, emerging from the ribosome, cross the membrane (*8, 9*). In 1972, Milstein *et al.* (*10*) showed that a cell-free reaction, programmed by myeloma messenger RNA, synthesized a larger form of immunoglobulin light chains than was finally secreted from the cell. They proposed that this extra piece might be NH_2-terminal and serve as a "signal" to direct secretion. Microsomes from these myeloma cells made mature-length protein, demonstrating that the signal sequence was cleaved during translocation into the RER. In 1975, Blobel and Dobberstein (*1*) showed that polysome-free dog pancreas microsomes would sequester newly made precursor of the immunoglobulin κ light chain and proteolytically process it to its mature molecular weight if the microsomes were present during protein synthesis. "Signal sequences" were discovered at the NH_2-termini of many nascent secretory proteins (*11*). They consist of 16–26 residues and have a polar, basic NH_2-terminus and a central, apolar domain (*12, 13*).

Rothman and Lodish (*14*) used a synchronized cell-free synthesis of vesicular stomatitis virus (VSV) G protein to show that translocation requires membranes early in nascent chain growth. Walter and Blobel (*15*) and Meyer *et al.* (*16*) isolated two receptor proteins, the signal recognition particle (SRP) and the docking protein (DP), which coordinate the synthesis of nascent pre-secretory proteins (with an NH_2-terminal signal peptide) with their insertion into microsomes (Fig. 3A). SRP is a complex of six polypeptides and 7*S* RNA (*17*). It binds to polysomes making pre-secretory or pre-membrane proteins and causes arrest of chain growth after approximately 80 residues. This is only a few more than the 40 residues buried within the ribosome plus the 25 (approximately) that comprise the average signal peptide. The SRP-polysome complex binds to DP, a 72,000-dalton integral membrane protein of the RER (*16*). Upon binding, the SRP is released and the polysome resumes chain elongation (Fig. 3A). The growing polypeptide chain passes through the endoplasmic reticulum membrane and into the lumen, where the signal peptide is cleaved and core glycosylation occurs.

In contrast to our present understanding of SRP-DP, less is known about the mechanism of polypeptide translocation through the apolar center of the endoplasmic reticular membrane. It is not known whether translocation is directly through lipid (*2*), as shown in Fig. 3, or through a proteinaceous pore. Unlike translocation in mitochondria and bacteria, translocation into the RER does not require a transmembrane electrochemical potential. We propose that binding of the complex of SRP and nascent chain to

the DP on the endoplasmic reticulum membrane would cause the membrane insertion of the signal sequence and the segment of amino acids adjacent to its COOH-terminus as a helical hairpin. SRP and DP would be dislodged as part of their recycling. Signal sequence cleavage of secretory proteins and many transmembrane glycoproteins occurs on the extracytoplasmic face of the membrane (Fig. 3A). This model incorporates features proposed previously by several investigators. Receptors to target nascent proteins as well as signal and "stop-transfer" or "membrane-anchor" sequences, and the idea that the information for the orientation of each membrane protein is contained in discrete, short sequences of the polypeptide chain that act independently, are from the signal hypothesis. (*1, 3, 18*). The membrane trigger hypothesis (*2*) emphasized assembly information in the mature sequence, the importance of protein folding, and direct, hydrophobic interaction between the polypeptide chain and the hydrocarbon core of the membrane bilayer. The importance of the energetics of transfer of polypeptide domains between water and hydrocarbon was emphasized in the direct transfer model (*19*) and the helical hairpin hypothesis (*20*). The loop models (*21, 22*) and the helical hairpin hypothesis proposed that pairs of helices insert into the membrane.

Synthesis of Membrane Proteins on the Endoplasmic Reticulum

Biosynthesis of single-spanning membrane proteins whose NH_2-termini face the extracytoplasmic face is similar to that of secretory proteins (Fig. 3A). These membrane proteins contain a sequence of about 20 hydrophobic amino acids that anchors the growing polypeptide in the phospholipid bilayer. For example, Yost *et al.* (*23*) and Guan and Rose (*24*) have shown that the apolar sequences at the COOH-terminus of immunoglobulin M (IgM) heavy chain and the VSV G protein stop translocation across the endoplasmic reticulum. These long stretches of hydrophobic amino acids could bind so tightly to the fatty acid core of the membrane that continued extrusion of the protein would be blocked; thus it would be considered a stop-transfer sequence. As the ribosomes continued elongation of the nascent chain, the newly added residues would remain on the cytoplasmic face. However, in some proteins long stretches of hydrophobic amino acids are translocated entirely across the bilayer (*25*); recognition of a stop-transfer sequence might also require an appropriate "receptor" protein in the endoplasmic reticulum.

