PROTEIN TRANSLOCATION ACROSS AND INTEGRATION INTO MEMBRANES

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I. INTRODUCTION

A. Directing Proteins to Different Sites in a Cell

An eukaryotic cell is divided into several distinct compartments by the presence of intracellular membranes. The organelles surrounded by these membranes perform specialized functions characterized by specific sets of proteins. A given polypeptide is generally found exclusively in only one cell compartment. The diverse, yet highly specific, final localization of proteins contrasts with the common site of their synthesis. With the exception of a few mitochondrially coded polypeptides, in an animal cell they all are synthesized in the cytoplasm, be it in membrane-bound or free polyribosomes. What sort of signals direct a polypeptide from the site of synthesis to its ultimate destination? Closely connected with this problem is the question concerning the mechanism of transport. In many cases, polypeptides have to traverse at least one membrane that is normally impermeable for such molecules. Thus, there must be mechanisms for the selective and vectorial translocation of proteins across membranes. Membrane proteins are not only found in different cell compartments but, in addition, have various orientations in a given membrane. They may span a phospholipid bilayer once or several times, can have their N-terminus on one or the other side of the membrane, or just be imbedded from either side. A given polypeptide chain has a defined and characteristic orientation in the membrane, and again one may ask, how this specific incorporation is coded for and brought about.

B. Translocation-Competent Membranes

The faculty of transporting proteins is limited to a few membranes in a cell; all other membranes receive their membrane-spanning polypeptides from these translocation-competent ones by either lateral diffusion or by vesicle budding and vesicle fusion. Most proteins do not change their orientation within the membrane after their initial incorporation into the phospholipid bilayer. For example, the extracellular part of a eukaryotic plasma membrane protein is initially found in the lumen of the rough endoplasmic reticulum (RER) which is topologically equivalent to the exterior of the cell. A notable exception appears to be the 5′-nucleotidase, which apparently changes its orientation in the membrane on its way from the RER to secretion vacuoles.[1,2] This interesting case certainly deserves further study.

In an eukaryotic cell, the main organelle which transports proteins across membranes is the RER. Secretory proteins, lysosomal enzymes, and membrane proteins of the ER and of the plasma membrane, to name just a few groups for which many examples are known, are all transferred at least in part across the RER. A second site of translocation of proteins is found in mitochondria. Most likely, the peroxisomal membrane has also such a competence, but little is known about details.[3,3a] In plant cells, the chloroplasts import many proteins

Table 1
SOME EXAMPLES OF HOW THE PRESENCE OR ABSENCE OF SIGNALS COULD EXPLAIN THE LOCALIZATION OF PROTEINS IN A CELL

Location of the protein	Signal for translocation	Signal for specific membrane incorporation	Signal for sorting
Cytoplasm	No	No	No
Exterior of the cell (secreted)	Yes (RER)	No	No
Lysosomal enzymes	Yes (RER)	No	Yes
ER membrane	Yes (RER)§	Yes	Yes (?)
Plasma membrane	Yes (RER)	Yes	No (?)
Mitochondrial matrix	Yes (mit.)&	No	No
Inner membrane of mitochondria	Yes (mit.)&	Yes	No
Intermembrane space of mitochondria	Yes (mit.)	Yes	Yes+

Note: § = some protein; & = the signals for import need not be the same for all proteins; + = some proteins; RER = rough endoplasmic reticulum; mit. = mitochondria.

from the cytoplasm. The general mechanism of protein import may be similar to that of mitochondria,[4] but discrimination between proteins destined to the two organelles must somehow occur. In bacteria, the cytoplasmic membrane is the predominant site of protein translocation,[5] but some proteins are able to cross both membranes in Gram-negative bacteria.[6-6b]

The present review is focused on those translocation systems of proteins which are best known: the RER membrane, the cytoplasmic membrane in bacteria, and the mitochondrial membranes.

C. Topogenic Signals — Contiguous Amino Acid Sequences or Conformational Domains?

It appears that, in general, each polypeptide has its own signal(s) which determine(s) its destination. It is, however, possible that in certain cases, cell polarity, spatial sequestration of mRNA, or "helper"-polypeptides guiding another protein which does not have its own signal is responsible for the sorting of proteins.

Theoretically, a protein would need up to three signals to reach its destination in a cell:

1. A signal directing the polypeptide to a translocation competent membrane; such a signal should distinguish, for example, between a cytoplasmic, mitochondrial, or secretory protein (see Table 1).
2. Signal(s) for the defined incorporation of a membrane protein into the phospholipid bilayer.
3. Sorting signal(s) for further transport to receiver organelles. It is possible that a fourth type of signal is required to keep a protein at a certain site. Of course, some of the different signals may coincide.

Table 1 shows that, assuming the presence of these signals, many different locations of proteins could be explained.

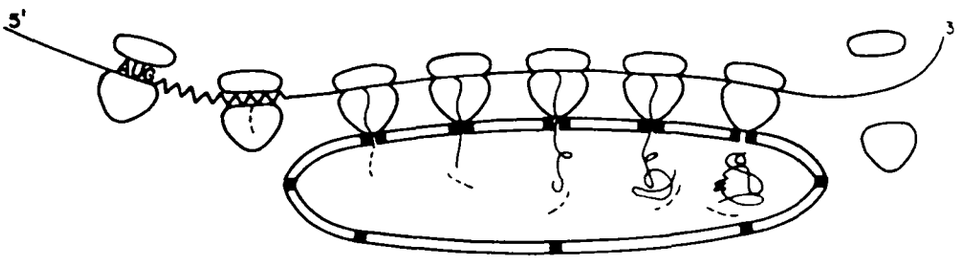


FIGURE 1. Schematic representation of the signal hypothesis. Codons after the initiation codon AUG which code for the signal peptide are indicated by a zig-zag region in the mRNA. The signal sequence is indicated by a dashed line. Proteolytic cleavage is indicated by the presence of short dashed lines within the intracisternal space. For details see text. (From Blobel, G. and Dobberstein, B., *J. Cell Biol.*, 67, 852, 1975. With permission.)

What is the nature of these topogenic signals? Ultimately, the signal(s) must be coded for in the gene of the corresponding polypeptide, and it appears that decoding only occurs at the protein level. Recognition of certain features of the mRNA has been claimed in old papers.[7-10] Recently, Richter et al.[10a] presented preliminary evidence that specific factors recognize classes of mRNA before initiation of translation in *Xenopus* oocytes. In general, however, it appears to be the polypeptide which contains the information for its final destination.

The topogenic determinant in a protein could either be segments of the polypeptide chain, so called "topogenic amino acid sequences",[11] or "topogenic sites" only expressed after folding into a tertiary structure. In fact, much evidence exists in favor of peptide segments directing proteins across the RER membrane or across the cytoplasmic membrane in bacteria (see Section II.D). On the other hand, it is conceivable that some signals require the interaction of amino acid residues distant in a polypeptide chain.

It should be kept in mind that the direct recognition marker may not always reside in the amino acid sequence. For example, lysosomal enzymes are sorted to the lysosome in fibroblasts by attachment of mannose-6-phosphate residues (see Reference 12). Of course, the information for this modification must somehow be contained in a specific amino acid sequence.

For the actual process of protein translocation across membranes, which this review concentrates on, covalent modification of proteins is not likely to play a decisive role. The attachment of carbohydrate[13] or fatty acid[14,15] chains occurs after translocation of at least part of the polypeptide chain across the phospholipid bilayer, and the membrane transfer proceeds normally if the modification event is blocked.[16]

II. TRANSLOCATION OF PROTEINS ACROSS THE RER MEMBRANE AND ACROSS THE CYTOPLASMIC MEMBRANE IN BACTERIA

A. The Biosynthesis of Secretory Proteins in Eukaryotes

1. Basic Facts Supporting the Signal Hypothesis

The signal hypothesis was formulated by Blobel and Dobberstein[17,18] to explain in molecular terms the basic observation that eukaryotic secretory proteins, in contrast to cytoplasmic ones, are synthesized in polyribosomes bound to the RER membrane and transported across the phospholipid bilayer in a cotranslational manner.[19-22] It was postulated that synthesis of a secretory protein starts with a free ribosome (Figure 1). A signal peptide located at the N-terminus of the growing polypeptide chain is recognized by a receptor in the RER membrane as soon as it emerges from the ribosome and triggers membrane binding. The nascent secretory polypeptide would then be transferred across the phospholipid bilayer

through a tunnel formed by the transient association of transmembrane proteins. Chain elongation would be strictly coupled to translocation. During or shortly after completion of the protein, the signal peptide is cleaved off by an enzyme called signal peptidase which is located at the luminal side of the RER membrane. When the ribosome reaches the stop codon on the mRNA, the ribosome is released from the membrane and the pore in the membrane disappears.

This scheme of events is supported by the following observations:

1. Cell-free translation of mRNA coding for immunoglobulin light chain yielded a product which was some 20 amino acid residues longer at the N-terminus than the mature polypeptide.[23,24] Similar extensions were found subsequently for many other secretory proteins and the precursors were called "preproteins" (some bacterial precursors are called "proproteins"). Contrary to the original expectation,[25] a common amino acid sequence for the cleaved-off peptide was not observed even for related proteins or proteins from the same cell.[26]
2. When rough microsomes (isolated from dog pancreas) were present during cell-free translation, the mature immunoglobulin light chain was synthesized and the product was inside the membrane vesicles as judged from its protection against the action of added proteases. Degradation occurred if detergents were also added to disrupt the membranes.[17,18] If the membranes were added after translation, no translocation or cleavage of the precursor polypeptides occurred. This result cannot be taken as a direct proof for an obligatory cotranslational mode of protein transport (see Reference 27). However, in later synchronized translation experiments, it was found that microsomes can only be added up to the point where about 70 to 100 amino acid residues are synthesized in order for translocation to occur; later addition only yields the nontranslocated precursor.[28,29] Cleavage in vitro of the precursor polypeptides by the signal peptidase was at the correct site. These data indicated the successful in vitro reconstitution of the translocation process. Szczesna and Boime[30] confirmed these observations by the use of ascites tumor microsomes.
3. When polysomes (isolated from myeloma cells and detached from the membranes by detergent treatment) were allowed to complete their nascent chains in vitro, mature light chains were found after short incubation times, and after longer times, precursors were found. This was consistent with the idea that ribosomes close to the 5′ end of the mRNA carry nascent chains not yet cleaved by the signal peptidase, whereas those towards the 3′ end carry processed nascent chains.[18] This experiment proved not only the cotranslational cleavage of the signal peptide, but also that the synthesis of precursors is not an in vitro artifact.
4. Coupling of translocation and elongation of the polypeptide chain is indicated by the fact that nascent polypeptide chains are protected against proteolytic attack when they are longer than about 50 residues.[31] Furthermore, in synchronized translation experiments, it was found that core glycosylation at Asn-residues, which occurs in the lumen of the RER, proceeds in vitro at the time expected from the extent of elongation of the polypeptide chain;[28-29a] this argues for a residue-by-residue transfer. Also, the disulfide-bridge formation in the immunoglobulin light chain appears to occur at a time point consistent with a rather extended conformation of the nascent polypeptide during passage through the membrane.[32]
5. Cleavage of the precursor polypeptides could be carried out in vitro in the absence of protein synthesis by disrupting microsomal vesicles with suitable detergents.[33,34]
6. The postulated time-dependent association of the ribosomes with the RER membrane was proved directly.[180] Also, ovalbumin mRNA became membrane bound only after some time of synthesis.[31]

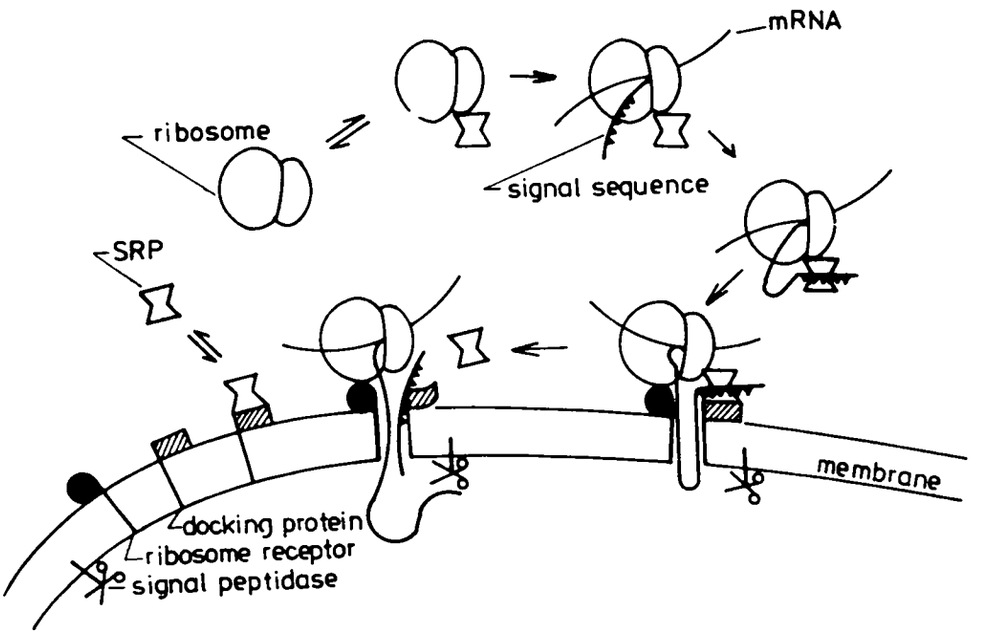


FIGURE 2. Schematic representation of the present view on the mechanism of protein transfer across the endoplasmic reticulum membrane. The scheme shows the early steps in the biosynthesis of a secretory protein. According to the loop model (Section II.F.3), it was assumed that the nascent polypeptide chain crosses the membrane with a hairpin structure. The existence of an aqueous tunnel in the membrane through which the polypeptide is transported is also assumed (see Sections II.F.4 and II.G). The involvement of ribosome receptor(s) is also largely hypothetical as yet. For details see text. (Modified after Walter, P. and Blobel, G., *J. Cell Biol.*, 91, 557, 1981.)

7. The demonstration of circulation of ribosomes between a free and membrane-bound state,[35] as well as the fact that cytoplasmic and secretory proteins are equally translated in cell-free systems and that only the addition of microsomes is required for translocation of the latter, indicates that ribosomes per se are not specialized in the synthesis of certain classes of proteins.

In the next section we shall discuss a current state model. Some parts of the original scheme have to be changed and others can be detailed (Figure 2). Still other predictions, including the transient formation of a tunnel, are under dispute as yet (see Section II.F.4). However, the signal hypothesis has been a stimulating guideline for many experiments and its general ideas remain valid.

2. An Updated Scheme of Translocation Across the RER Membrane

Figure 2 summarizes the present view of the translocation of proteins across the RER membrane.[36,37]

The process starts as in the older scheme (Figure 1) with a free ribosome initiating translation at the 5′ end of the mRNA. As soon as the signal peptide has completely emerged from the ribosome, it is recognized by a ribonucleoprotein particle, named signal recognition particle (SRP). SRP can be either free in solution or already bound to the ribosome before binding to the signal peptide. The elongation of the polypeptide chain is arrested, i.e., translation stops. The complex is then bound via SRP to its receptor (docking protein) present in the RER membrane. Simultaneously, the ribosome becomes membrane bound and SRP

is released from the ribosome. This completes the initiation of the translocation process; SRP can now bind to another signal peptide and begin a new cycle. The actual translocation proceeds without participation of SRP or its receptor. Details of this part of the process are as unclear as before (Figure 1). After reaching the termination codon, the ribosome falls off from the membrane, and thus a ribosome cycle is completed. For the last step, a protein factor may be required.[38] The signal peptide is cleaved off by the signal peptidase at the luminal side of the membrane and the peptide is degraded rapidly by peptidases. The final tertiary structure of the protein is probably achieved after removal of the signal peptide.[39]

We shall discuss the various aspects of this scheme in more detail in the following sections. However, one point should be mentioned here: the mechanism proposed assumes that each ribosome needs SRP to direct it to the membrane. It has not been excluded, however, that SRP is only required once to direct the mRNA to the RER membrane. In this case, only the first ribosome translating the mRNA would require SRP, but further ribosomes starting translation on the already membrane-bound mRNA may not need SRP for initiation of translocation.

B. Bacteria May Not Be Too Different from Mammals

Prokaryotic signal peptides were discovered much later than eukaryotic ones.[40,41] The first example was the major outer membrane lipoprotein, which was shown to have a cleaved-off N-terminal signal peptide. Signal peptides were subsequently found for the majority of exported proteins, which in Gram-negative bacteria include polypeptides of the cytoplasmic membrane, periplasm, and outer membrane. Rare cases of secretion across two membranes have also been reported.[6-6b] In Gram-positive bacteria, which lack an outer membrane, proteins are secreted directly into the medium.

It appears that the processes of translocation across the RER membrane and the cytoplasmic membrane in bacteria are basically similar. An *a priori* justification for our attempt to discuss both systems together is provided by the observation that rat proinsulin is secreted into the periplasm of *E. coli* with a prokaryotic or eukaryotic signal peptide,[42,43] and similar results have been obtained for ovalbumin[44-46] and carp proinsulin.[47] Although the efficiency of secretion appears to be low in these cases, and there are even examples where the mature eukaryotic proteins could not be detected in the periplasm (fibroblast interferon[48] and rat growth hormone[49]), it is evident that eukaryotic signal peptides may function in bacteria. The reverse has also been shown: the signal peptide of β-lactamase directs the prokaryotic protein across the RER membrane in vitro and in vivo.[50,51] Based on the ubiquitous recognition of signal peptides, we shall discuss similarities and differences of the translocation systems in more detail in the following sections.

C. Incorporation of Proteins into Membranes

1. Initiation of Translocation Appears Identical for Membrane and Secretory Proteins

Although originally proposed for eukaryotic secretory proteins, it is now clear that some membrane proteins are transported in a similar manner as suggested by the signal hypothesis. Many membrane proteins are synthesized in membrane-bound polyribosomes, and plasma membrane proteins have an overall pathway similar to that of eukaryotic secretory proteins (see References 52 to 54).