Membrane proteins such as erythrocyte Band III (*26*), the histocompatibility antigen (HLA)-DR–associated invariant chain (*27*) and the asialoglycoprotein receptor (*28*), as well as a few secreted proteins such as ovalbumin (*29*) are synthesized on the endoplasmic reticulum, yet are not cleaved during insertion. This led to the concept of an internal, uncleaved signal sequence (*3, 30*), namely a sequence that could be recognized by the same proteins [except the leader (signal) peptidase] and would perform all the same functions as its cleaved counterparts. Studies with ovalbumin (*26, 29*) indicate that it has an uncleaved signal sequence near its NH_2-terminus and have illustrated the difficulty in experimentally establishing the location of such an uncleaved signal sequence. One criterion is that such sequences be required for the insertion of a protein into microsomes (*31, 32*). Bos *et al.* (*33*) have provided the clearest demonstration of an uncleaved signal.

Uncleaved signal peptides, together with anchor sequences and other internal membrane insertion domains, can generate the complex topologies of different types of membrane proteins. For example, the bulk of the influenza virus neuraminidase is extracytoplasmic and is anchored to the membrane by a hydrophobic sequence at its NH_2-terminus. Neuraminidase does not undergo endoproteolytic cleavage during its biosynthesis, and the NH_2-terminal hydrophobic do-

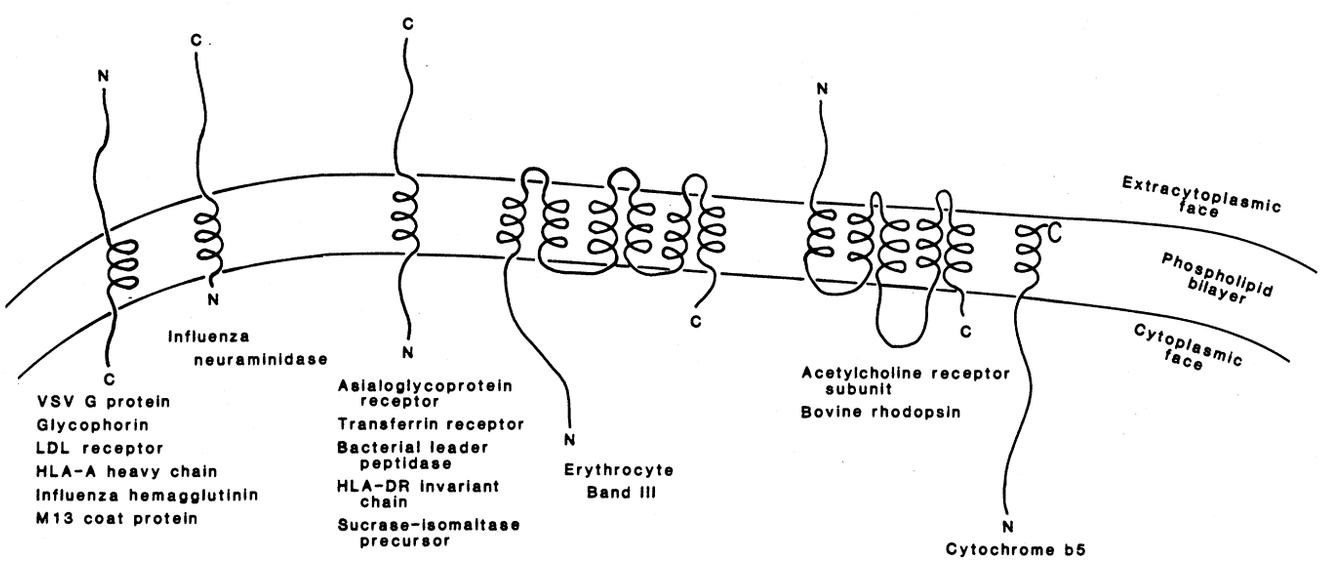


Fig. 1. Topologies of integral membrane proteins. Segments of the chain within the bilayer are depicted as helices. Extramembrane regions are drawn as lines, and no attempt is made to depict the folding of these segments of the proteins. References for the amino acid sequences and transmembrane topologies are: VSV G protein (*103*); glycophorin (*104*); LDL (low-density lipoprotein) receptor (*105*); HLA-A heavy chain (*106*); influenza hemagglutinin (*107*); M13 coat protein (*108*); influenza neuraminidase (*109*); asialoglycoprotein receptor (*28, 111*); transferrin receptor (*112*); bacterial leader peptidase (*68*); HLA-DR invariant chain (*27*); acetylcholine receptor subunit (*41*); cytochrome b5 (*113*); erythrocyte Band III (*37, 114*). Although only six membrane-spanning regions are drawn, recent data on the sequence of the entire Band III messenger RNA (*37*) indicates that there are 12 stretches of hydrophobic residues of length sufficient to span the membrane. Initial work suggested that the precursor of sucrase-isomaltase spanned the plasma membrane twice, with both the NH_2- and COOH-termini remaining exoplasmic (*110*). However, the complete complementary DNA sequence, as well as other data, indicate only a single membrane-spanning segment, with the NH_2-terminus facing the cytoplasm (*115*). N, NH_2-terminus; C, COOH-terminus.

main functions both as a signal and a membrane anchor sequence. Genetic fusion of this NH_2-terminal domain to a different viral glycoprotein that had its NH_2-terminal (cleaved) signal deleted restored translocation of this glycoprotein (*33*). This suggests that neuraminidase has an NH_2-terminal uncleaved signal sequence, and that all signal sequences, whether or not cleaved, function similarly in initiating translocation across the endoplasmic reticulum membrane. Similar studies (*34*) on the asialoglycoprotein receptor showed that the membrane spanning segment of 21 hydrophobic amino acids also functions as a signal sequence; SRP is required for its insertion into the RER. In Fig. 3B, we show how insertion of this protein might occur; as for secretory proteins, the signal peptide and the adjacent segment of the protein are postulated to insert as a helical hairpin (*20*).

Proteins such as cytochrome b5 are anchored to the membrane by apolar residues at the COOH-terminus, are synthesized on free polysomes, and probably insert into the endoplasmic reticulum membrane posttranslationally (Fig. 3C). SRP is not required for insertion of cytochrome b5 (*35*).

As proposed by Blobel (*3*), multi-spanning proteins such as sucrase-isomaltase and Band III could achieve their final topology by a succession of internal signal sequences and membrane-anchoring stop-transfer sequences. Hydrophobic side groups of the amino acids project outward to interact with the apolar fatty acyl core of the bilayer. However, many transmembrane sequences in multi-spanning proteins such as bacteriorhodopsin (*36*), Band III (*37*), or the acetylcholine receptor (*38*) have polar residues, which are not seen in the intramembrane segments of single-spanning proteins like VSV G protein, the asialoglycoprotein receptor, or M13 coat protein. These membrane-spanning helices may be amphipathic, with charged or polar residues confined to one face of the helix. Polar faces of several adjacent sequences could, in the mature protein, form a polar "pore" or "channel" through the membrane (*36, 37, 39–41*). These amphipathic helices may not be hydrophobic enough to function as simple signal or stop-transfer sequences. The proteins might utilize only one signal sequence, that would catalyze insertion of the most NH_2-terminal helical hairpin of the nascent chain into the endoplasmic reticulum membrane, and one membrane anchor. As the nascent chain continued to grow in the cytoplasm, domains with hydrophobic surfaces could form and insert spontaneously into the membrane (*2*) without involvement of SRP and DP (Fig. 3C). Several helices could associate with each other to shield their polar surfaces and form an "insertion domain" that could spontaneously insert into the phospholipid bilayer, presenting only an apolar face to the fatty acyl side chains of the phospholipids. Recombinant DNA techniques should allow the construction of novel membrane proteins that will test these concepts.

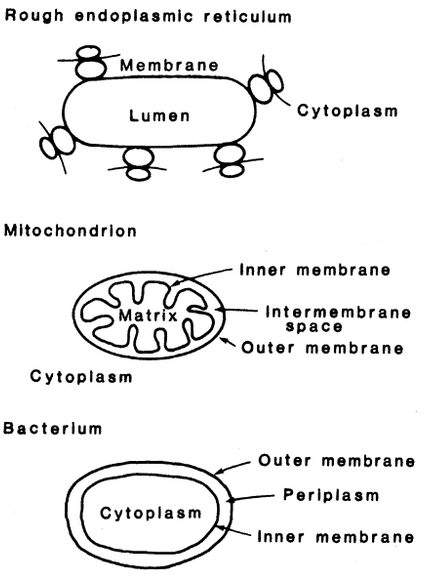


Fig. 2. Compartments of endoplasmic reticulum, mitochondria, and bacteria.

Demonstration of the close coupling between protein synthesis and membrane translocation in the endoplasmic reticulum has come from studies in a crude reticulocyte or wheat germ cell-free translation reaction supplemented with dog pancreas microsomes. Mutants, specific drugs, and in vivo studies, perhaps in a microorganism such as yeast, will be important to confirm or modify the current picture and to help in dissecting the crucial membrane translocation step.

Mitochondria

Mitochondria differ from the endoplasmic reticulum in almost every aspect of their biogenesis (*41, 42*). Except for the few proteins encoded by the mitochondrial DNA (*43*), all mitochondrial proteins are specified by nuclear genes. Each is synthesized in the cytoplasm and imported to one of the four mitochondrial compartments; outer membrane, intermembrane space, inner membrane, or matrix (Fig. 2). Isolated yeast and *Neurospora* mitochondria specifically imported only mitochondrial proteins from a cell-free translation reaction (*44*). Each of the proteins examined went to its correct compartment, and the uptake was just as efficient when the mitochondria were added posttranslationally to the protein synthesis reaction as when they were present throughout the reaction (*45*). In vivo pulse-chase studies in *Neurospora* mycelia and yeast (*46, 47*) have shown that mitochondrial proteins pass through a cytoplasmic pool prior to binding to the organelle. Isolated mitochondria have polysomes on their outer surfaces, and these polysomes are highly enriched in nascent mitochondrial preproteins (*48*). However, Suissa and Schatz (*49*) showed that these represent only a small fraction of the polysomes for any given protein and that the proportion is governed simply by the rate of protein synthesis. Protein uptake into the matrix, inner membrane, or (except for cytochrome c) into the intermembrane space requires an electrochemical potential across the inner membrane (*46, 50*).