The first direct evidence for a signal peptide directing membrane proteins across the RER membrane came from studies on the G-protein of the vesicular stomatitis virus (VSV).[28,29,55,56] This protein is originally inserted into the RER membrane before being transported to the plasma membrane of the infected cell. It was shown to have an extension at its N-terminus by 16 amino acids when synthesized in vitro. If dog pancreatic membranes were present, the signal peptide was cleaved off and most of the protein was protected against added proteases with the exception of about 30 amino acid residues at the C-terminus which were

accessible. This is, in fact, the orientation and arrangement of the mature G-protein, which is known to span the membrane once with a sequence close to its C-terminus.[28,57] The identity of the cleaved-off peptide with a signal peptide of a secretory protein was shown by competition in vitro for segregation into microsomes under conditions in which there was no limitation of translation of the two mRNAs.[56]

Nature itself has provided the most convincing proof that signal peptides directing secretion and membrane-incorporation are functionally the same. Secreted and membrane-bound IgM are synthesized from mRNAs derived from the same gene by differential splicing.[58,59] The coded amino acid sequence differs at the C-terminus, but the signal peptide for both proteins is the same.

Similar conclusions could be drawn from genetic manipulations of viral membrane proteins. If the membrane-spanning polypeptide segment was removed from the hemagglutinin of the influenza virus[60,61] or from the G-protein of the VSV,[62] the shortened proteins were secreted rather than membrane incorporated. If, on the other hand, the N-terminal signal sequence was removed, neither secretion nor membrane insertion occurred.

These data show that at least some parts of the translocation of the polypeptides are the same for secretory and some membrane proteins. The examples also demonstrate that information contained in the C-terminus of these membrane proteins prevents their secretion. It therefore appears that initiation of translocation and final membrane anchoring are consecutive events and independent of each other (see also References 56, 63, and 64).

Since initiation of translocation is similar for membrane and secretory proteins, we shall deal with them jointly. Further evidence for the similarity of the processes in molecular details is discussed in Section II.D through F.

2. Different Types of Membrane Proteins

a. Imbedding or Translocation?

As mentioned before, many integral membrane proteins require signal peptides for transfer of large structural domains to be exposed to the aqueous phase on the transverse side.

There exists a second class of integral membrane proteins which have large structural domains deeply incorporated into the phospholipid bilayer, with only small parts in the aqueous phase at the ectoplasmic side. This class includes many transport and channel-forming proteins. The structure of these proteins is poorly known (see next section), but they may span the membrane several times. Although not immediately obvious, these proteins appear to need signal peptides and use a translocation machinery for membrane insertion, as is the case for the simple membrane-spanning proteins. It is conceivable, however, that some parts of the polypeptide chain are only imbedded in the membrane (see below).

There is a third class of integral membrane proteins which do not need a signal peptide for their membrane incorporation. They have specialized "imbedding" domains (or sequences) which interact with the phospholipid bilayer and allow their membrane insertion without need of a translocation apparatus. It is likely that proteins of this class do not have major folded domains in the ectoplasmic aqueous phase. Nevertheless, they could conceivably span the membrane.

Of course, both translocation and imbedding could occur in a single polypeptide chain.[11] It may also be possible that a protein is first completely transferred across a membrane and then imbedded from the ectoplasmic side into the bilayer.

How can one distinguish between the two modes of membrane incorporation — translocation and imbedding? The best criterion for translocation of a protein across the RER membrane is certainly the requirement of SRP for its membrane incorporation. For example, cytochrome P-450, a protein of the ER membrane, is incorporated into microsomes only in the presence of SRP.[65] By contrast, cytochrome b_5, another protein of the ER membrane, is imbedded into natural and artificial membranes in the absence of SRP.[66-68]

Indeed, cytochrome b_5 is the best known example of an imbedded protein. The data already mentioned indicate that cytochrome b_5 does not carry a signal peptide that would trigger translocation. Furthermore, cytochrome b_5 is made without a precursor, synthesized in free polyribosomes, and appears to be post-translationally incorporated into membranes.[69,70] Its N-terminus is hydrophilic and stays in the cytoplasm, whereas a C-terminal domain of 35 mostly hydrophobic amino acids serves as membrane anchor.[66,71,72] In Strittmatter's laboratory, evidence was accumulated that indicated no part of the membrane-incorporated C-terminus reaches the other side of the bilayer (see Reference 73). Although the C-terminus of cytochrome b_5 resembles signal peptides in hydrophobicity, it does not compete with them even in a 1000-fold molar excess.[67] This result further supports the assumption that cytochrome b_5 does not carry a C-terminal signal peptide which, because of its location in the protein chain, could not support the translocation of the protein across the membrane. Rather, the C-terminus appears to interact directly with the phospholipid bilayer and may therefore be called an "imbedding" sequence.

Based on the information on the structural features of cytochrome b_5, we have tentatively proposed that for imbedding a protein must have a separately folded domain which is highly structured and has a hydrophobic surface.[67] If such a globular structure approaches a phospholipid bilayer, the latter may be perturbed, allowing the spontaneous incorporation without mediation of a membrane protein. Such a model would predict that an imbedded protein is found on the cytoplasmic face of all cellular membranes. Indeed, cytochrome b_5 has been found in different compartments.[74] However, its concentration in the smooth ER membrane is much higher than anywhere else, and a cytochrome species with a different amino acid sequence has been found in mitochondria.[75] One feels therefore inclined to invoke further factors determining the concentration of a protein in a membrane, such as transmembrane (translocated) proteins with binding affinity for cytochrome b5 or differences in the accessible lipid domains.

How general is the case of cytochrome b_5? Cytochrome b_5 reductase may be a second example of an imbedded protein in eukaryotes.[76-78] Other examples are the two main membrane proteins synthesized by rabbit reticulocytes, which are synthesized in free polyribosomes and incorporated into the plasma membrane without reaching the outside of the cell.[79] Most inner membrane proteins of *E. coli* are made without precursor. Immunological studies show that most of the proteins of the inner membrane are only exposed on the cytoplasmic face (see Reference 80). These proteins are therefore candidates for imbedded polypeptides.

b. Structure of Membrane-Spanning Domains

It is appropriate to distinguish between simple membrane segments which span a membrane once and more complex structures which involve extensive intramolecular protein-protein interactions typical for tertiary structures within the phospholipid bilayer.

The amino acid sequences of simple membrane segments have been determined for many proteins (for a rather comprehensive list, see von Heijne[81]). All these segments consist of a long hydrophobic amino acid sequence (about 20 residues) flanked by charged residues. If the membrane segment is at the C-terminus of a protein, the charged residues staying in the cytoplasm are, in general, basic. Charged amino acid residues and amide groups (Gln and Asn) are excluded from the hydrophobic domain except at its boundaries, where they sometimes occur but might reach the aqueous phase. These features indicate that complete intrachain saturation of all H-bonds of the protein segments within the membrane prevails. It is therefore likely that these simple membrane-spanning segments adopt a helical, presumably alpha-helical structure. Indeed, about 20 residues would be required to span a phospholipid bilayer in an alpha-helical conformation. The rarity of Pro-residues in the peptides may also be taken as supporting evidence for this assumption.

Things might be different for complex membrane proteins, although structural information is scarce as yet. Bacteriorhodopsin, the best known example so far, is believed to be composed of seven helical rods traversing the membrane almost perpendicularly[82,83] (see, however, Jap et al.[84] for an alternative view). Although the amino acid sequence[85,86] can be fitted into the structural model so that most charged amino acid residues are located in the links between the helixes in the aqueous phases to both sides of the membrane, there are still charged residues which must be buried in the bilayer. It has been postulated that these helixes form a channel with the charged residues, oriented toward its interior away from contact with the lipid.[83] The nonpolar amino acid residues would be directed outward to the surrounding hydrophobic environment. Such a structure has been called "inside-out" since it is the reverse of what is typical for soluble proteins.

A completely different structure is evolving for other complex membrane proteins. The channel-forming proteins phoE and ompF of the outer membrane of *E. coli* do not possess a hydrophobic amino acid sequence longer than 5 residues in their mature chains and are nevertheless deeply incorporated in the bilayer. The pore-forming proteins appear to have almost entirely beta-structures.[87-89] These data indicate that folded domains within the phospholipid bilayer may have different structures. Of course, very serious constraints are exerted by the hydrophobic environment: shielding of charged residues and saturation of H-bonds are likely to be basic principles in the structure building of membrane proteins. Conceivably, however, these requirements are met in part by association of different polypeptide chains.

It is obvious from this short discussion that many more structures of complex membrane proteins have to be known before generalizations can be made. The progress in the crystallization of membrane proteins will hopefully permit the more widespread application of the powerful X-ray-, neutron-, and electron-diffraction methods.[90]

Do membrane proteins assume the thermodynamically most stable state as generally accepted for soluble globular proteins? As far as the limited knowledge on the structure of membrane-incorporated domains goes, the answer is affirmatory. Furthermore, denaturation-renaturation experiments carried out for bacteriorhodopsin also support this conclusion.[91] However, it should be kept in mind that the asymmetrical arrangement of a protein in the membrane may be kinetically rather than thermodynamically determined: if both sides of the membrane were absolutely symmetrical (including the aqueous compartments) it would be thermodynamically the same if the orientation of a protein were reversed. The fact that the natural orientation of polypeptides can be reproduced faithfully by in vitro translation experiments in the presence of microsomal vesicles which are leaky to small molecules,[92] certainly argues in favor of a kinetic model determining the sidedness of protein incorporation.

Consequences of these structural considerations for models on the biosynthesis of integral membrane proteins will be discussed in Section II.G.

3. The Membrane-Trigger Hypothesis — An Alternative to the Signal Hypothesis?

Whereas the signal hypothesis implicates a translocation system of growing complexity, Wickner[93] proposed in his "membrane-trigger hypothesis" that a soluble precursor protein could spontaneously traverse a phospholipid bilayer by a conformational change, and that the signal peptide would merely alter the folding pathway of a protein. The model is based mainly on results from Wickner's laboratory on the M13 coat protein, a small polypeptide (50 amino acid residues) of the inner membrane of infected *E. coli* cells with its C-terminus spanning the phospholipid bilayer.[80] Experiments in vivo indicated that the pulse-labeled procoat protein (the precursor with a signal peptide at the N-terminus) was soluble in the cytoplasm and could be chased into the transmembrane coat protein.[94-97] A membrane potential was required for the insertion of the procoat protein into the bilayer (see Section II.F.1). Membrane insertion of the coat protein could be mimicked in vitro using purified components.[98-100] The signal peptidase was incorporated into liposomes, and enzyme located

outside the vesicles was proteolytically degraded. When procoat protein was added, it gained access to the internal signal peptidase and was cleaved to coat protein.[101,102] The protein appeared to be correctly inserted into the membrane. It remains obscure why in the in vitro reaction a membrane potential was not required for membrane incorporation of the coat protein. In fact, Russel and Model[103] have claimed that procoat protein is not soluble but represents an integral membrane protein in pulse-labeled intact cells, but the conclusion has been doubted.[80] It remains a possibility that procoat protein can spontaneously traverse the membrane, but that the process would be made more efficient in vivo by the help of a proteinaceous translocation machinery. It is also possible that the coat protein is an exception since it is inserted spontaneously into the target membrane during the infection of a cell with the M13 phage.[104] There is, however, evidence that the precursor of the *E. coli* ompA-protein may follow a similar pathway of membrane insertion.[104a] In any case, some steps involved in the procoat assembly pathway appear to be common to other exported proteins: the signal peptidase I is known to cleave several other precursors and a membrane potential is required for export of some proteins.[105,106] It would be interesting to know whether procoat protein accumulates in *E. coli* cells defective in secretion (e.g., sec mutants or in malE-lacZ and lamB-lacZ fusion strains, see Section II.D). Such experiments could perhaps clarify whether procoat protein really takes the same route as other exported proteins. In general, the involvement of protein factors other than the signal peptidase I in the translocation process of bacteria is beyond any doubt (see Section II.E).

D. Molecular Features of Signal Peptides

This section deals with the requirements within the polypeptide itself for translocation across the RER membrane or across the bacterial membrane. Section 2.E. is devoted to the components of the transporting apparatus.

1. Are Signal Peptides Necessary and Sufficient for Protein Export?

There is little doubt that signal peptides are absolutely required for protein translocation across the RER membrane or cytoplasmic membrane in bacteria.

If the nucleotide sequence coding for the signal peptide is removed, the shortened gene codes for a cytoplasmic protein both in eukaryotes and in prokaryotes.[42,60,107] Deletions in the signal peptides of bacterial proteins also block their export.[108-110]

Point mutations and deletions affecting the export of individual polypeptides in *E. coli* generally map in the signal peptide (see Table 3) (for an exception see below). Furthermore, if the signal peptide is modified by site-directed mutagenesis, some changes affect the translocation of the protein.[111,112] Of course, these results cannot be taken as direct proof for the role of signal peptides in protein transport.

Nature itself has provided an example which is more convincing. There are two forms of yeast invertase, a secreted one and a cytoplasmic one, which are coded by two different mRNAs originating from the same gene.[113,114] The only difference is that the signal peptide coding sequence is absent for the cytoplasmic protein, in accordance with its assumed role in protein translocation.

Actually, the most convincing proof for the essential role of a signal peptide would be if one could show that addition of such a peptide to the N-terminus of a protein normally located in the cytoplasm directs it across the membrane. Such an experiment has been carried out successfully by Lingappa et al.[107] These authors have constructed a plasmid which contained a globin gene preceded by nucleotides coding for a signal peptide. By use of in vitro transcription and translation, they showed that the globin polypeptide was entirely segregated into dog pancreatic microsomes. This result also indicates that secretory proteins need not require special properties to be compatible with the translocation system of the RER.

Similar experiments had been undertaken for β-galactosidase in the *E. coli* system, but with unexpected results. The work, which was originated in Beckwith's laboratory, dealt mainly with two proteins, the periplasmic maltose-binding (malE) protein and the lambda phage receptor (lamB) protein of the outer membrane.

Different portions of the N-termini of these proteins were fused with the β-galactosidase protein and they strongly influenced the location of the fusion proteins. The fusion polypeptides were divided into up to five classes, depending on the size of the exported protein in them and on their properties (see Table 2). Several conclusions could be drawn from these results:

1. No construction without signal peptide was exported (class I).
2. A signal peptide does not appear to be sufficient for protein export since fusion of a short piece of the lamB protein, including the entire signal sequence, with the β-galactosidase yielded a protein located exclusively in the cytoplasm (class II).[115]
3. It appears that protein translocation was in fact initiated in some cases even though the fusion product was not entirely transported to the expected site. Induction of the synthesis of some of these polypeptides severely inhibited the export of many other proteins[116] and evidence exists that the fusion products were located in the cytoplasmic membrane.[117] β-Galactosidase may in fact have several parts in its polypeptide chain which are difficult to translocate across a phospholipid bilayer.[118,119] One may therefore assume that secretory proteins in bacteria need a permissive information within their mature amino acid sequence.

Sequence parts within the mature exported proteins may influence the efficiency of the export and may even be decisive for the outer membrane localization of the lamB protein. The longer the portion of the exported protein in the fusion polypeptide, the more efficient is the process. For example, class III fusions of the malE and lacZ appear to be translocated much more slowly than class IV fusions (upper part of Table 2).[110a] Similarly, class II fusions of the lamB and lacZ are found in the cytoplasm, class III in the cytoplasmic membrane, and class IV in the (actually expected) outer membrane location.[120,121]

Recently, the segments of the polypeptides carrying this additional information have been identified more precisely. In the case of the lamB protein, an important sequence appears to be located within the first 39 residues of the mature protein.[122] A hybrid protein that includes an intact signal peptide and 27 residues of the mature lamB protein remains cytoplasmic, whereas a fusion product containing 39 residues of the mature protein is inserted into the inner membrane, at least partially. A second sequence appears to be located between residues 39 and 49 since a hybrid which includes the first 49 residues of the mature lamB protein is partially transported to the outer membrane with a similar efficiency as the original class III fusions (containing 173 residues of the mature part). The region between residue 39 and 49 corresponds to one which is homologous among various major outer membrane proteins.[123] It has therefore been termed the "outer membrane signal"[122] and can be considered as a sorting sequence. Finally, residues 235 to 240 of the mature lamB protein appear to be helpful since deletions comprising this region significantly reduce the efficiency of hybrid protein export.[124]

Even more convincing concerning the actual translocation process are results obtained for the maltose-binding protein. Signal peptide mutants of the malE protein (see next section) appear to interfere with the export of normal envelope proteins. This effect is not apparent in the growth properties of the cells, but can be seen in pulse-chase experiments where precursors were found to accumulate.[110a] Since the mutant proteins are not translocated by themselves, the interference must occur prior to translocation. It appears that the phenomenon

Table 2
PROPERTIES OF FUSION POLYPEPTIDES BETWEEN EXPORTED PROTEINS AND β-GALACTOSIDASE

Maltose-Binding Protein

Class	Portion of the exported protein	Localization of the fusion protein in the *E. coli* cell	Signal peptide cleavage	Rate of export	Mal^S-phenotype of the bacteria	Ref.
Wild type	SP (26 res.) + 370 res. mature protein	Periplasm	Yes	Co- and post-translational	—	117, 110a, 250, 381
I	14 res. of SP	Cytoplasm	No	—	No	
II	SP + 15 res. of mature protein	Inner membrane and cytoplasm	Yes	Post-translational	Weak	
III	SP + 23 res. of mature protein	Inner membrane (cytoplasm)	Yes	Post-translational	Weak	
IV	SP + 189 res. of mature protein	Inner membrane	Yes	Cotranslational	Strong	
V	SP + res. of mature protein	Inner membrane	Yes	Cotranslational	Strong	

Lamda-Receptor Protein

Class	Portion of the exported protein	Localization of the fusion protein in the *E. coli* cell	Signal peptide cleavage	Rate of export	Mal^S-phenotype of the bacteria	Ref.
Wild type	SP (25 res.) + 421 res. mature protein	Outer membrane	Yes	—	—	115, 122, 124, 347, 348
I	4 res. of SP	Cytoplasm	No	—	No	
II	SP + 15 res. of mature protein	Cytoplasm	No	—	No	
	SP + 20 res. of mature protein	Cytoplasm	No	—	No	
	SP + 27 res. of mature protein	Cytoplasm	No	—	No	

	SP + 39 res. of mature protein	Partially inner membrane	(Yes)	Strong
	SP + 49 res. of mature protein	Partially outer membrane	(Yes)	Very strong
III	SP + 173 res. of mature protein	Partially outer membrane	(Yes)	Strong
IV	SP + 240 res. of mature protein	Outer membrane	(Yes)	Weak

Note: SP = signal peptide; res. = residue(s).

is not related to the accumulation of mutant pre-maltose-binding protein, but rather to the concomitant synthesis of the export-defective polypeptide. Most importantly, interference was observed with a mutant lacking seven amino acid residues of the signal peptide (see Table 3). Furthermore, if this mutation was present in a hybrid gene of class II, class III, and class IV malE-lacZ fusion, only the latter showed the interference. These data indicate that interference is caused by a sequence present in class IV, but not in class III, i.e., between residues 23 and 189 of the mature maltose-binding protein. This region could be more confined with a deletion mutant lacking residues −20 to 89 which still interfered with the export of envelope proteins. Thus, the important sequence is believed to be somewhere between residues 89 and 189 of the mature malE protein. It should be stressed again, however, that export, albeit inefficient and slow, can be seen with shorter sequence segments (e.g., see Reference 125).