Many mitochondrial proteins are synthesized with a transient NH_2-terminal leader peptide, while others are made and imported without cleavage (*51*). Mitochondrial leader peptides (*52*) are basic and have a different sequence pattern from pre-secretory proteins. Removal of mitochondrial leader peptides is catalyzed by a soluble matrix protease that has a specificity distinct from its counterpart in the endoplasmic reticulum or in *Escherichia coli* (*53*) (Fig. 4). In some cases, for proteins of the outer surface of the inner membrane or of the intermembrane space, the pre-sequence is removed by two successive proteolytic cleavages, one of which is catalyzed by the matrix protease (Fig. 4) (*50, 54*). Precursors of these proteins are thought to span the inner membrane transiently, with their NH_2-termini facing the matrix space. This model would explain the puzzling requirement for an electrochemical potential across the inner membrane for translocation of several proteins ultimately located in the intermembrane space. As shown in Fig. 4, the second cleavage of these proteins is thought to occur at the outer surface of the inner membrane (*52*).

Mitochondrial pre-proteins use several outer membrane receptors for import (*52, 57*). Cytochrome c, a protein of the intermembrane space, is made as a precursor (apocytochrome) without the heme group and without a cleaved presequence. Uptake requires heme addi-

tion. Microgram amounts of apocytochrome (but not holocytochrome) block uptake of radioactive apocytochrome c, but not uptake of inner membrane or matrix proteins, suggesting the involvement of a specific receptor (*58*). A chimeric protein with 350 amino acids of the β subunit of yeast F1 adenosine triphosphatase at its NH_2-terminus and a large portion of β-galactosidase at its COOH-terminus can be inserted into yeast mitochondria. This is also true for a gene fusion of the NH_2-terminal 53 amino acids of the precursor to subunit IV of cytochrome oxidase and a different cytosolic enzyme, demonstrating that part of the NH_2-terminus is sufficient to target a protein to the mitochondrion (*59*). Recently, several laboratories have reported that a soluble, cytosolic protein fraction is also necessary for import (*60*) (Fig. 4).

The outer membrane of mitochondria is distinct from the three other compartments in its biogenesis. Outer membrane proteins do not have cleaved leader sequences (*61*), and their membrane insertion does not require an electrochemical potential across the inner membrane. All of the information for targeting and anchoring the 70,000 molecular weight outer membrane protein is contained within the NH_2-terminal 41 amino acids (*62*). Like the proteins of the internal mitochondrial compartments, assembly into the outer membrane is not coupled to translation.

Several fundamental questions of mitochondrial biogenesis are unanswered. What is the role of the electrical potential? Does import into the matrix involve separate steps of traversing the inner and outer membrane or, as illustrated in Fig. 4, does this occur at adhesion zones between the two membranes as previously suggested (*41, 42*)? What is the number of outer membrane receptors, how do they catalyze translocation, and what other proteins are needed? Genetic identification of elements needed for import (*63*) may be vital to answering this question.

Bacterial Cell Surface

The cell surface of Gram-negative bacteria consists of three layers: the plasma membrane, an aqueous periplasm, and the outer membrane (Fig. 2). The mechanisms of membrane assembly and protein secretion in bacteria include those found in both mitochondria and RER. As in import of proteins into mitochondria, translocation of bacterial proteins across the plasma membrane requires a transmembrane electrochemical potential and, in different cases, can occur cotranslationally or after translation is complete. However, leader sequences of bacterial membrane and of exported proteins closely resemble those of the eukaryotic RER in structure (*12, 64*). Protein products of the bacterial *sec* (secretion) genes may, in some respects, have a function similar to that of the RER SRP. There is selective arrest of the synthesis of secretory proteins in *sec*C mutants (*65*), even though translocation occurs late in translation

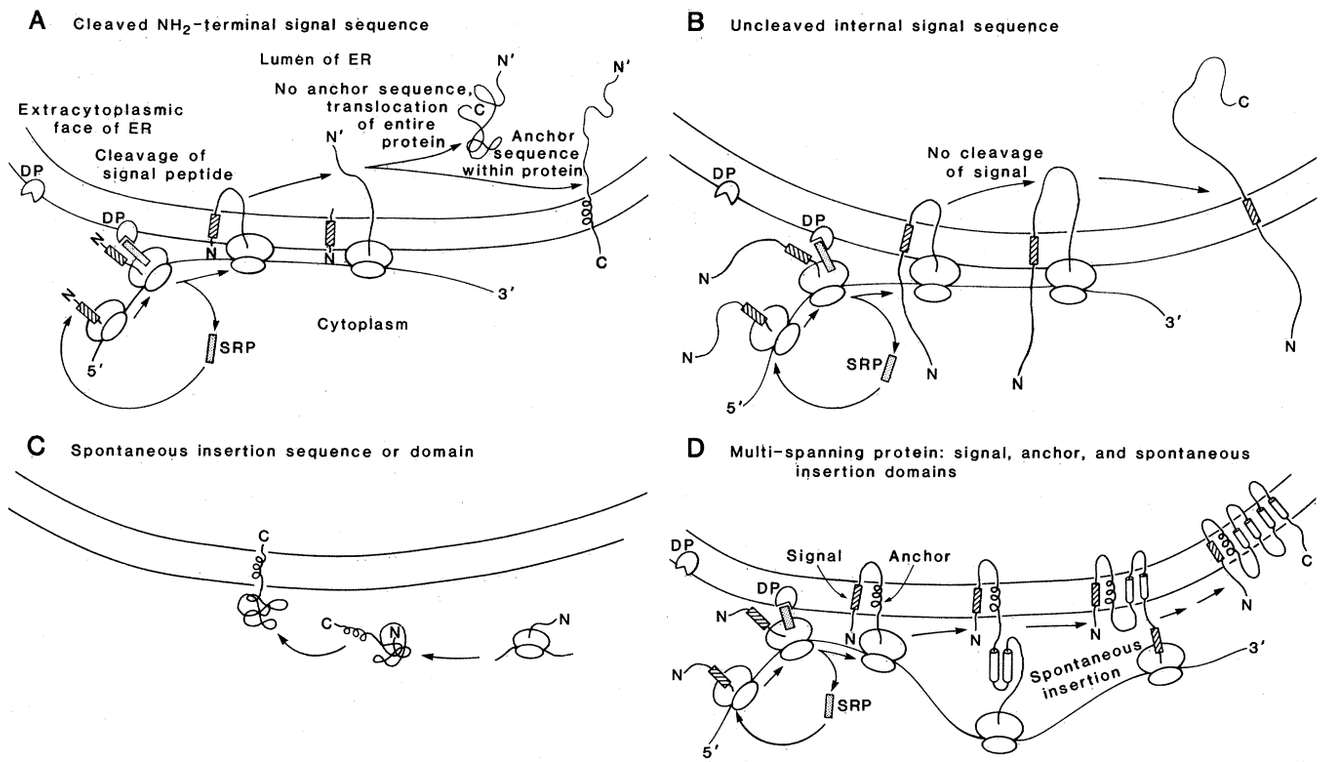


Fig. 3. A model for cotranslational insertion of membrane and secreted proteins into or through the endoplasmic reticulum membrane. (A) Secreted proteins and VSV G protein. Binding of the complex of SRP and nascent chain to the DP on the endoplasmic membrane would cause the insertion into the endoplasmic reticulum membrane of the signal sequence (hatched box) and the segment of amino acids adjacent to its COOH-terminus as a helical hairpin. After the nascent chain is cleaved, continued extrusion across the endoplasmic reticulum membrane would generate a secretory protein or, if there is an anchor (or stop-transfer) sequence, a single-spanning transmembrane protein with the same conformation as the VSV G protein. (B) Asialoglycoprotein receptor. If the signal sequence is uncleaved and there is no anchor sequence, continued growth and translocation of the nascent chain across the endoplasmic reticulum membrane would generate a protein with its NH_2-terminus facing the cytoplasm and its COOH-terminus in the lumen of the RER. (C) The synthesis of proteins such as cytochrome b5 occurs on cytoplasmic polysomes. The completed protein then inserts spontaneously into the RER, without mediation of SRP or DP, by means of an insertion sequence or domain. (D) Multi-spanning membrane proteins. The first helical hairpin could result from a combination of signal and anchor sequences; subsequent helices could fold against each other, forming a domain that would insert spontaneously as the peptide grows in the cytoplasm. The cylinders represent a possibly alternative structure of a membrane-spanning α-helix.

or posttranslationally (as discussed below).

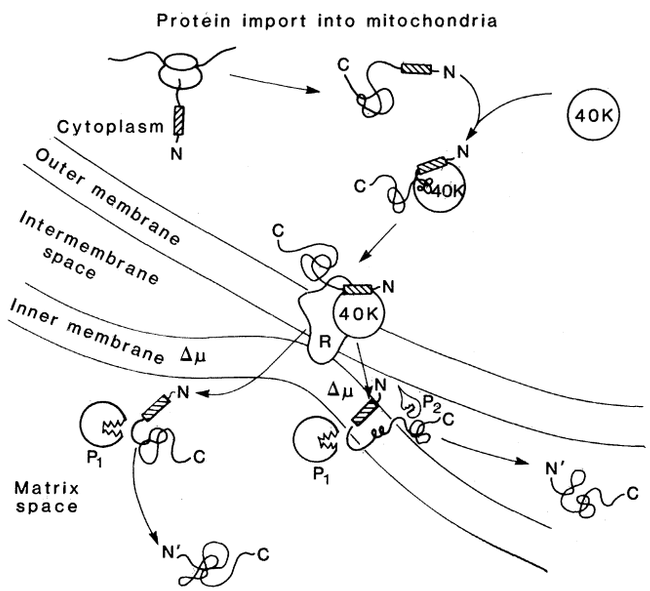
All known proteins of the periplasm and outer membrane, and at least several of the inner membrane, are made with NH_2-terminal leader (signal) sequences (*66*). The majority of inner membrane proteins are made without a cleaved leader sequence (*67, 68*). Bacterial leader sequences are processed by a membrane-bound leader peptidase whose active site is on the periplasmic face of the plasma membrane (*68*) and whose substrate specificity is identical to that of the RER enzyme (*69*). However, unlike the RER, efficient bacterial protein export requires the membrane electrochemical potential (*70*). In this regard, bacterial export resembles the mitochondrial import process. Bacteria and mitochondria are also similar in the relative timing of protein synthesis and protein translocation across a membrane.