There also has been found a second site mutation in the amino acid residue 19 of the mature malE protein which partially restores the export of a mutant carrying a deletion of seven amino acid residues in the signal peptide.[110] The mutated amino acid may be part of another region which is involved somehow in protein translocation.

All these data show that sequence parts in mature exported proteins influence the efficiency of the translocation process. It should be noted, however, that one possible interpretation of the results is the assumption that the mutated signal peptides still contain some information which is recognized by the system, and that proper folding of the polypeptide chain is required for efficient translocation across the membrane.

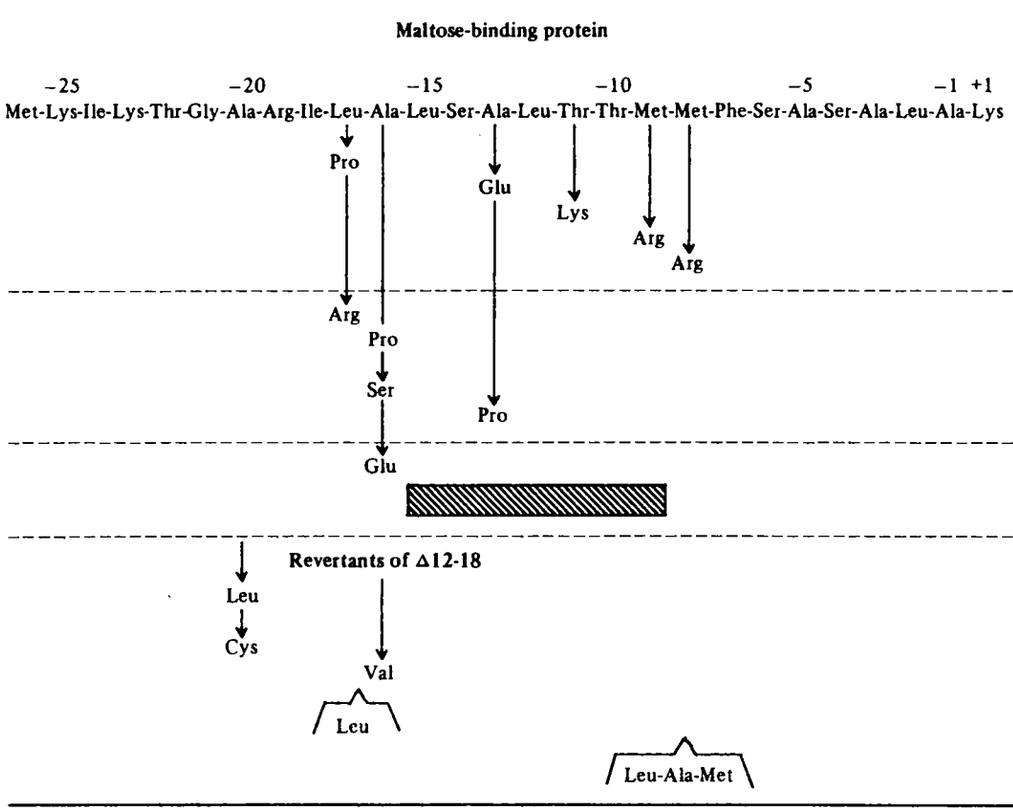
Summarizing the discussion, it can be concluded that all data are compatible with the idea that signal peptides are essential for protein translocation. However, more experiments on artificially designed export proteins would be desirable to further prove this important point. Data for bacterial proteins definitely show that a signal peptide is insufficient information and point to an active role of internal sequence parts. Similar evidence for the eukaryotic system is not available.

2. Features of Signal Peptides Required for Translocation

Most secretory proteins and many membrane precursor proteins contain cleavable signal peptides at their N-terminus (for exceptions see Section II.D.4). The signal peptides bear no sequence homology and are variable in length from 15 to 35 amino acid residues. The variability is found even if the proteins stem from a single cell. Studies on the evolutionary tree of preproinsulin showed that the divergence in the signal peptide is no less than in the C-peptide, indicating few constraints on its primary structure.[126,127] The most striking common feature of signal peptides is their hydrophobicity, or more precisely, a stretch of consecutive hydrophobic amino acid residues. This hydrophobic core is uninterrupted and has a minimum length of six to seven residues. Things may be different, however, for extreme conditions, as in halophilic bacteria. Bacteriorhodopsin has a N-terminal peptide which is cleaved off, but is not particularly hydrophobic.[128] Many, but not all, signal peptides have basic amino acid residues at their extreme N-terminus preceding the hydrophobic domain. Prokaryotic signal peptides appear a little less hydrophobic than eukaryotic ones and always have at least one basic residue at their N-terminus.[81,129]

The great variability of the signal peptides raises the question whether they all function in the same manner. Competition experiments carried out in vitro[56,130-132a] and the fact that SRP functions with all exported proteins tested so far (see Section II.E) argues in favor of a common recognition mechanism. In bacteria, a single point mutation affects the export of many proteins (see Section II.E). Furthermore, the recognition mechanism of signal peptides appears to be ubiquitous in nature. Not only can eukaryotic signal peptides direct secretion in *E. coli* (e.g., see Reference 42) and a prokaryotic one effect segregation into mammalian

Table 3
THE EFFECT OF MUTATIONS IN THE SIGNAL PEPTIDE ON THE TRANSLOCATION PROCESS

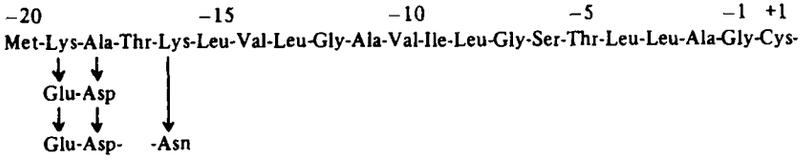


Designation	Strength of mutation	Ref.
10-1	weak	205
14-1	intermediate	
16-1	weak	
18-1	strong	
19-1	strong	
10-2	very weak	110a
11-1	very weak	
11-2	very weak	
14-2	weak	
11-3	weak	110
Δ12-18	very strong	
R2	strong	110
R3	strong	
R5	weak	
R4	intermediate	
R1	strong	

Table 3 (continued)
THE EFFECT OF MUTATIONS IN THE SIGNAL PEPTIDE ON THE TRANSLOCATION PROCESS

	Designation	Strength of mutation	Ref.
Lambda Receptor protein			
−25 −20 −15 −10 −5 −1 +1			
Met-Met-Ile-Thr-Leu-Arg-Lys-Leu-Pro-Leu-Ala-Val-Ala-Val-Ala-Ala-Gly-Val-Met-Ser-Ala-Gln-Ala-Met-Ala-Val-			349
Asp	S71	strong	
Glu	S70	strong	
Glu	S99	strong	
Arg	S69	strong	
	S60	very strong	
	S78	strong	
Arg	S96	almost no effect	108
Lys	S73		
Revertants of S78			
Leu	R2		146
Cys	R1		
Alkaline phosphatase			
−20 −15 −10 −5 −1 +1			
Met-Lys-Gln-Ser-Thr-Ile-Ala-Leu-Ala-Leu-Leu-Pro-Leu-Leu-Phe-Thr-Pro-Val-Thr-Lys-Ala-Arg-			213
Gln		weak	
Arg		strong	

Lipoprotein (*E. coli*)



I4	weak	111
I7	weak	112

Note: Boxed regions indicate deletions of the corresponding residues.

microsomes,[50] but also cell-free translation systems derived from mammalian or plant cells can be supplemented with microsomes from a variety of sources.[18,30,133,134]

Xenopus leavis oocytes can secrete proteins from many different origins when injected with the corresponding mRNA (for a review see Lane[135]). Thus, it is likely that all signal peptides are recognized by the same ubiquitous mechanism.

What feature of the signal peptides is recognized given their large differences in length and amino acid sequence? Several lines of evidence point to the importance of the hydrophobic core for the function of the signal peptide. Hortin and Boime[136] have shown that incorporation of β-hydroxy-leucine (HO-Leu) instead of leucine into secretory polypeptides containing signal peptides with many leucine residues prevents translocation across the RER membrane. Signal peptides with few leucine residues were not impaired.

It is therefore possible that the reduced hydrophobicity is responsible for the effect observed. Simulation of evolution on a computer indicated that signal peptides may have been selected on the basis of hydrophobicity with few other rules of constraint.[137] Most evidence for the role of the hydrophobic core is derived from bacterial exported proteins. Table 3 lists some point mutations in signal peptides of the four proteins best analyzed (the maltose-binding protein, the lambda-receptor protein, the alkaline phosphatase, and the outer membrane lipoprotein of *E. coli*). Deletion mutants of the maltose-binding protein and of the lambda-receptor protein and pseudorevertants of them are also included in the table. The following conclusions emerge from these data:

1. In the majority of cases, the mutation abolishing the function of the signal peptide is a change of a hydrophobic residue in the core to a charged residue. This clearly underlines the importance of the hydrophobic domain for protein translocation. The deletion of residues from this region has also a deleterious effect, as expected.
2. Changes of a hydrophobic residue to a Pro-residue or a hydrophilic, but uncharged, one (where these have been found) have a moderate effect on the function of the signal peptide. A change of a Thr-residue to a Lys-residue (mutant 16-1 of the maltose-binding protein) has only a slight effect. These data are in line with the conception that hydrophobicity is the main requirement.
3. Pseudorevertants of deletion mutants mostly map in the signal peptide and appear to elongate the hydrophobic portion of the remaining signal peptide. Mutations in which the charged Arg-residue at position −19 of the pre-maltose-binding protein is removed (R2 and R3) or three hydrophobic residues between residue −8 and −7 are introduced (R1), are stronger suppressors of the export deficiency than mutations in which an Ala-residue is changed for a Val-residue or an extra Leu-residue is added (see Table 3).
4. Not all residues within the hydrophobic core of the signal peptide appear of equal importance. Examples include a mutation at position −11 of the pre-maltose-binding protein (mutant 14-1) in which introduction of a Lys-residue has only a slight deleterious effect, and even more impressive, the silent mutations found at position −9 of the pre-lamB protein in which introduction of a charge has no effect, whereas mutation at position −7 to a charged residue interferes strongly with the function of the signal peptide (see Table 3).

Are there any parts in the signal peptides of importance for translocation other than the hydrophobic core? Inouye's group has addressed the question as to whether or not the basic residues at the N-terminus of the precursor of the lipoprotein have a role in protein transport. The two lysine residues were systematically altered to change the charge from +2 to +1, 0, −1, and −2.[111,112,129] No change in the rate of translocation was observed for a charge of +1 and 0, indicating that a basic residue is not absolutely required for translocation. The

Table 4
SIGNAL SEQUENCE OF CARP PREPROINSULIN AND VARIATIONS OF IT WHICH ARE FUNCTIONAL IN *E. COLI*[126,138]

Wild Type:

```
                                    -20                 -15
                          Met-Ala-Val-Trp-Ile-Gln-Ala-
```

Variants:

```
-30                 -25                 -20                 -15
Met-Thr-Met-Ile-Thr-Pro-Ser-Leu-Ala-Ala-Gly-Arg-Arg-Ile-Gln-Ala-
                    -25                 -20                 -15
          Met-Ser-Ile-Gln-Ala-Ala-Gly-Arg-Arg-Ile-Gln-Ala-
```

Continuation of the Sequences:

```
          -10                 -5                  -1  +1
Gly-Ala-Leu-Leu-Phe-Leu-Leu-Ala-Val-Ser-Ser-Val-Asn-Ala-Asn-Ala-Gly
                                                      ↑
```

Note: The cleavage site is indicated by an arrow.

rate of export was, however, significantly slowed down if the N-terminus had a negative charge. In all mutants, the rate of synthesis was reduced, perhaps indicative for a coupling between synthesis and transport. In agreement with the nonessential role of the positive charges, we found carp proinsulin to be secreted into the periplasm of *E. coli* cells even though it has no basic amino acid residue in its signal peptide (Table 4). Introduction of positive charges does not affect the efficiency of protein export.[138]

Summarizing this discussion, it appears that the hydrophobic core of the signal peptides is of overwhelming importance for directing translocation. Taking into account the great variability of the amino acid sequences of the signal peptides, a direct interaction with the phospholipid bilayer has been proposed.[118,139] This assumption was strongly supported by the results of Wickner's group on the spontaneous insertion of the M13 procoat protein into membranes (see Section II.C.3). On the other hand, evidence against a direct interaction of signal peptides with the phospholipid bilayer was provided by the use of preproteins synthesized in vitro in a cell-free system.[67,130,131] It was found that completed carp preproinsulin and human placental prelactogen are bound by RER membranes and compete with each other, whereas proinsulin or globin are not bound. Binding of the precursor blocked the sites for cotranslational translocation of nascent secretory proteins across the membrane. There was a saturable number of binding sites which were sensitive to trypsin. Binding was observed only for RER membranes, not for smooth membranes or artificial phospholipid bilayers.[67] Similar results were obtained with preproparathyroid hormones using a synthetic peptide containing a signal sequence.[132,132a] More recently, a synthetic peptide containing typical features of signal sequences was shown to block cotranslational translocation of secretory proteins and to bind in a saturable manner to RER membranes.[139a] If a photoreactive group was introduced into the radioactively labeled peptide, a 45-K cross-linking product could be seen after irradiation of the membrane-signal peptide complex. The interacting component appeared to be an integral membrane protein.[139b]

These results indicate that there is a protein receptor in the RER membrane which recognizes signal sequences. It is, however, unlikely that the binding entity is identical to SRP. Perhaps this poorly characterized receptor is involved in later steps of the translocation process.

The discovery of the SRP has provided proof for a signal peptide receptor involved in

the initiation of translocation in higher eukaryotes. In bacteria, the existence of a similar particle is also likely (see Section II.E.1). Although the possibility that some proteins, like the M13 procoat protein, use a different pathway cannot be dismissed, most signal peptides appear to be recognized by a common receptor (SRP). How is this possible if the sequences are so different?

It has been repeatedly suggested that they all preform a common secondary structure.[119,140-142] However, contradictory results were obtained by the use of the rules of Chou and Fasman;[143] a common structure could not be predicted for all signal peptides, as is also found with a different prediction method.[144] Experiments on a synthetic peptide also yielded ambiguous results for the secondary structure of a signal peptide.[145] On the other hand, a correlation was found between the export of the lamB protein and the predicted tendency of signal sequence mutants to adopt an alpha-helical conformation.[146] For example, the potential for formation of an alpha-helix was predicted to be in the order wild type > revertants of S78 (R2 and R1) > deletion mutant (S78) (see Table 3). The secondary structure prediction has been confirmed experimentally by CD-spectra of synthetic peptides.[147] However, it was also found that the signal peptides contain little secondary structure in aqueous solution but that it can be induced in micellar solutions of SDS or lysolecithin.

Contrary to the idea of a preformed alpha-helix in signal peptides, the introduction of charged residues into the hydrophobic core generally does not change the predicted secondary structures. We have therefore proposed that the structure of the signal peptide is not preformed, but induced by deep immersion of the hydrophobic core into a hydrophobic pocket of SRP.[144] The hydrophobic environment imposes saturation of all H-bond donors and acceptors. If the pocket itself does not present any hydrophilic groups, H-bond formation must occur within the signal sequence alone and a helical, presumably α-helical, conformation is induced. If the pocket contains polar groups, H-bonds may be formed with the signal peptide and a different structure can be induced. In any case, identical backbone conformations are induced for all sequences.

It is assumed that immersion into a hydrophobic environment is so deep — and this is only possible for entirely apolar sequences — that side-chain interactions are unimportant. Of course, the differences in the side chains would prohibit complete complementarity between the interacting molecules. However, a similar case has been proposed for intermediates in the unfolding of proteins in which the van der Waals contacts are loosened while water is still excluded from the interior of the molecule so that the hydrophobic interactions remain.[148] The proposed mechanism differs from other known ligand-receptor interactions for which side chains of amino acid residues play an important role.

The hypothesis of deep immersion of the hydrophobic core of signal peptides into a hydrophobic pocket of the receptor could explain several facts: (1) the sequence variability; (2) the effect of introduction of hydrophilic or charged residues into the hydrophobic domain; (3) the effect of more specific changes in this region if interaction by H-bonds with the receptor is essential; and (4) the minimum length of the uninterrupted stretch of hydrophobic residues is estimated to be six to seven amino acid residues,[144] in agreement with the actual data.

It should be noted that it is conceivable that signal peptides may play a role beyond the initial recognition step as well. For example, a direct interaction of the signal peptide with the lipid phase is possible for a later step of the translocation process.

3. Cleavage of Signal Peptides

As stated before, most signal peptides are cleaved by signal peptidases. Is this a necessary requirement for their function? It appears that translocation and cleavage of the signal peptide are not strictly coupled. Mutants of secretory and membrane proteins have been found or

constructed which are transported, but not cleaved.[149-153] Many membrane proteins and some secretory proteins are synthesized without precursor but appear to contain signal sequences (see Section II.D.4). In fact, one may raise the question as to why cleavage of the signal peptide is so often observed. Several ideas have been put forward:

1. Cleavage of the signal peptide may be one mechanism to make the translocation process unidirectional.
2. Signal peptides may serve as membrane anchors and prevent complete detachment of the polypeptide, if not cleaved off.
3. The long hydrophobic sequence may be without function in the mature protein and difficult to shield from the water environment.