There has, until recently, been some confusion over whether bacterial proteins must begin crossing the plasma membrane early in their synthesis in order to be exported, as is seen in the RER, or whether their export is not coupled to polypeptide chain growth, similar to posttranslational import by mitochondria. Much of this confusion is semantic. "Cotranslational" refers to any event that occurs before the end of translation. However, it has been confused with an obligate coupled extrusion of the nascent chain through the bilayer as it emerges from the ribosome, as occurs in the eukaryotic RER. Such extrusion is apparently not seen in bacterial protein export, yet there are important cotranslational events, such as folding and interaction with *sec*-encoded proteins, which are necessary for later translocation.

Bacterial membrane fractions are enriched in polysomes coding for certain pre-secretory proteins (*71*). Davis and co-workers found that nascent chains of alkaline phosphatase could be labeled by a membrane-impermeant radioactive reagent added to intact cells or spheroplasts (*72*). M13 procoat, synthesized in a cell-free reaction (*73*), was shown to assemble efficiently into plasma membrane vesicles present during the synthesis of the protein (*74*). However, other experiments have shown that bacterial protein export normally does not occur early in the growth of the polypeptide chain. Ito *et al.* (*75*) observed that the pulse-labeling of the periplasmic or outer membrane proteins in intact cells was dramatically delayed relative to the labeling of cytoplasmic or inner membrane proteins. The export of β-lactamase to the periplasm is entirely posttranslational (*76*), as is the insertion of M13 procoat protein into the plasma membrane in vivo (*77*). With the discovery that a membrane potential is needed for export, it was possible to experimentally separate the synthesis of both pro-OmpA and M13 procoat protein from their translocation (*70*).

These apparently contradictory observations, membrane-bound polysomes and membrane-spanning nascent chains on the one hand and two-step export on the other, were resolved by the experiments of Randall (*78*). She demonstrated that nascent chains grow to at least 80 percent of their final size before they begin translocation through the plasma membrane. This critical chain length is different for each exported protein. Translocation does not then begin synchronously at the critical molecular weight, but, instead, a stochastic "race" ensues between polypeptide chain growth and protein translocation. Thus ribose-binding protein and pre-β-lactamase are exported entirely posttranslationally, while only a fraction of the other protein species are exported cotranslationally. The remainder are completed as full-length pre-proteins within the cell, then are translocated entirely posttranslationally (*79*). These data are consistent with membrane-bound polysomes and with membrane-spanning nascent chains, yet clearly show that translocation is not strictly coupled to polypeptide chain elongation in bacteria.

Our current concept of protein export in bacteria is shown in Fig. 5. As with uptake of proteins into mitochondria, more than one receptor system targets different proteins to the bacterial plasma membrane. Mutations in any of several *sec* genes abolish export of a number of inner membrane, periplasmic, and outer membrane proteins but do not affect export of other proteins to these compartments (*80*). Since nascent pre-secretory proteins are not extruded through the bacterial membrane as they emerge from the ribosome, as in the RER, the *sec* proteins may serve functions distinct from those of SRP and DP. The *sec*



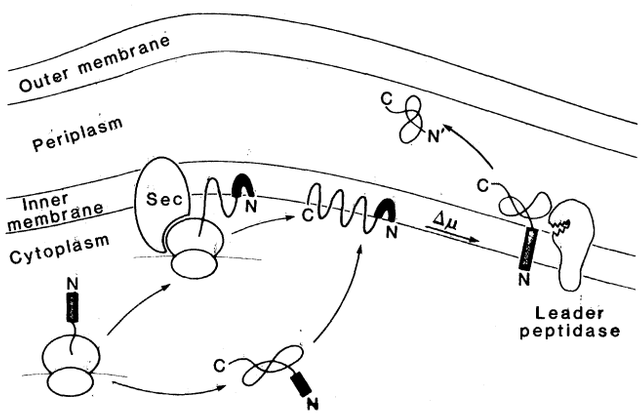


Fig. 4 (left). Protein import into mitochondria. The import of a matrix protein and an intermembrane protein with cleaved leader sequences is depicted. The function of the cytoplasmic import element, a 40-kilodalton protein (*60*), is not known. Proteins of the intermembrane space may partially insert across the inner membrane and undergo two-step proteolysis. R represents the outer membrane receptors for pre-mitochondrial proteins. P_1 and P_2 are proteases that remove the mitochondrial leader peptide. Fig. 5 (right). Assembly of the bacterial cell surface. The leader peptide is indicated by a hatched rectangle. As discussed in the text, many (but not all) pre-proteins require functional *sec* genes for export. Proteins reach critical molecular weight (*78*) or full length prior to beginning potential-dependent translocation.

proteins might stabilize certain pre-proteins until they can begin translocation. Alternatively, as illustrated in Fig. 5, they may bring specific proteins to the membrane early in their synthesis, analogously to SRP and DP. In bacteria, this may not lead to immediate translocation but may allow the polypeptide to grow at the interface between the aqueous cytoplasm and the apolar membrane.