In eukaryotes, cleavage of the signal peptides usually occurs during the synthesis of the protein so that preproteins can only be found in very small amounts in pulse-labeled cells.[154-157] Cleavage of precursors may occur post-translationally in eukaryotes if a Thr-analog is incorporated into the secretory protein.[158] Preproteins appear to be rapidly degraded if present in the cytoplasm.[159] In prokaryotes, processing of the precursors can be either co- or post-translational, depending on the specific protein.[160,161]

Signal peptidases appear to be very specific endopeptidases (see Section II.E.3). How do these enzymes recognize the cleavage site given the extreme sequence variability of the signal peptides? It is immediately obvious that these peptidases are basically different from other endopeptidases which recognize a specific amino acid sequence close to the site of cleavage. For signal peptidases, the only rule so far established is the presence of a small aliphatic amino acid residue, in most cases alanine, at the -1 position. This feature may be essential for cleavage since its substitution by Thr abolishes processing.[158] Of course, a single residue is insufficient to determine specificity. "Consensus" sequences of many signal peptides have been proposed, but the mechanism by which the enzyme recognizes its substrate remains unclear.[81,161a] It is known that even the amino acid residues flanking the cleavage site are not the only determinants. When the glycine at the -1 position of prolipoprotein was replaced by an alanine residue, no effect on the processing was seen. If the -1 position was deleted, the mutant prolipoprotein was not cleaved, although the residue at the -2 position is again alanine.[152] In carp preproinsulin, the sequence around the cleavage site is Asn-Ala-Asn-Ala, but cleavage is exclusively after the first alanine residue of the tetrapeptide sequence[126,162] (see Table 4). These data suggest that not only the immediate vicinity of the cleavage site is of importance. How distant can a residue be to have an influence on the cleavage reaction? It appears that cleavage mutants map to both sides of the splitting point. A -7 position deletion affected the cleavage of prolipoprotein.[129]

Taking the facts together, it is likely that not a precise amino acid sequence but rather a specific conformation, comprising perhaps up to ten amino acid residues, acts as recognition marker for a signal peptidase. The existence of a beta-turn close to the cleavage site is predicted by the rules of Chou and Fasman,[143] but such a structure can only be marginally stable if formed by the signal peptide alone. Nevertheless, the rule that small, neutral residues are abundant at positions -1 and -3 can be used as the empirical method for prediction of the cleavage site.[162a]

4. Position of Signal Sequences in Polypeptides

It came as a great surprise when Palmiter et al.[163] discovered that ovalbumin, a secreted protein, is synthesized without a cleavable signal peptide. Studies in vitro showed, however, that ovalbumin was sequestered into microsomal vesicles and competed for translocation with other secretory proteins with a N-terminal, cleavable signal sequence.[164,165] At first, the possibility was considered that a N-terminal signal peptide was just not cleaved off.

However, ovalbumin is not conspicuously hydrophobic at its N-terminus and has a charged residue at position 9. The first four amino acid residues are not required for secretion in *E. coli*.[44,45] Lingappa et al.[166] claimed that the signal peptide of ovalbumin is internally located on the tryptic peptide 229 to 276. However, their synchronization experiments were later shown to be faulty.[167] Revision of the data placed the signal peptide within the first 150 residues. Meek et al.[31] provided evidence that initiation of translocation of ovalbumin started already when the nascent chains had a length of 45 to 90 amino acid residues. They proposed that the signal peptide in ovalbumin is located between position 26 and 45, a stretch which is indeed hydrophobic.

Recently, more direct support for this hypothesis was provided.[167a,167b] Plasmids containing modified ovalbumin genes or fusions between ovalbumin and alpha-globin sequences were injected into *Xenopus* oocytes nuclei, and the localization of the translation products was investigated. It was found that ovalbumin lacking either 8 or 21 N-terminal amino acid residues was sequestered in the ER, although the latter product was not secreted out of the cells and was strongly associated with membranes. Also, deletion of the amino acid residues 231 to 279 did not affect translocation across the ER membrane, but affected secretion. Most importantly, fusion polypeptides of residues 9 to 41 or 22 to 41 of ovalbumin with alpha-globin were sequestered in the ER, but not secreted. One may conclude from the data that ovalbumin contains a signal peptide between residues 22 and 41. The sequence between residues 9 and 21 may be important for membrane detachment of the hydrophobic signal peptide and therefore for completion of the translocation process. It is also evident that secretion from a cell exerts further constraints on the sequence of the protein.

So far ovalbumin has remained an exception for eukaryotic secretory proteins. In prokaryotes, colicin E1 may be a secretory protein with an internal signal peptide.[168] The only hydrophobic sequence is located close to the C-terminus.[169] Genetic engineering experiments have provided evidence that this region is required for export and mediates the initial membrane attachment.[170] Colicin E1 is unusual since it is secreted post-translationally at a very slow rate. Furthermore, another gene product appears to be required for its secretion.[170] Nevertheless, its pathway may be similar to that of other exported proteins since it also accumulates inside the cytoplasm in secretion-blocked *E. coli* cells.[171]

An internal signal peptide was constructed by Talmadge et al.[172] by placing nucleotides coding for a few residues of the β-galactosidase and some linker residues in front of a slightly shortened rat preproinsulin gene. The length from the expected site of start of translation up to the cleavage site of preproinsulin was 39 amino acid residues. Proinsulin was found in the periplasm of *E. coli* cells, but whether the signal peptide was indeed internally located in the polypeptide chain during its function remained unresolved. Similar results were obtained by Rapoport et al.,[47] who constructed a plasmid in which the signal peptide of carp preproinsulin was preceded by 54 amino acid residues. In the case of the *E. coli* lipoprotein, the signal peptide was fully active if preceded by 145 amino acid residues.[173] By inhibiting the action of the lipoprotein signal peptidase by globomycin,[174] the authors were able to provide evidence that the fusion gene, containing the signal peptide as an internal sequence, is the precursor for the exported lipoprotein.

These data give preliminary evidence that signal peptides, normally located at the N-terminus, remain active after internalization. Things may be different, however, in eukaryotes. Kozak[175] placed repeats of a sequence in front of the signal peptide of rat preproinsulin and tested for secretion of insulin-like material from mammalian cells. It appears from her results that internalized signal peptides no longer were functional in secretion. It is, however, unclear where interference occurred.

Whereas uncleaved and internal signal sequences appear to be exceptions in secretory proteins, they may be frequent in membrane proteins, at least in eukaryotes. Some examples are listed in Table 5. In all these cases, precursor polypeptides do not exist. This raises the

Table 5
SOME EXPORTED PROTEINS WITHOUT PRECURSORS CONTAINING PRESUMED UNCLEAVED AND INTERNAL SIGNAL PEPTIDES

Protein	Location of the signal peptide	Ref.
Eukaryotic Secretory Proteins		
Ovalbumin	Translocated	163
Procaryotic Secretory Proteins		
Colicin E1	Translocated	168, 169, 171
Eukaryotic Membrane Proteins		
p62-Protein of Sindbis and Semliki Forest virus	Translocated	350, 351
Opsin	Translocated (?)	352—354
Cytochrome P-450	Membrane-spanning	355
Epoxide hydratase	Membrane-spanning	356
Isomaltase	Membrane-spanning	357
Ca^{2+}-ATPase	?	358
BandIII of erythrocytes	Membrane-spanning	359, 360
MP26 of lens membranes	?	361
δ-subunit of histocompatibility antigen	Membrane-spanning	64
Neuraminidase	Membrane-spanning	278, 362, 363
Procaryotic Membrane Proteins		
Signal peptidase I (leader peptidase)	Membrane-spanning (?)	229
LacY-protein (*E. coli*)	Membrane-spanning (?)	364, 364a

question whether they contain signal sequences at all. For some proteins the requirement of SRP for their membrane incorporation in vitro has been demonstrated which provides good evidence for the existence of signal peptides (see Section II.E.1). For the band III protein of erythrocytes, it has been shown that dog pancreatic membranes can be added much later than for proteins with N-terminally located signal peptides (up to the point where about 500 amino acid residues were synthesized), and translocation still occurs.[176] Indeed, the N-terminus of this protein remains in the cytoplasm, and a membrane-spanning segment has been localized close to residue 500.[177]

For some of the proteins listed in Table 5, the amino acid sequences are known and contain hydrophobic segments reminiscent of signal peptides. However, definite proof for their function is lacking as yet. The sequence in question should transport a cytoplasmic polypeptide when added to its N-terminus and should replace a cleavable signal peptide in its function, and certain mutations in it should affect the translocation process. The size of an SRP-arrested polypeptide fragment, if it exists, may also indicate the location of a signal peptide in a protein. Nevertheless, the data already existing indicate that signal peptides need not be located at the N-terminus of a polypeptide chain and therefore in that case they are not cleavable.

E. Components of the Translocation Apparatus

The requirement of a translocation apparatus for protein transport across membranes remains a matter of dispute in some cases (see Section II.B.3). It is, however, beyond any doubt that in general a number of proteins are instrumental in the translocation process. This section discusses the various components known so far.

1. The Signal Recognition Particle (SRP)

The discovery of SRP can be considered to be a milestone in the elucidation of the molecular details of the translocation process across the RER membrane.[36,178-180]

The basic observation was that extraction of rough microsomes with high concentrations of KCl rendered the membranes inactive for protein segregation, but that readdition of the salt extract reconstituted their function.[181] Initial confusion was created by the fact that high salt-washed rough microsomes (K-RMs) were active in the reticulocyte lysate system (see Reference 182) until it was realized that it contains, unlike the wheat germ system, SRP.[196] Furthermore, Jackson et al.,[184] in early experiments, were unable to inactivate RER membranes by extraction with high salt, even though they used the wheat germ system for assay and thought that the deviating results of Warren and Dobberstein[181] were due to proteolysis. It is now clear that both KCl and protease (+KCl−) treatment abolish the translocation competence of rough microsomes.

Walter and Blobel[185] isolated a protein complex from the high salt extract of RER membranes by hydrophobic chromatography and sucrose gradient centrifugation and showed that it is the only component required to reactivate K-RMs. It was later realized that the complex also contained a 7S-RNA; it was therefore renamed signal recognition particle (SRP).[186]

SRP from dog pancreas has a sedimentation coefficient of 11S and contains 6 polypeptide chains of molecular mass 72, 68, 54, 19, 14, and 9 kdalton.[185] The 7S-RNA was shown to be identical with the 7SL-RNA known before as a metabolically stable RNA-species. More than 75% of the total cellular 7SL-RNA is contained in the SRP.[187] The 7SL-RNA has a unique structure: it contains, at both ends, segments which correspond to highly repetitive sequences of the genome (Alu-sequences) and a middle part (S-sequence) which is repeated less frequently in the genome or is unique.[187-189]

The RNA is relatively well protected in the particle against the action of nucleases justifying the practice to lower the endogenous mRNA-content of rough microsomes by treatment with low concentrations of micrococcal nuclease. A detailed analysis with micrococcal nuclease indicated that the SRP on treatment with high concentrations of the enzyme separates into two domains, one containing the four largest polypeptides associated with the S-segment of the 7S-RNA and the other containing the two smallest bound to the Alu-fragments.[190] It appears therefore that the 7SL-RNA folds back in the particle allowing the 5′- and 3′-Alu segments to pair bases with each other.

The structure of SRP is stabilized by Mg^{2+} ions and can be disrupted by lowering their concentration. In this manner, Walter and Blobel[191] dissassembled the particle into native protein and RNA components. When recombined under suitable conditions, the original structure and function of SRP was reconstituted. A highly cooperative process of self-assembly was found, similar to that observed for the reconstitution of ribosomal subunits. By modifying isolated components and reconstituting into SRP, it should be possible to probe their role in the particle. Such an approach has already been taken by reassociation of 7SL-RNA from different species with the polypeptides from dog pancreas. *Xenopus leavis* and *Drosophila* 7SL-RNA yielded functional particles with sedimentation coefficients of about 11S, although the latter RNA does not cross-hybridize with mammalian 7SL-RNA.[191,192] *E. coli* 6S-RNA, the possible equivalent of the eukaryotic 7S-RNA (see below), does not reconstitute SRP. It is an intriguing possibility that the 7SL-RNA interacts with other RNA-species of the translocation apparatus, but experimental evidence is lacking as yet.

Although originally purified on the basis of its ability to reactivate K-RMs in their translocation competence, SRP turned out to have a second function: in the absence of K-RMs, the addition of SRP to the wheat germ cell-free translation system led to a severe inhibition of the translation of preprolactin and pregrowth hormone, whereas alpha- and beta-globin synthesis remained unaffected.[179] The inhibition was released by addition of K-RMs to the assay. This indicated a role for SRP in the coupling of synthesis and translocation of secretory proteins. This conclusion was clearly borne out by synchronized translation of pituitary mRNA in the presence of SRP.[36] A 70-amino acid residue long peptide fragment accumulated, but disappeared when K-RMs were added. The processed and translocated polypeptides were synthesized simultaneously, indicating a precursor-product relationship. It remains to be directly shown by sequencing that the arrested peptide fragment is related to preprolactin, the main translation product of the pituitary mRNA. The length of the arrested fragment may be explained by a direct interaction of SRP with the signal peptide: about 40 amino acid residues would be buried within the ribosome[193,194] and 30 would have emerged from it, including the complete signal peptide.

It has been shown that the two smallest polypeptides of SRP are responsible for the translational arrest of the particle and dispensable for its function in translocation.[259a]

An interaction between SRP and the nascent chain was further supported by binding experiments.[179] SRP binds to polyribosomes synthesizing secretory proteins, but not to those synthesizing globin. It binds to monosomes by four orders of magnitude less strongly. The binding of SRP to the signal peptide of the growing secretory polypeptide is mainly suggested by the effect of HO-Leu incorporation: not only was the translocation abolished,[136] but also binding of SRP to polyribosomes and the translational arrest exerted by SRP.[36] It was therefore concluded that SRP recognizes the Leu-rich signal peptide. Of course, HO-Leu is incorporated throughout the protein and its effect may not be entirely due to a change in the signal peptide. Also, these data do not exclude the possibility that other parts of the nascent chain, which are somehow influenced by the signal peptide, contribute to the recognition process. It therefore appears that more definitive proof for the interaction of SRP with the signal peptide is still desirable. Nevertheless, the data provide most convincing evidence for an interaction of SRP with the nascent polypeptide chain as opposed to a recognition of the mRNA-template.

Although SRP was originally purified from rough microsomes, this cannot be its exclusive location in a cell. Indeed, cell fractionation studies indicate that SRP can be found in various fractions, depending on the concentration of KCl in the homogenization buffer.[187] At low salt concentration, SRP is primarily located in the microsomal fraction. With rising KCl concentrations, SRP is found on free ribosomes until, at high concentrations, it is entirely in the supernatant fluid. At physiological salt concentrations, SRP is present in all three compartments, indicating its potential to circulate between them. All these data led to the scheme shown in Figure 2 and explained in Section II.A.2.

The role of dog pancreatic SRP for the segregation of proteins into microsomal vesicles in vitro is now well established for secretory proteins from various sources. Examples include apolipoprotein AI,[195] immunoglobulin light chain,[196] human placental lactogen,[197] carp proinsulin,[381] and β-lactamase.[50] Storage globulin polypeptides from legumes (Bassuener et al.)[197] and lysosomal enzyme precursors (Erickson et al.)[198] also require SRP for membrane transfer. Membrane proteins for which the essential function of SRP for membrane insertion has been shown include the δ-subunit of the acetylcholine receptor,[199] the γ-subunit of the histocompatibility antigen,[64] cytochrome P-450,[65] the major lens membrane protein (MP26), and the Ca^{2+}-ATPase of the sarcoplasmic reticulum.[68] In the latter two cases, however, a translational arrest by addition of SRP alone to the cell-free translation system was not observed (to be further discussed in Section II.F.2). It can be concluded from these examples that proteins

in general require SRP for translocation across the RER membrane. The data also provide indirect evidence for the existence of SRP in lower eukaryotes and even prokaryotes.

Does SRP exist in bacteria? A search comparable to the approach taken for the eukaryotic system cannot be made as yet for bacteria since an efficient in vitro translocation system does not exist. Only partial processing of precursor polypeptides could be obtained with inside-out *E. coli* plasma membrane vesicles.[27,200] Recently, some progress has been made in the improvement of a prokaryotic in vitro system, but the translocation efficiency still did not exceed 25%.[200a]

The genetic approach, however, has proved very powerful for *E. coli*, although definite identification of polypeptides contained in the bacterial SRP or functioning as docking protein has not yet been achieved.

Based on the observation that some fusion polypeptides of the maltose-binding protein and the β-galactosidase were held up in the membrane and conferred a lac-minus phenotype to the bacteria (Reference 201; see also Table 2), temperature-sensitive mutants mapping outside this gene were looked for, which prevented initiation of translocation and therefore caused a lac-plus phenotype (presumably the lac-minus phenotype is caused by inhibition of tetramerization of the β-galactosidase if the enzyme is incorporated into membranes). The mutants mapped in two loci, called secA and secB. When present in otherwise wild-type *E. coli* cells, they lead to growth inhibition at 42°C, and precursors to some proteins accumulate. When both mutations were present simultaneously, the effects were enhanced.[202] However, some periplasmic proteins were normally secreted in these mutants.[201] Nevertheless, the pleiotropic phenotype of the sec mutants suggests that the genes play a role in protein export. When amber (peptide-chain terminating) mutants in the secA gene were analyzed, it turned out that complete absence of the gene product did not lead to the accumulation of some precursors but to a total block in the synthesis of exported proteins. Other proteins were not affected.[203] It was recently demonstrated that signal sequence mutations in the maltose-binding protein precursor, which were previously shown to abolish the secretion of this protein,[204,205] also prevented the blocking of synthesis when introduced into the secA amber strain.[206] These data clearly indicate that the signal sequence interacts with a protein factor either directly or indirectly involving the secA gene product. This is, of course, consistent with the SRP-model for protein secretion in eukaryotes. In favor of this idea, the secA gene product has been identified as a 92-kdalton protein associated with the periphery of the cytoplasmic membrane of *E. coli*.[203,207] Antibodies raised against the secA protein were claimed to precipitate a portion of the 6S-RNA[208a] from cell homogenates.[208] However, by using a reconstituted cell-free system, a soluble activity was recently described that is required for the export of proteins in *E. coli*. This factor sediments at about 12S, but does not contain the 6S-RNA.[208b] These data may suggest the presence of a SRP-like factor in bacteria, but the possibility remains that some exported proteins take another route since they are not affected by the sec mutations.

Extragenic suppressor mutations of the secA-ts mutants were recently found which restored normal growth of the bacteria at 37°C.[210] They were called secC and showed a cold-sensitive phenotype. At 23°C, there was a defect in the synthesis of exported proteins, whereas cytoplasmic ones remained unaffected. A marked allele specificity was found with different secA alleles, which was taken as evidence for a direct interaction of the secA and secC proteins.