Steps of Protein Translocation

Protein translocation in bacteria, mitochondria, and RER are summarized in Table 1. There are clearly no universal themes, or even completely consistent groupings of export themes. For example, bacterial pre-proteins have leader sequences like those of the endoplasmic reticulum but require a potential, as is seen for mitochondrial import. A new framework is needed for coherent organization of our knowledge of protein insertion into and across membranes. We suggest that the common features of protein translocation are its three necessary steps, the association of the protein with receptors on the correct membrane, the translocation through the membrane, and covalent modifications and folding on the opposite membrane surface. Individual proteins have evolved to use different combinations of the translocation themes to accomplish each step in export.

Protein binding to receptors on the correct membrane is essential to provide accurate protein sorting within the cell. In eukaryotic cells, recognition is mediated by specific soluble and organelle-bound elements. Bacteria, in which all exported proteins initially cross the same membrane, may only require sorting after translocation is complete. In addition to sorting, the binding step may stabilize pre-proteins against denaturation or possibly folding into a "dead-end" structure. This may be one function of the bacterial *sec* genes and of the mitochondrial cytoplasmic assembly component. This may even be viewed as a role of the SRP, which prevents elongation of the nascent chain unless DP is present. Receptors must facilitate translocation by either stabilizing the protein, catalyzing its refolding into a competent conformation, or transferring it to other elements that catalyze translocation.

Leader peptides of bacteria and RER (*12, 13*) average approximately 23 residues in length and have three characteristic domains. The NH_2-terminal domain is short (1–5 residues), basic, and polar. The central domain is nonpolar and contains a "core" of 4–8 strongly hydrophobic residues. Mutations that alter the charge at the NH_2-terminus (*81*) or introduce charged residues into the apolar domain (*82*) strongly inhibit protein export. The third domain begins with a helix-breaking residue (usually proline) and has small residues, characteristically glycine or alanine, at positions −3 and −1 relative to the cleavage site. These residues are not essential for translocation but serve as a leader peptidase recognition site (*76, 83*). Despite these conserved features, there is no true conservation of sequence.

Table 1. Characteristics of the three steps of protein insertion into membranes.

Assembly system	Recognition step			Translocation step		Maturation step	
	Signal sequence	Soluble receptor	Membrane receptor	Membrane potential	Translation-coupled	Proteolysis	Other
Endoplasmic reticulum	RER/bacterial type	Signal recognition particle	Docking protein	None	Yes	Signal peptidase	Core glycosylation
Mitochondria							
Outer membrane	None			None	No	No	None
Inner compartments	Distinct structure	40K protein	Distinct receptors in OM	Required	No	One- or two-step matrix protease	Addition of prosthetic group (heme)
Bacteria							
Inner membrane	Usually none	*secC**	*secY**	Required	No	Leader peptidase (ER-like)	
Outer compartments	RER-bacterial type	*secC**	*secY**	Required	No	Leader peptidase and lipoprotein signal peptidase	Coupling to peptidoglycan

*Sec gene function is required for approximately half of the exported proteins.

The leader peptide is clearly essential for export (*84, 85*). It is not clear whether the leader is sufficient to specify export, that is, whether the match between leader sequence and the mature protein is critical, or whether part of the information for export lies in the sequence of the rest of the protein. Despite the similarities of the leader sequences, the answers to this question may be different for bacterial protein export and for protein secretion into the lumen of the RER. In bacteria, the fact that proteins grow to 80 percent of their final molecular weight before beginning translocation across the plasma membrane (*78*) suggests that there is information late in the protein sequence that is needed for secretion. This idea is supported by genetic studies; a fusion protein of the *lam*B leader joined to β-galactosidase is not secreted across the plasma membrane (*86*), while a fusion product that contains virtually the entire *lam*B protein is efficiently secreted (*87*). A fusion of the β-lactamase leader sequence to a foreign cytoplasmic protein (*85*) produced a chimeric protein that also failed to be secreted. Lipoprotein was still secreted when its leader sequence was replaced by that of OmpF (*88*); however, replacement of the rat pre-proinsulin leader sequence with the leader sequence from pre–β-lactamase inhibited its secretion from *E. coli* (*89*). Mutations have been described in the mature portions of bacterial prolipoprotein (*90*), M13 procoat (*91*), and pre-maltose binding protein (*92*) that affect their export. On balance, it seems that the leader sequence is necessary, but not sufficient, for bacterial protein export.