A different genetic approach has led to other candidates of constituents of SRP. Starting with a mutant with a deletion in the signal sequence of the lambda-phage receptor (lamB protein), second site mutations were searched for, in which the export of this protein was restored.[211] Three loci were found, called prlA, B, and C (the prlB mutant most likely does not affect any component of the export machinery).[109] The prlA mutants, which have been most extensively studied, restored the translocation of many export-deficient signal peptide

mutations including those of the maltose-binding protein and alkaline phosphatase.[211-213] They appeared to have no effect on normal signal peptides.[212] The mutation maps on the promotor-distal end of the ribosomal protein gene cluster, which suggests that it is regulated in coordination with the synthesis of ribosomes.[214] Different alleles of the prlA locus showed different efficiency of suppression of signal peptide mutations. Furthermore, selectivity with respect to the various signal peptide mutations was observed. Again, these data may be interpreted as a direct interaction of the prlA protein with the signal peptide. Another locus, very close to that of prlA, but not identical with it, has recently been found with a similar selection procedure using a deletion mutant of the maltose-binding protein (delta 12 to 18, see Table 3).[215] This gene (prlD1) is only a weak suppressor, but if present simultaneously with the prlA mutation, there is a strong growth defect and many precursors of exported proteins accumulate.[110a] Again, allele selectivity was found, indicative of an interaction of the prlA and prlD1 proteins.

The secY gene described by Ito et al.[217] most likely is identical to the prlA gene locus. Mutations were found, however, either in a preceding gene, which had a polar effect on the prlA protein,[217] or in the protein itself,[218] which affected the export of many proteins. These experiments clearly show that the prlA protein is involved in the normal export process of cells rather than providing an alternative route. It should be noted that suppression of the signal-peptide mutations by the prl mutations, though restoring the export process in principle, led to significant retardation of translocation.[110a]

A number of recent results indicate that synthesis and translocation are coupled in *E. coli* as they are in the eukaryotic system. Mutational alterations in the hydrophilic segment of the *E. coli* lipoprotein[111] or of the lamB protein[219,220] significantly reduce the translation efficiency. Total lack of the secA protein stops the synthesis of the maltose-binding protein, but cytoplasmic proteins remain unaffected.[203] The secC mutants are defective in the synthesis of exported proteins at the nonpermissive temperature, but again other proteins are still synthesized. These data suggest that a SRP-induced translational arrest is also operational in *E. coli*.

2. The Docking Protein — the SRP-Receptor

It was predicted by the signal hypothesis that integral membrane proteins would be required for recognition and translocation of proteins across the RER membrane.[18] It was therefore a logical attempt to proteolytically dissect and reconstitute microsomal vesicles. If RER membranes are incubated with low concentrations of trypsin or elastase, a component is cleaved off which can be removed from the membranes by raising the KCl concentration above 0.2 *M*. When added back to protease-inactivated membranes, the extract restores the translocation activity.[182-184,221] The active component in the extract was purified and was shown to be a 60-kdalton polypeptide.[222] The original component in the membrane, giving rise to the 60-kdalton fragment after proteolysis, was identified as a 72-kdalton protein. It appears that this protein consists of two domains, a membrane and a cytoplasmic (60-kdalton) one. Functional reconstitution only requires an ionic interaction, but it has not been proved that the 60-kdalton fragment interacts with the residual peptide in the membrane to yield translocation competence.

The function of the polypeptide was demonstrated by use of the SRP-induced arrest of translation as an assay. Meyer et al.[196] found that the purified 60-kdalton fragment released the translational arrest as did the K-RMs from which it is derived. They named the peptide "docking protein" on the assumption that it binds (docks) SRP to the membrane.

A similar, but not exactly identical, conclusion was reached by Gilmore et al.[223,224] These authors isolated the 72-kdalton protein from a detergent extract of salt-extracted rough microsomes by affinity chromatography with SRP coupled to Sepharose. Identity with the 72-kdalton protein of Meyer et al.[222] was shown by peptide mapping and immunological

means. However, Gilmore et al.[224] found that only the 72-kdalton protein and not the 60-kdalton fragment was able to release the translational arrest exerted by SRP. They explained the deviating results of Meyer et al.[196] by contamination of their 60-kdalton preparation with the 72-kdalton protein. This assumption has been confirmed by Dobberstein's group, who showed that the 72-kdalton protein is more active than the 60-kdalton fragment.[225] On the basis of its function, Gilmore et al.[223,224] have termed the 72-kdalton protein the "SRP-receptor". Such a receptor has been predicted before.[36]

A SRP-receptor has not yet been identified in bacteria. Any of the genes found to influence the protein export in *E. coli* could in fact be either a constituent of SRP or the receptor. One possibility, for example, would be that secA is the bacterial docking protein, whereas secB, secC, prlA, and prlD1 could be constituents of the SRP.[109] It is obvious that identification of the polypeptide chains and antibodies raised against them would greatly contribute to further progress.

3. Signal Peptidases

The signal peptidase(s) from eukaryotes has not been completely purified as yet. Assay of the enzyme, although possible after treatment of microsomes with detergent and completed preproteins as substrate,[33,34] is complicated by the requirement of testing by sequence analysis for correct cleavage. Using prepromellitin as a model, Mollay et al.[226] achieved a separation of the signal peptidase from signal peptide-degrading enzymes and proved thereby that the former enzyme is a neutral endopeptidase of a specific type. Neither the molecular weight nor the amino acid residues constituting the active center are known.

The signal peptidase is probably located at the luminal side of the RER membrane since it is resistant to extensive proteolysis.[33] The signal peptidase not only requires detergent for its solubilization, but also for the assay.[226]

In *E. coli*, it is clear that there exist at least two signal peptidases, called signal peptidase I and II.[129] Signal peptidase II is apparently specific for precursors of lipoproteins, whereas signal peptidase I (also called "leader peptidase")[93] is believed to be required for all other precursors of export proteins in *E. coli*.

The signal peptidase I is coded by the gene lepI located in the *E. coli* map at a place not identical to any other known component of the translocation apparatus.[227,228] The amino acid sequence of the signal peptidase I predicted from the nucleotide sequence of the gene agrees with the amino acid composition of the purified enzyme.[229] Its molecular mass is about 35 kdalton. By proteolytic digestion of *E. coli* spheroplasts, it was shown that the majority of the polypeptide chain is exposed to the outside and that the amino terminus anchors the enzyme in the membrane. Interestingly enough, the signal peptidase I, which is itself an exported protein, does not possess a cleavable signal peptide.[229] The enzyme is mostly found in the cytoplasmic membrane, although a small percentage may also be present in outer membranes.[229,230]

It has been shown that the signal peptidase I cleaves a variety of secretory precursor proteins, including some from eukaryotes.[80] Conversely, the eukaryotic signal peptidase cleaves pre-β-lactamase correctly.[50]

The gene for the signal peptidase II has also been sequenced recently and is unrelated to that of signal peptidase I.[231,232] This fact is demonstrated further by the isolation of a temperature-sensitive signal peptidase II mutant in which the processing of prolipoprotein can be blocked, but not that of other secretory proteins.[233,234] Interestingly enough, the promotor of the gene also controls a tRNA synthetase gene (Ile-tRNA), but its relation to protein export, if any, remains unclear.[235] The signal peptidase II is predicted to contain 164 amino acid residues. The amino acid sequence contains several hydrophobic domains, but the orientation of the enzyme in the membrane is not yet known. The enzyme cleaves prolipoprotein only after addition of glycerine to the cysteine residue at position + 1, which

is a prerequisite for attachment of fatty acids.[236,237] Treatment of *E. coli* cells with globomycin leads to accumulation of the glycerine-modified prolipoprotein.[174,236] Globomycin does not inhibit the signal peptidase I. The signal peptidase II appears to be temperature-stable (up to 80°C) when assayed in vitro.[238] This permitted the detection of the cleaved-off signal peptide of prolipoprotein since the signal peptide-degrading enzymes were inactivated under these conditions. The degradation of the signal peptide could be prevented in vitro by addition of antipain or other peptide aldehydes which inhibit proteases.[239] The intact signal peptide was found in the membrane fraction. Signal peptide degradation cannot be prevented in a similar way in the eukaryotic system.

4. Ribosome Receptors

Both the synthesis of secretory and some membrane proteins in membrane-bound polyribosomes and the cotranslational mode of transfer across the RER membrane suggest an active role of ribosomes. Evidence for a specific binding of ribosomes to the RER membrane is old.[240-243] Ribosomes were found to bind only to the rough and not to the smooth ER. Binding was saturable and sensitive to protease treatment of the microsomes. The interaction was sensitive to high salt concentrations. It appears from electronmicroscopic data that the large ribosomal subunit contains the interaction site.[243,244] However, the exit site of the nascent protein chain on the ribosome, which should be close to the binding site to the RER membrane, seems to be in the neighborhood of the interface between the two ribosomal subunits.[245] The ribosomal protein(s) interacting with the membrane is not known as yet. Two membrane proteins of the RER, termed ribophorins I and II, have been suggested as ribosome receptors.[246-248] This hypothesis is based on their occurrence in rough, but not smooth, ER membranes of different cell types, their close association with ribosomes, and their ability to be cross-linked to ribosomal proteins. However, direct evidence for an interaction of the ribophorins with ribosomes during the translocation process is lacking as yet.

One may raise the question as to whether ribosomes are linked to the membrane via SRP which is known to interact both with ribosomes and the membrane (see Section II.E.1). This possibility can be excluded since there are about tenfold more membrane-bound ribosomes per cell than SRP and SRP-receptor molecules.[224] Furthermore, Gilmore and Blobel[37] have shown that SRP is released from the ribosomes when K-RMs are added, even in the absence of chain elongation. Since the SRP-receptor does not have a measurable affinity to ribosomes, the result indicates that the ribosomes are transferred to a different membrane receptor.

The role of ribosomes in bacteria is less clear. Membrane-bound ribosomes do exist and appear to play a role in protein export.[249,250] This conclusion has been strengthened recently by showing that the signal peptide mutants of the maltose-binding protein and alkaline phosphatase are primarily synthesized in free polyribosomes in the cytoplasm,[110a] whereas the wild-type proteins are synthesized in membrane-bound polyribosomes.[249] The result demonstrates that, as in eukaryotes, the signal peptide directs the ribosome to the translocation competent membrane.

In contrast to the eukaryotic system, there is no firm binding of ribosomes to the cytoplasmic membrane.[251] Whereas there is little doubt that translocation across the RER membrane is strictly cotranslational, for bacteria, conflicting data exist. Some proteins can be translocated late in their biosynthesis or even after their completion.[80,252,253] In *Bacillus subtilis*, a protein of molecular mass 64 kdalton has been identified which is associated with the inside of the cytoplasmic membrane.[254] In the absence, but not in the presence, of ribosomes this protein was sensitive to proteases and accessible to antibodies prepared against it. These data would be compatible with its presumed role as a ribosome receptor.

5. What Role Do the Phospholipids Play?

This question cannot be answered yet and has not been studied extensively. It is clear that lipids play at least a permissive role. In bacteria, changes in the composition of the phospholipids and their mobility influence the process of protein export.[255-257] However, it is possible that this is an indirect effect on membrane proteins involved in translocation. It cannot be excluded at the moment that special areas of the membrane with nonbilayer structure of the phospholipids (inverted micelles) could be the sites of protein transfer. These regions could provide an internal, hydrophilic environment within the membrane equally favorable as a protein channel for transport of the growing polypeptide.

F. Functional Aspects of the Translocation Process

1. Where Does the Energy Come From?

The source of the energy for protein translocation is not yet established for the eukaryotic system. In bacteria, however, the post-translational export of some proteins can be inhibited by drugs which dissipate the electrochemical potential across the inner membrane.[94,95] ATP itself does not appear to be required. By use of specific drugs, it was further shown that the asymmetrical charges at both sides of the membrane are required rather than a proton gradient.[106,258] How a membrane potential influences protein transfer across a bilayer is not clear. It is conceivable that a "protein pump" is coupled to an ion pump in a comparable manner as is known for the coupling of transport systems of small molecules. A membrane potential is also required for the post-translational import of many proteins into mitochondria (see Section III.D.4). On the other hand, evidence has been provided recently that ATP is needed for protein translocation in *E. coli* in vitro.[258b]

The transport system across the RER membrane almost certainly cannot take the energy from a membrane potential. It is doubtful that an ion gradient can be built up in microsomal vesicles which are leaky for small molecules, including many ions. Thus, a membrane potential, if it exists, must be small. Uncouplers and ionophores have no effect on protein translocation in vitro.[258a] It is possible that in eukaryotes the mechanical link between the ribosome and the RER membrane pushes the growing chain. In fact, calculations by von Heijne[119] based on this assumption explained fairly well why certain amino acid sequences are translocated across the bilayer, whereas others are not. Arguments were provided that it is probably not the translation energy per se which is used to "push" the polypeptide across the membrane. It should be noted that the energy required for the initial establishment of a transmembrane orientation of the growing polypeptide chain should be much greater than for the subsequent residue-by-residue transfer. Significant energy compensation is expected for the latter process since for each amino acid residue incorporated into the membrane, another residue would appear in the luminal aqueous phase of the RER. If an aqueous tunnel is transiently formed within the membrane through which the polypeptide is transported, there would be even less energy required once the pore has been established (see Section II.G).

Perhaps the energy is provided by an as yet unknown modification mechanism. For example, it is conceivable that SRP is modified, e.g., by phosphorylation or adenoribosylation, and thereby activated for signal recognition and membrane binding. Demodification at the membrane could provide the energy required for the initiation of translocation.

2. Regulation of Protein Transfer — Obligatory Coupling of Synthesis and Translocation?

The discovery of the SRP-induced translational arrest by Walter and Blobel[36,180] raises the interesting question as to whether protein transfer could be regulated. It was proposed that the chain elongation arrest would prevent the misdirection of proteins destined for export, in the case where membrane-binding sites are limiting.[36] One could also imagine that synthesis and export of a translocated protein are regulated at this level. Of course, in

the latter case, a special mechanism for discrimination among different translocated proteins has to be postulated.

Does a translational arrest occur in vivo? The answer is not yet known with certainty. Data by Richter and Smith[259] and Richter et al.[10a] do not support this assumption. mRNA coding for secretory proteins is found in the postribosomal supernatant when injected in excess into *Xenopus* oocytes. The high salt extract of rough microsomes rather than K-RMs stimulated the synthesis of secretory proteins. These data indicate that membrane-binding sites are not limiting and that an insignificant amount of mRNA carries elongation-arrested ribosomes. A translational arrest has not been observed in vitro in the reticulocyte lysate system which contains low amounts of endogenous SRP,[196] even if more SRP is added.[382] The same observation has been made with a Hela-cell-free system.[225] Two membrane proteins with uncleaved signal peptide, the MP26 of the eye lens and the Ca^{2+}-ATPase of the sarcoplasmic reticulum, require SRP for membrane incorporation in vitro, but a translational arrest is not found in the wheat germ system in the absence of microsomes.[68] Ovalbumin only shows a transient SRP arrest.[209] Perhaps the absence of a translational arrest is related to the presumed internal location of the signal peptide in these cases. Recently, a partially reconstituted SRP-molecule was constructed which lacked the two smallest polypeptides of the original particle, but showed nevertheless full translocation activity. The translational arrest was, however, abolished.[259a] All these observations cast doubt on the idea of an obligatory coupling of translational arrest and translocation. Conversely, it is possible that other factors, e.g., other RNPs, interact with the ribosome at the same regulatory site as used by SRP, without playing a role in the translocation process.

Recently, we have theoretically analyzed factors which could dissociate the translational arrest from the translocation process.[260] A mathematical model which faithfully describes available experimental data (Figure 3) was used to analyze the following parameters: concentrations of SRP, of microsomal SRP-receptor, of ribosomes and mRNA, location of the signal peptide in a protein chain, and size of the "window" during which SRP can interact with the nascent polypeptide chain. The "window" is defined as the distance in amino acid residues between the initial and final point for an interaction of SRP with the growing chain emerging from the ribosome.

As expected, the size of the "window" is important for the extent of the translational arrest and of protein translocation: the longer the chance for SRP to interact with the nascent chain, the more SRP is bound. It should be noted that, depending on the size of the "window", the translational arrest is more pronounced at shorter times than after longer periods of incubation (ribosomes are slowed down by the continuous interaction with SRP), i.e., inhibition is partially released with time. The size of the "window" may be a specific property of a given polypeptide. It could depend, for example, on the folding of the protein chain. One may speculate that the "window" may be greater for N-terminally located signal peptides that for internal ones since in the latter case the peptide may be rapidly buried in the already synthesized protein part. For very small proteins, like prepromellitin (73 amino acid residues) the "window" must be small and the translational arrest should be weak. Indeed, this is observed.[225]

Since the inhibitory effect of a small "window" can be overcome by a moderate increase of the concentration of membrane receptors for SRP, a case may exist in which the translational arrest is absent, but translocation (and signal peptide removal) occurs. An example is given in Table 6.

Another important factor influencing the translational arrest and the translocation to a different extent is the constant for the binding of SRP to ribosomes carrying an exposed signal peptide. If the constant is lowered, the arrest is reduced more than the translocation efficiency (calculations not shown). This result may explain different efficiencies of signal peptides.

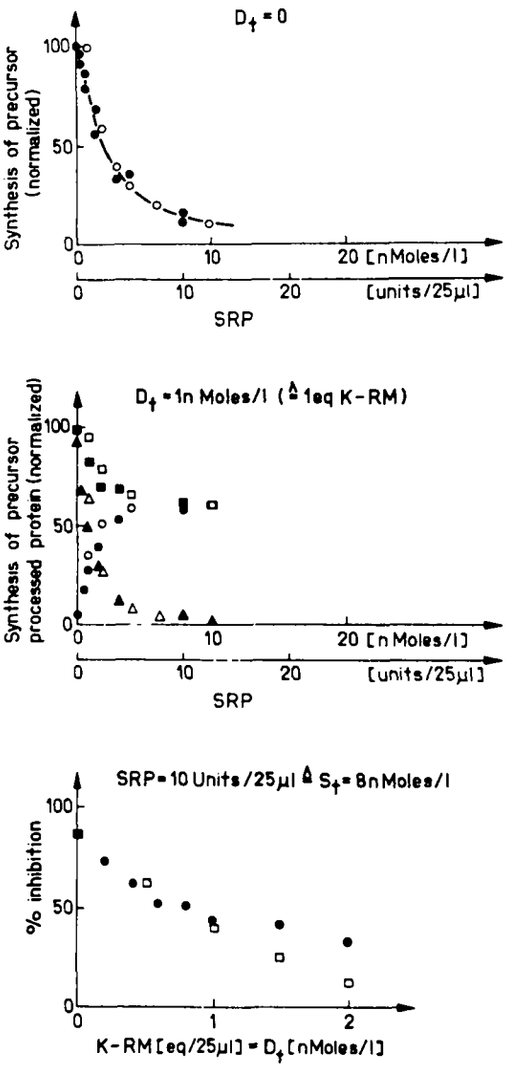


FIGURE 3. Mathematical modeling of the effect of SRP and its receptor on the in vitro synthesis and translocation of a secretory protein. In the figure, experimental data are compared with predictions made on the basis of a mathematical model.[260] The upper panel shows the inhibitory effect of SRP on the translation of preprolactin mRNA in the absence of microsomal membranes. In the presence of KCl-washed membranes (K-RMs) (middle panel), addition of SRP leads to the conversion of precursor molecules (triangles) to translocated and processed ones (circles). Squares give the total protein synthesized. The lowest panel shows the effect of addition of K-RMs on the total protein synthesis in vitro. The release of the translational arrest exerted by SRP is clearly seen. The experimental data (filled symbols) are taken from the literature.[179,180,223] Calculated steady-state data (open symbols) were obtained by the use of the following parameter values:[260] concentrations of mRNA and ribosomes: 1 and 100 nmoles per liter, respectively; rate constants of initiation, elongation, and termination of translation: 0.012 ℓ/min × nmol, 20 min^{-1}, and 20 min^{-1}, respectively; association constant for binding of SRP to ribosomes carrying exposed signal peptides: 2.5 ℓ/nmol; association constant for binding of the complex to the SRP-receptor (docking protein): 0.03 ℓ/nmol; number of codons occupied by a ribosome: 12; and size of the "window": 10 amino acid residues. D_f gives the concentration of the docking protein. Equivalents of K-RMs and units of SRP are defined in Reference 179.

Table 6
MODELING OF A CASE WHERE DISSOCIATION OF SRP-INDUCED TRANSLATIONAL ARREST AND TRANSLOCATION OCCURS

SRP concentration (nmoles per liter)	Translational arrest (docking protein = 0) synthesis of precursor (nmoles × 100/ℓ × min)	Translocation (docking protein = 5)	
		Synthesis of precursor	Synthesis of mature protein (nmoles × 100/ℓ × min)
0	3.26	3.26	0
1	3.26	2.42	0.84
2	3.26	1.91	1.35
4	3.25	1.34	1.92
8	2.75	0.83	2.43

Note: The values given in the table are steady-state rates obtained after integration of a system of more than 200 differential equations. The mathematical model is based on the treatment of Heinrich and Rapoport[165] as extended recently.[260] The following parameter values were used for the calculations: size of the "window": 1 amino acid residue; residue at which SRP binds to the nascent polypeptide chain: 70; concentrations of mRNA and ribosomes: 1 and 100, respectively; number of codons occupied by a ribosome: 12; association constants for binding of SRP to ribosomes carrying nascent chains: 2.5; and association constant for binding to the SRP receptor (docking protein): 0.03. All concentrations are expressed in nmoles per liter.

The position of the signal peptide in a protein chain should not per se dissociate the two effects (calculations not shown). An increase of the mRNA-concentration, however, weakens both the extent of the arrest of translation and of translocation, and the latter process can be made more efficient by higher levels of SRP-receptors, leading again to a dissociation of the two processes. It may be concluded that dissociation of the two effects need not be caused by specific properties of the polypeptide in study.

In general, processing and translocation of a polypeptide can be observed even at low concentrations of SRP where a translational arrest in the absence of microsomes is insignificant (see Table 6). This is easily explained: a SRP arrest requires that ribosomes pile up in front of the arrest site at the mRNA so that eventually the initiation site where translation starts becomes blocked. Translocation, on the other hand, only depends on the situation at the arrest site and can occur when the piling up of ribosomes does not extend all the way back.

Rough extrapolations to the situation in a cell show that inhibition of translation by SRP is negligible and translocation essentially complete. Such a situation appears sensible and is in agreement with the preliminary data discussed above.

An exciting new perspective in the regulation of protein synthesis by SRP has been opened recently. Proinsulin biosynthesis in pancreatic B-cells is known to be stimulated by glucose at the translational level during the first hour.[261] Welsch et al.[262] recently gave preliminary evidence that glucose increases the percentage of SRP bound to microsomal membranes. If these findings can be verified, they may indicate that SRP, its receptor, or some other part of the translocation machinery can be modified and is subject to regulatory influences. Furthermore, one may raise the question as to whether the translocation of all proteins is blocked in the absence of glucose (which seems unlikely) or whether and how preproinsulin synthesis is affected specifically.

3. The Loop Model — a Likely Hypothesis

How does the signal peptide cross the membrane? According to the signal hypothesis in its original form, its N-terminus should cross the membrane first (Figure 1).[18] In this manner, positively charged residues usually located at the terminal part of the signal peptide would have to pass the hydrophobic lipid environment first. Furthermore, the succeeding hydrophobic domain, which has a high affinity for the lipid environment, would have to be translocated entirely across the membrane so that the mature peptide can follow. Obviously, translocating the signal peptide to the other side of the lipid bilayer in this manner would require a great deal of energy. In order to circumvent these difficulties, a loop model was proposed,[14,41,141] according to which the N-terminal, hydrophilic, or charged part of the signal peptide would stay in the cytoplasm (Figure 2). Several predictions of the loop model have been verified in prokaryotes, these include:

1. If signal peptide cleavage is prevented, the resulting precursor protein should be anchored with its uncleaved signal peptide in the membrane. Indeed, prolipoprotein, accumulated in the presence of globomycin, was located in the cytoplasmic membrane of *E. coli* cells; most of the protein was translocated through the bilayer and lysine residues −19 and −16 were inaccessible to modification from outside unless the cells were disrupted.[263-265]
2. Signal peptides transposed to an internal location are functional in *E. coli* for protein secretion.[47,172,173] Obviously, such results are difficult to explain if the hydrophilic N-terminus were to cross the membrane first. However, a direct demonstration that the N-terminal part stays in the cytoplasm is missing as yet.
3. The location of the cleaved-off signal peptide in the membrane fraction and the facilitating role of the positive charges at the N-terminus of prolipoprotein are consistent with the loop model.[111,112]

For eukaryotes, evidence for the loop model is scarce. Internal signal peptides would fit the loop model. Some membrane proteins have a N-terminal membrane-anchoring sequence presumed to be the uncleaved signal peptide (see Table 5) and most of their polypeptide chain translocated across the RER membrane. Again, such an orientation would be difficult to explain on the basis of a linear extrusion model.

Other preliminary evidence indicated that the hydrophilic extreme N-terminal part of signal peptides of eukaryotic secretory proteins stays in the supernatant fluid of microsomal vesicles after translocation in vitro.[266]

Taking evidence and plausibility together, the loop model appears an attractive hypothesis which needs, however, more experimental support, particularly for eukaryotic systems.

4. Tunnel vs. Direct Transfer Across the Phospholipid Bilayer

Blobel and Dobberstein[18] have proposed that the growing polypeptide chain is transferred across a hydrophilic pore transiently formed in the RER membrane. Theoretical arguments, on the other hand, indicate that the transport could proceed directly through the phospholipid bilayer without excessive energy demand[118,139] (see also Section II.B.3).

At present there are no data which prove or disprove the existence of tunnel proteins. Recently, Ferro-Novick et al.[267,268] have found a new class of secretory mutants in yeast (sec53 and sec59) in which the translocation process appears to be halted after shift to the nonpermissive temperature and the protein is completed in the cytoplasm. If the transmembrane orientation of the secretory proteins can be convincingly demonstrated, these data would provide evidence for the involvement of a protein component in the translocation process itself, but not necessarily for a tunnel protein. Another promising approach should be the cross-linking of nascent polypeptide chains with membrane components during their

transport across the RER membrane. It is also conceivable that the opening of an aqueous tunnel could be monitored by an increase of conductivity across the bilayer or by an increased ion exchange, provided that suitable experimental conditions can be worked out.

It is obvious that the actual mode of protein transfer across membranes is a crucial point not yet solved. It is not even known whether the protein chain meets a hydrophobic or hydrophilic environment during passage.

5. Linear or Domain Transfer?

Translocation across the RER membrane is believed to proceed linearly as the nascent chain is elongated.[28-29a,32] It is conceivable, however, that larger parts of the growing polypeptide are buried within a tunnel in the membrane (see Section II.G). For *E. coli*, evidence exists that entire polypeptide domains can be translocated at once across the membrane. Randall[253] has used the accessibility of nascent chains of periplasmic proteins to externally added protease as a criterion for the transport across the inner membrane of *E. coli*. Limited proteolysis was employed to identify specific regions of the polypeptide chain. It was concluded that translocation of the polypeptide is a late event relative to the extent of chain elongation, occurring in some cases only after completion of the protein. It appears that this important result should be further strengthened by classical peptide mapping experiments.

Late cotranslational or post-translational transport of proteins appears to be quite usual in *E. coli* and may reflect the efficiency of translocation.[80,200a,252,253] Fusions of the malE and lacZ gene have recently been shown to be transported post-translationally if they contain only short portions of the mature exported polypeptide (class II and III, see Table 2) and cotranslationally, if the portion is long (class IV and V).[110a] Mutations in the positively charged segment of the lipoprotein signal peptide lead to a slow, post-translational transport.[111,112] It is possible that in these cases folded polypeptide domains cross the phospholipid bilayer.

It should be pointed out that post-translational membrane transfer of a protein does not exclude the possibility that a cotranslational event is required. For example, a pore in the membrane could be opened which is used by the exported protein only after its completion. Signal recognition may also be a cotranslational event. Nevertheless, the requirement of a membrane potential for protein export and the possible domain transfer are clearly properties distinct from those known for the eukaryotic system.

G. Sequential Insertion Model and Amphipatic Tunnel Hypothesis — Two Extensions of the Signal Hypothesis

1. Description of the Models

Both extensions of the signal hypothesis have been suggested in order to explain, in particular, the biosynthesis of complex membrane proteins. In both models, it is assumed that signal peptides initiate translocation of a polypeptide by the transient involvement of integral membrane proteins. An aqueous tunnel, through which the nascent chains are transported across the membrane, is postulated as before in the signal hypothesis.[18] In the requirement of a complex translocation system, both models differ from the membrane-trigger hypothesis[93] (see Section II.B.3).

The model, which we shall call "sequential-insertion model", has been proposed by Blobel[11] and Sabatini et al.[268a] It extends the signal hypothesis by making two assumptions:

1. A second type of topogenic sequence is postulated: stop-transfer peptides. These sequences are assumed to trigger a translocation stop, i.e., reverse the previous function of a signal peptide.

2. It is postulated that more than one signal peptide can be present in a single polypeptide chain. Assuming the presence of alternating signal and stop-transfer sequences, the polypeptide would be incorporated in a stepwise manner by repeated initiation and termination of translocation.

In its simplest formulation, the model assumes that each topogenic sequence determines the location of the following, not the preceding, part of the nascent polypeptide chain. Some hypothetical examples are shown in the schemes of Figure 4. It should be noted that after each stop-transfer sequence the synthesis of the protein would continue with a free ribosome until a following signal peptide redirects it to the membrane. If a second signal peptide existed, the protein would span the membrane twice, and so on. The membrane-spanning segments would be signal and stop-transfer peptides in an alternating manner. In a more sophisticated version of the model, it is assumed that a signal peptide may also transport a polypeptide segment previously synthesized. In this case, a stop-transfer peptide may precede a signal peptide (see Figure 4, Case 1d).

Finally, the model may be supplemented by assuming the existence of imbedding sequences in a polypeptide chain (see Section II.B.2) in addition to signal and stop-transfer peptides (Figure 4, Case 2b).

The recently proposed amphipatic tunnel hypothesis,[269] assumes only the presence of one signal peptide in an exported polypeptide chain, regardless of its complexity. No other topogenic sequences triggering the translocation process are postulated.

The model is an extension of the signal hypothesis by making more specific assumptions on the properties of the transient tunnel in the membrane. It is assumed that it contains an aqueous environment but that its inner surface has both hydrophilic and hydrophobic areas. The tunnel would provide binding sites for parts of the polypeptide chain transported through it. Hydrophobic portions would have a tendency to be retained at the hydrophobic surface area. Hydrophilic portions would normally not be retained unless a special charge distribution or H-bonding pattern would be complementary to the polar parts of the channel. Binding is assumed to be in equilibrium after each step of elongation of the polypeptide chain. If several parts of a protein retained within the tunnel can interact with each other so as to form a hydrophilic surface, they are able to pass through it without further hindrance. When the protein is completed, the ribosome falls off from the mRNA and dissociates into subunits. It is assumed that, concomitantly, the tunnel is disassembled. Any parts of the polypeptide retained within the tunnel during its biosynthesis are then being tested for compatibility with an absolutely hydrophobic environment. If charges cannot be compensated or H-bonds cannot be formed between donors and acceptors, the polypeptide segment(s) is (are) expelled from the membrane. Expulsion would occur so as to minimize the energy demand, i.e., the segment would generally be liberated to the aqueous phase where most of the polypeptide chain is already located. If compatibility is achieved, the protein will be retained in the membrane. Compatibility requires hydrophobicity of the surface of the membrane-incorporated polypeptide domain. It should be noted that the constraints on membrane compatibility are greater than for retainment in the tunnel: a short hydrophobic sequence may be bound to the tunnel, but could be expelled from the phospholipid bilayer.

It should also be noted that a special case exists when the ribosome has reached the termination codon of the mRNA. In general, the segment of the polypeptide chain actually in the tunnel will not be compatible with the hydrophobic environment and will be expelled. A membrane anchor which is able to interact with the phospholipid bilayer in a stable manner may still be buried in the ribosome and could stop "pulling" the polypeptide chain across the membrane.

The application of the hypothesis to several cases is illustrated in Figure 5. Different secretory proteins could have different modes of membrane transfer. They could be trans-

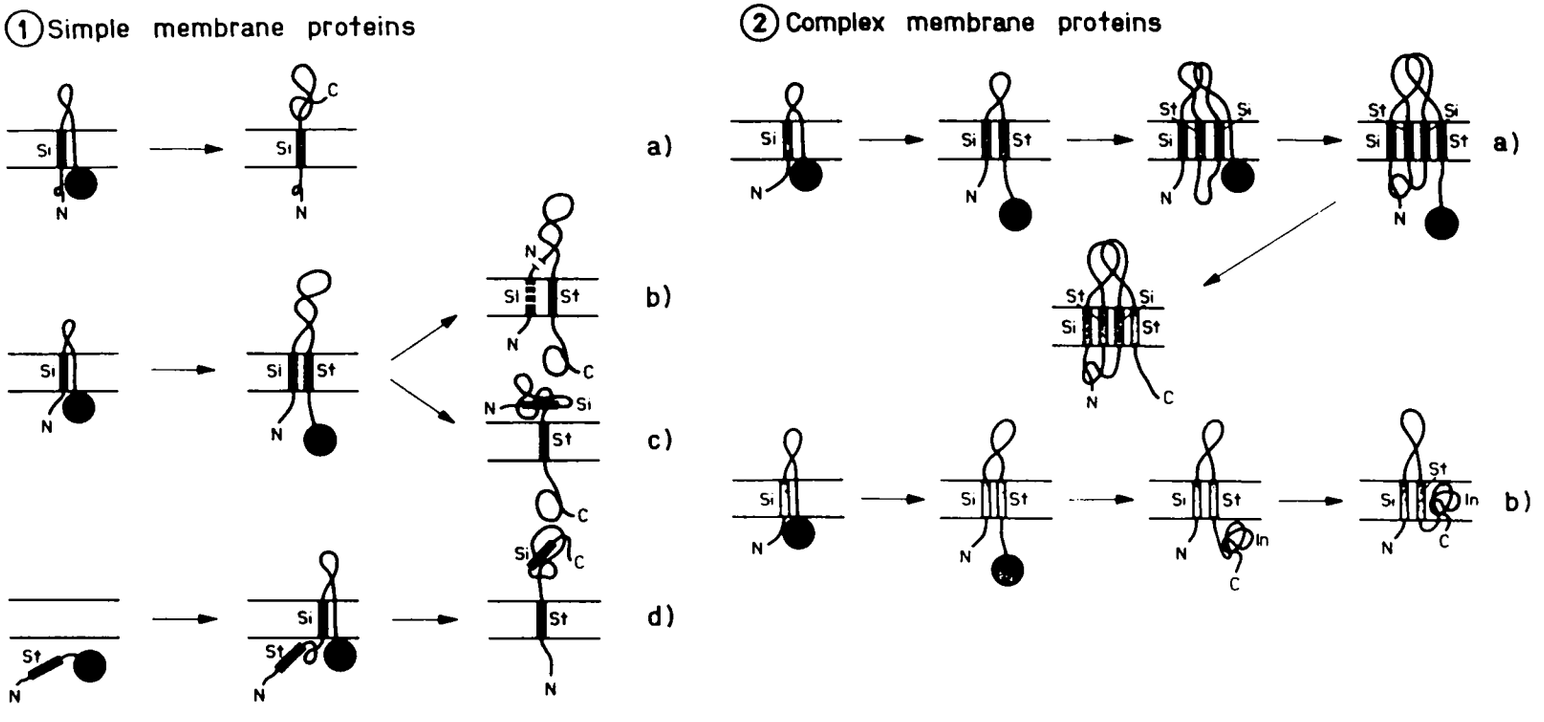


FIGURE 4. Hypothetical cases of sequential membrane insertion of proteins. The schemes show the cotranslational incorporation of hypothetical polypeptides into membranes. The upper side corresponds to the ectoplasmic side. The filled circles indicate the ribosomes synthesizing membrane proteins. They can be either bound to the membrane or "dangling" at the nascent polypeptide chain. Si, St, and In denote signal, stop-transfer, and insertion (imbedding) sequences, respectively. (1a): The signal peptide is uncleaved and serves as membrane-spanning segment. In the final state, the N-terminus remains in the cytoplasm. (1b) and (1c): In the final state, the protein spans the membrane once with a stop-transfer sequence. The N-terminal signal peptide can be cleaved off (1b) or translocated across (1c). (1d): The stop-transfer peptide precedes the signal peptide. (2a): A polytopic membrane protein is inserted into the membrane by alternating signal and stop-transfer peptides; (2b): the N-terminal part of a complex membrane protein is inserted by a signal and stop-transfer peptide and the C-terminal part by an insertion (imbedding) sequence (or domain). For details see text.