In contrast, Yost *et al.* (*23*) have shown that the leader sequence of pre–β-lactamase, fused to the membrane-spanning segment of IgM and the COOH-terminal portion of globin, will direct the insertion of the hybrid protein into dog pancreas microsomes. A chimeric protein consisting of the NH_2-terminus of IgM heavy chain or of the asialoglycoprotein receptor (containing the internal

signal) and the COOH-terminus of globin is completely translocated across the endoplasmic reticulum (*23*, *34*). This suggests that insertion into the RER does not require information from the mature protein sequence. This difference between bacterial secretion and that in the RER is in accord with the differences in coupling between translation and translocation in these two systems.

The translocation step is not as well understood. Eukaryotic ribosomes appear to form a very tight junction with the RER membrane, and pre-secretory proteins may never contact the cytoplasm or fold prior to translocation. Thus, translocation of secretory proteins may be insensitive to the exact sequence of the polypeptide. In contrast, stop-transfer and insertion sequences are important information in the mature region of membrane proteins. Proper folding of soluble, secreted proteins that are synthesized on the RER may be essential for completion of translocation. Immediately after completion of synthesis, ribosomes release the nascent chain and dissociate into subunits. This leaves the COOH-terminal 25–35 amino acids (those formerly embedded in the large ribosomal subunit) exposed on the cytosolic face, and approximately 20 amino acids spanning the endoplasmic reticulum membrane. Since translocation of these last 45–55 residues cannot be coupled to chain elongation, some other process must provide a driving force. This could be the folding of the rest of the chain on the lumenal side of the membrane. This may be responsible for the SRP-independent translocation of short proteins such as the 70-residue precursor of bee venom mellitin (*93*) across microsomal membranes. The translation of this protein is virtually complete by the time the entire signal sequence has emerged from the ribosome; its assembly into the microsome must therefore be essentially posttranslational.

What are the energetics of translocation and what is the role of the electrical potential? While answers to these questions must await further experiments, several facts are noteworthy. Bacterial pre-proteins are exported in a direction from the negative to positive with respect to the transmembrane electrochemical potential, while the opposite is true for the import of mitochondrial pre-proteins. If the mechanism underlying these requirements is the same, then it becomes difficult to envision a simple electrophoretic model. A mutant of M13 procoat has been described which, while unaltered in net charge in the translocated region, displays dramatically less dependence on the electrochemical potential for export (*94*). Bakker and Randall (*95*) showed that the chemical portion of the potential can substitute for the electrical component in driving bacterial export. This also casts doubt on simple electrophoretic models. Other possible roles for the potential include affecting the lipid structure, governing the concentration of other critical solutes, or even driving a protein-proton transport system. The well-studied voltage-dependent translocation of diphtheria toxin (*96*), mellitin (*97*), and asialoglycoprotein receptor (*98*) across lipid bilayers may be analogous to the translocation of pre-proteins; translocation of premitochondrial proteins and bacterial pre-secretory proteins need to be assayed in these systems.

When translated in a cell-free extract, the human erythrocyte glucose transporter can insert into the endoplasmic reticulum entirely posttranslationally (*99*). This glycoprotein probably spans the membrane as 12 α-helixes (*100*) and bears a single *N*-linked oligosaccharide. The observation that its insertion into the endoplasmic reticulum membrane and its glycosylation requires SRP, but not concommitant translation, indicates that the binding and translocation steps need not be obligatorily coupled even in the RER. Its insertion thus resembles that of bacterial membrane proteins, except that there is no obvious requirement for a membrane potential.

The maturation step, which follows translocation, may be essential to the operation of additional sorting steps. Import of apocytochrome c across the mitochondrial outer membrane requires heme addition in the intermembrane space. When heme addition is blocked, the apocytochrome c remains bound on the outer mitochondrial surface (*55*). Explanations other than the reversibility of the insertion steps in the absence of maturation are, of course possible. For example, proteins catalyzing translocation might also need to donate pre-proteins to the appropriate maturation enzyme in order to catalyze another translocation event.

Prospectus

Further progress will depend on: (i) genetic and in vivo studies to define the physiological pathways and provide strains that are optimal for biochemical analysis, (ii) development of specific drugs to interrupt the pathway, reveal intermediates, and assist studies of enzymology, and (iii) analysis of cell-free reactions that are amenable to fractionation and reconstitution from their purified components. Bacterial export has benefited from intensive genetic study, while this approach is only beginning (in yeast) to be used in investigations of translocation into the RER and for mitochondrial biogenesis. Cloning has allowed the isolation of substantial quantities of the bacterial leader peptidase (*101*); it will allow preparation of large quantities of other catalysts of protein translocation in the near future. Mutants have been isolated to test the functions of different domains of pre-secretory and mitochondrial proteins. Pulse-labeling of a microorganism such as yeast may allow detection of predicted complexes, such as cytoplasmic SRP-polysomes, and may reveal new intermediates.

Cell-free translocation reactions (*102*) may provide assays for the products of (*sec*) and protein localization (*prl*) genes (*64*) and allow the study of the role of the electrical potential. Submitochondrial translocation across isolated inner or outer membrane has not yet been reported, nor has a soluble detergent extract of RER been reconstituted to yield a translocation-competent liposome. Each of these cell-free reactions is the focus of intensive research and will provide further insights into the molecular mechanisms of translocation.

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