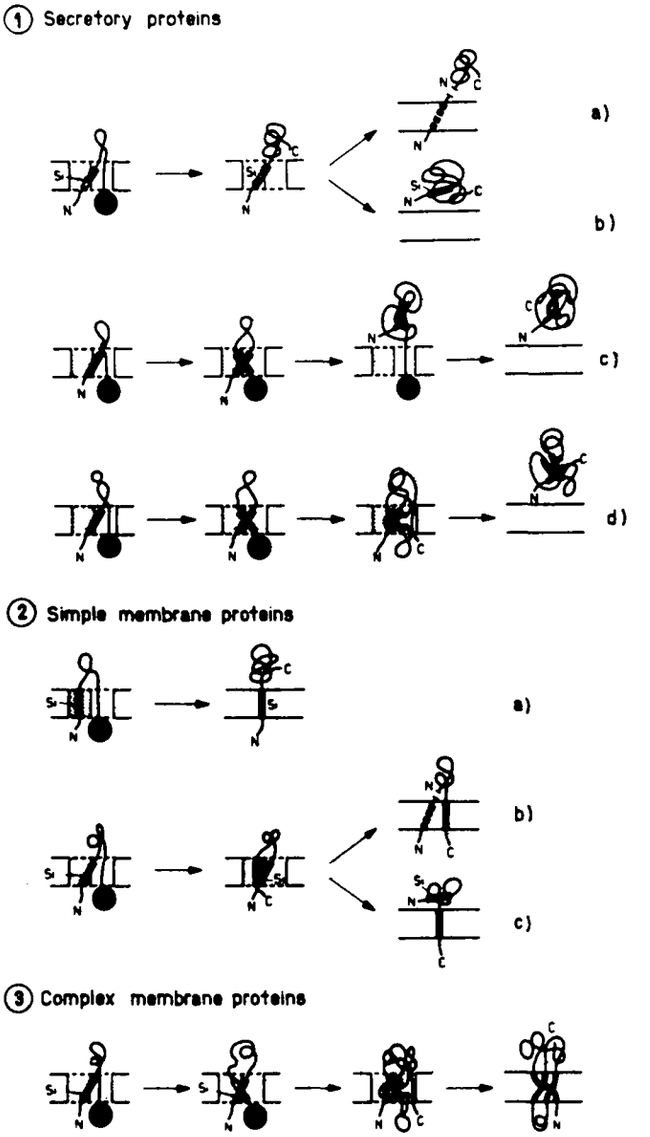


FIGURE 5. Translocation of proteins across membranes according to the amphipatic tunnel hypothesis. The schemes show the cotranslational translocation of hypothetical polypeptides. The hydrophobic and hydrophilic parts of the presumed tunnel in the membrane are indicated by dotted and blank areas, respectively. Cylinders indicate portions of the polypeptide chain retained in the tunnel. Hydrophobic and hydrophilic parts of the retained segments are indicated in grey and black, respectively. Si denotes the signal peptide, N and C the NH_2- and COOH- termini, respectively. The filled circles are the ribosomes which remain membrane bound until completion of the polypeptide chain (see Figure 4). The signal peptide is either cleaved off (1a) or translocated across the membrane (1b) since it is not compatible with membrane insertion (the hydrophobic portion is too short). (1c) and (1d): The protein contains several segments transiently retained in the tunnel. These parts fold into a hydrophilic globular structure capable of traversing the membrane either before (1c) or after (1d) completion of the protein. (2a): The signal peptide is compatible with membrane insertion (the hydrophobic portion is long enough, (cf. 1b) and serves as a membrane-spanning segment (2b) and (2c): The signal peptide is followed by a second hydrophobic segment retained in the tunnel. The signal peptide can be either cleaved off or translocated across the membrane. In both cases, the C-terminus would remain in the cytoplasm. (3): One example of a complex membrane protein is considered with several segments retained in the amphipatic tunnel. These parts of the protein could fold to yield a hydrophobic surface compatible with the hydrophobic membrane environment. Note that the incorporated polypeptide segments need not traverse the membrane perpendicularly. For further discussion see text.

located linearly through the membrane (Case 1a or b) or even after completion of the protein if the parts transiently retained in the membrane remain unstable in the bilayer (Case 1d). It is also obvious that the segments of the polypeptide retained in the tunnel could associate in a very complex manner typical for tertiary structure formation, both for formation of a hydrophilic or of a hydrophobic surface.

It should be emphasized that we have adopted the loop model for all schemes in Figures 4 and 5. Fixation of the signal peptide in this manner defines the final orientation of a protein in the membrane: the N-terminus should be in the cytoplasm unless the signal peptide is cleaved off or is translocated across the bilayer.

2. Comparison of the Models

Both models are obviously able to cope with simple cases such as proteins having a N-terminal, cleaved signal peptide, and a C-terminal membrane-spanning segment (Figures 4 and 5). Difficulties arise, however, for the sequential-insertion model if the amino acid sequence staying in the cytoplasm is very short (for example, immunoglobulin M has only three amino acid residues following the membrane anchor).[59] Since about 20 to 40 amino acid residues are buried within the ribosome,[193,194] translocation would occur in some cases after termination of the polypeptide synthesis. Such a post-translational positioning would be fully compatible, on the other hand, with the amphipatic tunnel hypothesis. It is striking that many simple membrane proteins have 20 to 40 amino acid residues staying in the cytoplasm, which would correspond to an almost simultaneous stop of translocation and protein synthesis. There are, however, exceptions where the C-terminal tail in the cytoplasm is long.[270,271] Furthermore, Yost et al.[271] have inserted the nucleotide sequence coding for the membrane-spanning segment of immunoglobulin M between sequences coding for the β-lactamase and alpha-globin, and showed that after translation in vitro, the artificial protein had the expected orientation, with the β-lactamase part in the lumen of RER vesicles and the α-globin part outside. This experiment shows that the membrane anchor need not be located at the C-terminus. This result is, however, compatible with both models.

A touchstone for the models is provided by complex membrane proteins spanning the membrane more than once. According to the sequential-insertion model, multiple signal and stop-transfer sequences should exist. However, the presence of only one signal peptide, as postulated in the amphipatic tunnel hypothesis, is supported by the occurrence of cleaved-off peptides in complex membrane proteins. For example, the major envelope proteins of *E. coli* ompA,[272] ompF,[273] and lamB[274] possess cleaved signal peptides with typical structures. Bacteriorhodopsin also has a cleaved-off peptide which, although not particularly hydrophobic, may be the signal peptide in the halobacterium.[128] There is no reason to believe that further signal peptides are required for membrane insertion. Indeed, for the lamB protein it is clear that mutation of the cleaved-off signal peptide converts the protein into a cytoplasmic one (see Section II.D). If there were additional signal peptides, one would expect a protein partially incorporated into the membrane.

According to the sequential-insertion model, at least some of the seven helixes of bacteriorhodopsin, which traverse the membrane almost perpendicularly, should be alternating signal and stop-transfer peptides. Functional differentiation between the different helixes is, however, difficult to justify by their primary structures. According to the amphipatic tunnel hypothesis, they are all merely membrane-spanning segments. In other membrane proteins, there are no long hydrophobic or uncharged segments capable of spanning a membrane so that internal signal and stop-transfer peptides cannot be identified.[274,275]

According to the sequential-insertion model, the folding pathway of a complex membrane protein would be determined by the order of the topogenic sequences. Each membrane-spanning segment, or at most two consecutive ones, would be incorporated separately into the membrane. This follows from the assumption that the translocation system disassembles

after each stop-transfer sequence. The intermediates should be stable in the phospholipid bilayer.

On the other hand, according to the amphipatic tunnel hypothesis, folding would generally yield a membrane-compatible structure only when all sequence parts required for the final conformation have been synthesized. Such a folding pathway is more similar to that of typical soluble, globular proteins. In fact, there is no reason to assume that sequential insertion will result in a structure corresponding to a global energetic minimum. It appears, however, that the final conformation of complex membrane proteins is thermodynamically determined (see Section II.B.2.b).

As discussed before (Section II.F.5), evidence exists for a domain transfer across the cytoplasmic membrane in *E. coli*.[253] Such data are difficult to reconcile with the sequential insertion model but are compatible with the amphipatic tunnel hypothesis. The requirement of sequences in the mature part of an exported protein for efficient transport (see Section II.D.1) is easily understood within the framework of the amphipatic tunnel hypothesis, in contrast to the sequential-insertion model. On the other hand, the fact that a cytoplasmic protein (globin) can be translocated across the RER membrane would be more consistent with the sequential-insertion model, although it does not contradict the alternative model.

The amphipatic tunnel hypothesis also provides a clue as to why some hydrophobic amino acid sequences are able to cross the membrane. For example, the hydrophobic peptides of some viral envelope proteins which are responsible for fusion of the virion with cellular membranes (e.g., hemagglutinin HA2 of the influenza virus) are transferred across the RER membrane during biosynthesis of the proteins in the infected host cell.[276-278] Some fusion peptides are known to be buried in the interior of the protein molecule until activated by low pH.[279,279a] We would predict that these hydrophobic sequences are transiently retained within a tunnel in the membrane and transferred across it when folding of the polypeptide permits shielding of the hydrophobic regions by hydrophilic residues. The sequential insertion model would assume that stop-transfer sequences must have additional features besides hydrophobicity and length.

As mentioned before, many membrane proteins have uncleaved signal peptides which are either serving as a membrane-spanning segment or translocated across the bilayer (see Tables 5 and 7). The amphipatic tunnel hypothesis makes specific predictions on the structural differences between membrane-spanning and translocated signal peptides: the latter ones should be incompatible with membrane incorporation. As shown in Table 6, such peptides appear to have shorter hydrophobic and uncharged regions or proline residues within them. Furthermore, in some examples of translocated signal peptides, there is a carbohydrate chain attached to a neighboring amino acid residue. These features may indeed prevent a stable membrane anchoring of these sequences.

The two models of protein translocation must also explain the results obtained in *E. coli* with fused polypeptides of exported proteins and β-galactosidase (see Section II.D.1).

Why is a signal peptide insufficient for protein export in some cases? This fact appears difficult to explain by the sequential insertion model. On the other hand, according to the amphipatic tunnel hypothesis, the amino acid sequence of the β-galactosidase, which is not designed to be transported across a membrane, may contain many parts transiently retained within the tunnel, so that only small portions of the protein are translocated. After dissassembly of the tunnel, the incompatible parts are expelled from the membrane into the cytoplasm.

The fact that some fusion polypeptides interfere with normal export of the cell and remain in the membrane could be explained by the sequential-insertion model by assuming that the β-galactosidase part does not contain a stop-transfer sequence so that disassembly of the tunnel cannot occur. This would eventually use up all the tunnel proteins available in the cell and stop protein export. It is less obvious why interference is dependent on the length

Table 7
SEQUENCES PRESUMED TO BE MEMBRANE-SPANNING OR TRANSLOCATED SIGNAL PEPTIDES

Membrane-Spanning Signal Peptides

Protein	Sequence	Ref.
Influenza (Lee) neuraminidase	$H_2\dot{N}$-MLPSTVQTLTLLLTSGGVLLSLYVSASLSYLLYS...	366
Influenza (Victoria) neuraminidase	$H_2\dot{N}$-MNPNQKIITIGSVSLTIATICFLMQIAILVTTVT...	367
Influenza (WSN) neuraminidase	$H_2\dot{N}$-MNPNQKIITIGSICMVVGIISLILQIGNIISIWI...	367
Cytochrome P-450 LM_2	$H_2\dot{N}$-MEFSLLLLLAFLAGLLLLLF...	368
Cytochrome P-450 PB	$H_2\dot{N}$-MEPSILLLLALLVGFLLLLV...	355
Cytochrome P-450 b	$H_2\dot{N}$-EPTILLLLALLVGFLLLLVRG...	369
Cytochrome P-450 a	$H_2\dot{N}$-MLDTGLLLVVILATLTVMLLLTL...	369
Epoxide hydratase	$H_2\dot{N}$-MWLELVLASLLGFVIYWFVS...	356

Translocated Signal Peptides

Protein	Sequence	Ref.
Semliki Forest virus p62	$H_2\dot{N}$-SAPLITAMCVLA*NATFPCFQPPCVPCCYENAEA...	370
Sindbis virus p62	$H_2\dot{N}$-SAAPLVTAMCLLG*NVSFPCDRPPTCYTREPSRA...	371
Ovalbumin	H_3C-CONH-GSIGAASMEFCFDVFKELKVHHANENIFYCPIAIMSALAMVYLGAKDSTR...	31
Bovine opsin	$H_2\dot{N}$-MNGTEGPNFYVPFS*NKTGVVRSPFEAPQYYLAEP...	353

Note: Amino acid residues are given in the one-letter code. Hydrophobic residues are underlined. Stars indicate sites of attachment of carbohydrate. Proline residues are emphasized by dots. Charges residues are also indicated.

of the portion of the exported protein in the fusion protein (see Table 2). According to the amphipatic tunnel hypothesis, the N-terminal part would be translocated with increasing efficiency the longer it is, whereas the C-terminal (β-galactosidase) part may have too many segments retained within the tunnel. After completion of the fusion protein, it would be forced into a transmembrane orientation which does not allow a stable membrane anchoring. Such a situation may alter the membrane structure and interfere with protein export of the cell. In the case of the lamB protein, beyond a certain length of the portion of the exported protein, a stable membrane incorporation would be possible and the β-galactosidase part would be expelled into the cytoplasm. Such a situation would not interfere with normal protein export (see Table 2).

How could one distinguish experimentally between the sequential insertion and the amphipatic tunnel hypothesis? According to the latter hypothesis, SRP would be needed only once, whereas according to the alternative model, it would be required as many times as there are signal peptides in a polypeptide chain. The dependence on SRP may be testable in synchronized translation experiments. The cycling of ribosomes between a free and a membrane-bound state during the synthesis of a membrane protein, postulated in the sequential-insertion model, could also be tested in experiments.

In summarizing, it appears that the amphipatic tunnel hypothesis provides a better understanding of several facts, but that crucial assumptions remain to be verified. It should be noted that the model can also be used to explain the post-translational transport of proteins across membranes other than the RER membrane and the cytoplasmic membrane of bacteria.

III. PROTEIN IMPORT INTO MITOCHONDRIA

A. An Organelle with Different Compartments — Additional Problems for Protein Sorting

The mitochondrial genome encodes only a small number of polypeptides. The majority of mitochondrial polypeptides are encoded on nuclear DNA, synthesized in the cytoplasm, and transported into the organelle.[280,281] How is this large group of different proteins directed specifically to the mitochondria? Further differentiation is also needed since mitochondria are delimited by two membranes giving rise to two aqueous compartments: the matrix and the intermembrane space. Specific sets of proteins are found in all four possible compartments: matrix, inner membrane, intermembrane space, and outer membrane. Furthermore, membrane proteins can have all conceivable orientations in at least the inner and, possibly also, the outer membrane. They can be anchored, for example, to the inner membrane from either side, or they can traverse the membrane more than once. Obviously, one would like to know not only the signals directing a protein to a specific mitochondrial compartment, but also the mechanism by which these polypeptides selectively and vectorially traverse one or two membranes. Compared with the protein transport across the RER or the cytoplasmic membrane of bacteria, relatively little is known concerning the molecular details of the transport into mitochondria, but progress is quickly being made. We shall discuss shortly the present stage (for more comprehensive reviews see Hay et al.[282] and Reid[283]).

B. Post-Translational Import

It was initially believed that the import of mitochondrial proteins occurs cotranslationally, mainly based on data showing the attachment of cytoplasmic 80S ribosomes to mitochondria of yeast.[284-287] However, although there was enrichment found in mRNA coding for some mitochondrial proteins in the mitochondria-bound polysome fraction,[289] at most, 60% of the total mRNA for a mitochondrial polypeptide is recovered, and some imported proteins were entirely synthesized in free polysomes.[290]

It is clear now that there is no obligatory coupling of protein synthesis and translocation. Mitochondrial polypeptides completed in vitro in cell-free translation systems can be post-translationally taken up by isolated mitochondria.[291,292] Post-translational import has also been described in vivo. Cytoplasmic precursor polypeptides can be found in pulse-chase experiments in *Neurospora* and yeast,[293,294] and reversible inhibition of the import process permits the accumulation of precursor polypeptides in the cytoplasm and their post-translational uptake into mitochondria.[294] Post-translational import of proteins into mitochondria of higher eukaryotes has also been amply described.[295,296] Whether it is the exclusive way, or whether post- and cotranslational import coexist is not certain as yet.

C. Most Imported Proteins Have Precursors — Do These Contain Signal Peptides?

The majority of imported mitochondrial proteins, with the exception of those located in the outer membrane, are synthesized as larger precursors. The extension has been found invariably at the N-terminus for all cases so far studied, although additional extensions at the C-terminus which are cleaved cannot yet be excluded. The size difference between the precursor and the mature form can be as large as 10 kdalton.

On the other hand, there exist proteins in all mitochondrial compartments which are not synthesized as larger precursor polypeptides. Examples include the 2-isopropyl-malate synthase located in the matrix,[298] the ADP/ATP-translocator located in the inner membrane,[299] and cytochrome c[300,301] and adenylate kinase,[302] components of the intermembrane space.

Of course, based on the knowledge of protein translocation across the RER membrane one would be inclined to assume that the N-terminal extensions normally found are signal peptides guiding the polypeptide to the mitochondrial compartment. In the exceptional cases, where proteolysis is not required for protein import, the signal peptide could remain with the mature protein.

A number of nuclear genes coding for mitochondrial proteins have recently been cloned and sequenced. A list of deduced amino acid sequences of the N-termini is given in Table 8, together with the cleavage sites so far determined. Even this limited information indicates that there is no obvious common sequence property among N-terminal extensions even if the proteins are transported to the same submitochondrial compartment and presumably carry functionally identical signal peptides. It appears, however, that the N-terminal peptides are predominantly basic. The 17-kdalton subunit of the cytochrome c reductase seems to be an exception with a high content of acidic amino acid residues (see Table 8). The import of this polypeptide has not yet been studied in detail. Unfortunately, the limited number of representatives for each group and the unknown location of the cleavage site in some cases do not yet permit the deduction of further regularities.

Once the mitochondrial genes have been cloned, the way is open to manipulate them by in vitro mutagenesis and gene fusion. This is particularly easy for yeast for which plasmid vectors and mutants are available. Douglas et al.[303] have constructed a fusion protein between the N-terminal part of the β-subunit of the F1-ATPase and a major portion of the β-galactosidase, an approach which proved successful for the study of protein transport in bacteria (see Section II.D). The artificial protein was found at the location of the natural mitochondrial polypeptide, arguing for the existence of some topogenic information in the N-terminal portion of its sequence. However, it appears that at least 139 residues of the F1-ATPase are required to target the fusion protein into the mitochondria, although the cleaved-off peptide only comprises about 20 amino acid residues. Parts of the mature β-subunit may therefore be required for the targeting process.

Convincing evidence for a cleavable signal peptide in the precursor of yeast cytochrome oxidase subunit IV was provided recently by Hurt et al.[303a] A fusion protein was constructed by gene technology, containing 22 (out of 25) amino acid residues from the cleavable prepiece of the subunit IV precursor and the entire polypeptide chain of the cytosolic mouse dihy-

Table 8
N-TERMINAL SEQUENCES OF PROTEINS IMPORTED INTO MITOCHONDRIA

Location of the mature protein	Protein	N-terminal sequence of the precursor	Ref.
Outer membrane	70-kdalton protein	MKSFITRNKTAILATVAATGTAIGAYYYYNQLQQQQQRGKKNT...	317
Intermembrane space	Cytochrome c	MTEFKAGSAKKGATLFKTRCLQCHTVEKGGPHKVGPNLHGIFGRH...	373
	Cytochrome c peroxidase	MTTAVRLLPSLGRTAHKRSLYLFSAAAAAAAAAAATFAYSQSHKRSSSSPG...	374
		GGSNHGWNNWGKAAALASTT...	
Inner membrane	ATP synthase (subunit 9)	MASTRVLASRLASQMAASAKVARPAVRVAQVSKRTIQTGSPLQTLKRTQM	305
		TSIVNATTRQAFQKRAYS...	
	Cytochrome c reductase (14-kdalton subunit)	MPQSFTSIARIGDYILKSPVLSKLCVPVANQFINLAGYKKLGL...	375
	Cytochrome c reductase (17-kdalton subunit)	MDMLELVGEYWEQLKITVVPVVAAAEDDDDNEQHEEKAA...	376
Matrix	EF-Tu	MSALLPRLLTRTAFKASGKLLRLSSVISRTFSQTTTSYAAA...	377
	Citrate synthase	MSAILSTTSKSFLSRGSTRQCQNMQKALFALLNARHYSS...	378
	Ornithine transcarbamylase (human)	MLFNLRILLNNAAFRNGHNFMVRNFRCGQPLQNKV	379
	(rat)	MLSNLRILLNKAALRKAHTSMVRNFRYGKPVQSQV	380

Note: Cleavage sites are indicates by arrows.

drofolate reductase. The fusion protein was imported into the mitochondria and cleaved by the matrix protease (see Section III.D.3). These results show that the cleavable prepiece contains all the necessary information for importing an attached cytosolic protein into mitochondria. Also, the cleavage site appears to be determined by the conformation of the signal peptide rather than by a specific amino acid sequence, since the fusion protein lacks the cleavage site of the authentic subunit IV precursor.

Recently, Hurt et al.[303b] have extended their work and have shown that even the first 12 amino acid residues of the subunit IV precursor are sufficient to guide the protein to the matrix in vitro and in vivo. Cleavage does not occur, however, if only 12 or 16 amino acid residues are linked to the dihydrofolate reductase. Similar results were obtained for the alcohol dehydrogenase. Again, the cleavable presequence directed the linked cytosolic enzyme to the matrix space.

Even more exciting are experiments with the inner membrane protein cytochrome c_1.[303c] If the entire presequence (61 amino acid residues) was fused with the dihydrofolate reductase, the enzyme was correctly transported to the inner membrane. If only the first 32 residues were used, the enzyme ended up in the matrix space. These data indicate that a signal sequence (guiding the protein to the matrix) is followed by a stop-transfer signal, keeping the polypeptide in the inner membrane. A similar case, except that the stop occurs already in the outer membrane, is the likely mechanism of biosynthesis of outer membrane proteins (see below).

An "addressing signal" for mitochondrial import appears to be located in cytochrome c close to its C-terminus, comprising the heme-attachment region.[304]

The proteins of the outer membrane of the mitochondria appear to be generally made without precursor. This has been clearly demonstrated for the pore-forming polypeptide porin, a protein of about 30-kdalton mass. There is no shift in the electrophoretic mobility when the primary translation product is compared with the mature, membrane-bound form, and the N-terminal methionine residue remains uncleaved.[312-314] At least three additional outer membrane proteins of unknown function are also not proteolytically processed.[314] On the other hand, a 35-kdalton polypeptide of rat liver outer mitochondrial membranes has been described to be synthesized via a precursor (35.5 kdalton).[315] It is not yet clear whether this small size difference reflects proteolytic processing of the protein.

For the 70-kdalton protein of the outer mitochondrial membrane of yeast, it is likely that the membrane-anchoring sequence is at the N-terminus. It has a long stretch of uncharged amino acid residues (residues 10 - 37),[317] and removal of 203 amino acid residues from the C-terminus does not prevent its import into mitochondria.[318]

Recently, it has been shown that a fusion protein consisting of the first 12 residues of the 70-kdalton protein and the dihydrofolate reductase is partially targeted to the matrix and inner membrane.[303b] It therefore appears that even an outer membrane protein contains a signal sequence for targeting to the matrix space and that a stop-transfer sequence keeps it in the outer membrane.

In summary, it can be concluded that a positively charged N-terminal amino acid sequence functions as a signal for import into mitochondria and is sufficient information. Import does not depend on cleavage, which is specified by another sequence part (possibly overlapping with the targeting signal).

Directing a protein to the correct mitochondrial compartment may not be the only function of the N-terminal extensions. For example, the subunit 9 of the ATP-synthase of *Neurospora* is an extremely hydrophobic protein of 81 amino acid residues.[304a] The N-terminal extension, on the other hand, is remarkably polar, comprising 66 mostly hydrophilic amino acid residues.[305] Since the precursor is soluble in the cytoplasm, it may be suggested that one role of the extension is to keep the protein in solution.[306,307] Interestingly enough, this subunit is synthesized in yeast inside the mitochondria without precursor.[308,309] Nevertheless, yeast

mitochondria can import and process the *Neurospora* polypeptide.[310] Presumably, the *Neurospora* precursor carries a signal similar to that of imported yeast precursor polypeptides.

In keeping with the membrane-trigger hypothesis of Wickner,[93] several precursors of mitochondrial proteins appear to be different in conformation from their mature counterparts. Such changes have been documented for the *Neurospora* ATP/ADP-translocator,[299] cytochrome c,[300] rat ornithine transcarbamylase,[311] and the α- and β-subunits of the F1-ATPase of yeast (cited in Reference 283).

D. Components Involved in Protein Import

We shall now summarize the knowledge on the protein components involved in recognition, membrane transfer, and proteolytic processing of imported proteins.

1. Receptors for Mitochondrial Precursor Polypeptides

It appears plausible to assume the presence of receptor proteins which guide precursor polypeptides into the mitochondria. The best example is the import receptor for apocytochrome c. This apoprotein can be prepared in milligram amounts by chemically removing the heme group from cytochrome c, or in trace amounts by cell-free translation.[319,320] Labeled apocytochrome c is very tightly bound to mitochondria, but is not internalized if the attachment of heme is inhibited by addition of deuterohemin. The labeled precursor can be specifically removed from the mitochondria by excess of unlabeled protein. Furthermore, the bound precursor can be converted to holocytochrome c if the inhibitory effect of deuterohemin is abolished by addition of protohemin,[320] indicating that binding is indeed essential for the import process. There is a limited number of binding sites and they are sensitive to proteases. Import of apocytochrome c was also studied with rat liver mitochondria.[304] Similar import receptors have been found for other precursor proteins. If oxidative phosphorylation is inhibited, many precursor polypeptides accumulate at the outer surface of the mitochondria and can be internalized when the block is released.[321] It could be shown that dilution of the mitochondria did not slow down the import of the ADP/ATP-translocator, indicating that the protein does not dissociate from the binding sites before being imported.

Further evidence was provided by Riezman et al.,[316,322] who isolated right-side-out vesicles of the outer mitochondrial membrane which were able to bind various precursors including precytochrome b_2, but not cytosolic enzymes. Mature cytochrome b_2 was not bound. Vesicles isolated from the inner mitochondrial membrane did not show the binding phenomenon.

Do all precursor polypeptides bind to the same receptor? The answer is probably "no". Addition of excess of unlabeled apocytochrome c did not affect the uptake of the ADP/ATP-translocator or of the subunit 9 of the ATP-synthase under conditions in which the import of labeled apocytochrome c is completely prevented.[323] Even more interestingly, the import of cytochrome c_1 is not blocked by excess of cytochrome c, although both proteins end up in the same submitochondrial compartment, the intermembrane space.[324] It appears at present that apocytochrome c uses a pathway different from that of all other precursors. Unfortunately, similar competition experiments have not been feasible as yet for other precursor proteins due to the lack of sufficient material. Some precursors may, however, be obtained in vivo in substantial amounts if their processing is prevented.[294,325] The recent cloning of genes for some imported proteins also provides the possibility of synthesizing sufficient amounts of these polypeptides.

The apocytochrome c receptor has been purified recently as a 20-kdalton protein.[325a] Surprisingly, the receptor appears to be located in the intermembrane space; this is not in contradiction to the protease experiments cited above, since relatively high concentrations of trypsin had to be used in order to inactivate the receptor. It is possible that apocytochrome c dissolves into the outer membrane and is "pulled" into the intermembrane space by its receptor.

2. Soluble Factors in the Cytosol

Recent studies indicate that soluble factors present in reticulocyte lysates and yeast cytosol stimulate the in vitro import of proteins into mitochondria.[326-328] Indeed, it has been known for some time that uptake of proteins synthesized in the wheat germ cell-free system is inefficient in comparison with the reticulocyte lysate. The soluble factor in the yeast cytoplasm has a molecular mass of 40 kdalton.[328] The function of these factors in the translocation process is unknown as yet. Recently, evidence was provided that in addition to the mentioned proteins, a cytosolic RNA-species present in a ribonuclear protein particle is required for the import of several precursor proteins into mitochondria.[328a] After cell-free translation, the precursors were found in a 400-kdalton complex in close association with a RNAse-sensitive component. In analogy to SRP, such a factor may recognize the signal peptides in mitochondrial precursors and guide them to "docking protein(s)" in the outer membrane.

3. Proteolytic Processing of Mitochondrial Precursors

As stated before (Section III.C), most mitochondrial polypeptides (with the exception of those destined to the outer membrane) are synthesized as larger molecules which have to be processed proteolytically.

In most cases, there is a single cleavage event: removal of the N-terminal extension by an endopeptidase. This enzyme has been identified as a soluble component of the matrix in yeast, rat, and maize mitochondria.[329-333] It appears to be a neutral metalloprotease which can be inhibited by chelators of divalent metal ions such as 1,10-phenanthroline or EDTA. The inhibition can be reversed by addition of Zn^{2+}, Co^{2+}, or Mn^{2+} ions, but it is not known which ion is the physiological cofactor of the enzyme.[330,333,334] The protein appears to be a dimer of molecular mass of 110 to 115 kdalton. The partially purified enzyme cleaves many different mitochondrial precursors in the absence of mitochondria, yielding the correct N-terminus of the mature forms.[335] On the other hand, it exhibits a remarkable substrate specificity as it does not cleave nonmitochondrial proteins nor denatured precursors. It appears therefore that the enzyme recognizes some spatial structure in the precursor molecules rather than a specific amino acid sequence or peptide bond.

At least one further protease is required for the processing of some intermembrane proteins. Cytochrome b_2, cytochrome c_1, and cytochrome c peroxidase appear to be processed in two steps. The matrix protease cleaves these polypeptides not to the mature forms, but to intermediates.[297,336,337] The same intermediates have been found in vivo under certain conditions[336] and in vitro with isolated mitochondria.[324,338] For cytochrome b_2, it has been shown that the intermediate is membrane bound in contrast to both the precursor and the final form which are soluble. Similarly, cytochrome c peroxidase precursor which had been taken up by the mitochondria appears membrane bound. For cytochrome c_1, which is anchored in the outer surface of the inner membrane, it has been shown that covalent attachment of a heme group is a prerequisite for the action of the second protease.[337] The enzyme is most likely located in the intermembrane space.

A two-step processing for the precursor of rat liver ornithine transcarbamulase, which is a matrix enzyme, has been described by Mori et al.,[331] but is under dispute (see Reid).[283] A two-step processing of the precursor of the subunit 9 of the ATPase has also been described recently, but the similarity of the sequences around the two cleavage sites may suggest that the matrix enzyme does both jobs.[338a]

Is proteolysis coupled to translocation or is it a subsequent step? Proteolytic processing is in general a rapid reaction with a half-life in the order of 0.5 to 2 min. The only exception is cytochrome c peroxidase which is only slowly processed so that intramitochondrial precursor polypeptides could be observed.[294] Precursors apparently localized inside the mitochondrion have also been found for other cases in vitro.[339] These results would indicate that translocation and processing are not necessarily coupled, a fact which might have been

anticipated from the existence of uncleaved precursors. On the other hand, these cases may be exceptional or in vitro artifacts. It has been shown recently that rhodamin G, which inhibits the matrix protease, prevents the in vivo uptake of a subunit of the F1-ATPase, but not of the ADP/ATP-translocator which is not proteolytically cleaved.[340] These results would therefore suggest that in cases where proteolysis occurs, uptake and processing are tightly coupled. It is, however, not known whether imported mitochondrial proteins can be processed *during* their translocation.

4. Energy Requirement of Protein Import

The import of proteins to the matrix or to the inner membrane of mitochondria requires energy. This was first shown by Nelson and Schatz[341] on intact cells. On the basis of results with inhibitors of oxidative phosphorylation, and with a rho-minus mutant of yeast which lacks a functional ATP-synthase, it was claimed that ATP, and not an electrochemical gradient across the inner mitochondrial membrane, is required. However, later work both with yeast and *Neurospora* demonstrated that this conclusion was erroneous.[342,343] Oligomycin, an inhibitor of the proton-translocating ATPase, blocks the protein import in vitro, although the ATP-concentration is expected to increase inside the mitochondrion. This result was confirmed with a combination of different inhibitors and substrates of respiration, showing that even if the ATP-concentration remained unchanged, variation of the electrochemical gradient influenced the protein import. The original error may be explained by the ability of rho-minus mutants to generate a significant membrane potential by the action of the electrogenic ADP/ATP-translocator, which may have been sufficient to support protein import into the organelle. The energy appears to be required for the translocation process and not for proteolytic processing. Accordingly, the import of several proteins which are not cleaved is also dependent on the electrochemical gradient. Also, the matrix protease is able to catalyze the cleavage reaction in a partially purified form. Furthermore, the precursor of cytochrome c peroxidase is taken up by an energy-consuming step before it is cleaved,[336] and precursors of the mitochondrial proteins accumulate outside the mitochondrion if oxidative phosphorylation is inhibited.[294]

At present, it is not clear whether energy is actually consumed or if energy is needed to keep the membrane in a certain state.[344] The need for a membrane potential has been mentioned before the export of proteins across the cytoplasmic membrane in bacteria (Section II.F.1). The polarity of the potential is, however, reversed: in mitochondria, transport occurs towards the more negatively charged matrix and in bacteria, towards the more positively charged periplasm. Proteins transported to the outer membrane of mitochondria do not require a membrane potential, as clearly shown for porin which is incorporated into isolated vesicles of the outer mitochondrial membrane. The insertion cannot be prevented by inhibitors of oxidative phosphorylation under conditions in which the import of matrix and inner membrane proteins is blocked.[313,314] The outer mitochondrial membrane itself is unable to generate a membrane potential since it is leaky to small ions.

Proteins located in the intermembrane space fall into two classes. Cytochrome b_2, cytochrome c_1, and cytochrome c peroxidase do require a membrane potential for import.[297,324,336,338,341,342] On the other hand, cytochrome c does not require an electrochemical gradient.[323] Taking into account that the former class is initially processed by a matrix protease (Section III.D.3) and has membrane-bound intermediates, it is very likely that they are translocated in part across the inner mitochondrial membrane. In contrast, cytochrome c would reach the intermembrane space by a pathway which does not involve the inner membrane, and therefore a membrane potential is not required.

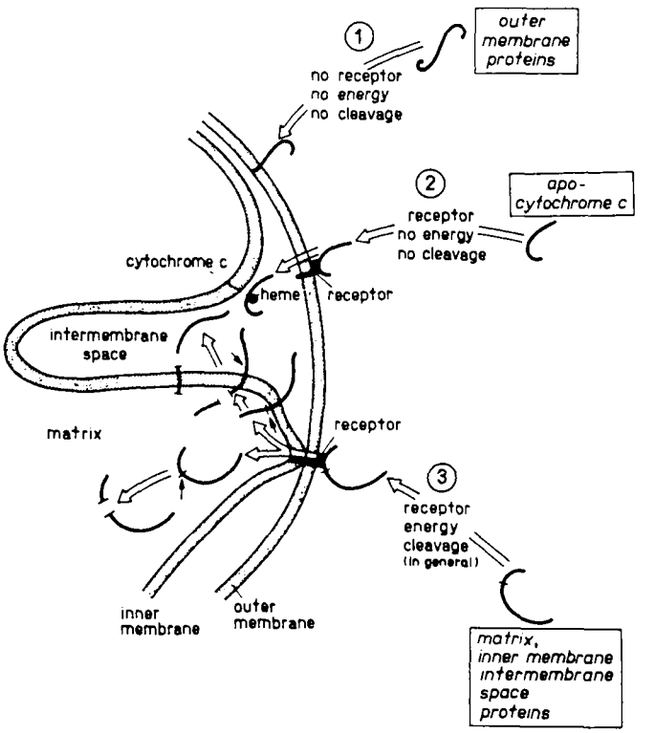


FIGURE 6. Three different pathways of protein import into mitochondria. For explanation and details see text. Cleavages of the imported polypeptide chains are indicated by small arrows. The absence of receptors for incorporation of proteins into the outer membrane is hypothetical.

E. Different Pathways for Different Mitochondrial Proteins

Figure 6 summarizes our present knowledge on different pathways for protein import into mitochondria. There are at least three, and their characteristics are as follows

1. Transport of proteins to the outer mitochondrial membrane — these proteins may carry a targeting sequence for mitochondrial import followed by a stop-transfer signal which keeps them in the outer membrane. They do not need energy for membrane incorporation. Generally, there is no proteolytic processing involved.
2. Import of cytochrome c — this protein appears to take a route different from that of all other proteins so far tested. It uses a separate receptor probably located in the intermembrane space, does not require a membrane potential for its import, and is not proteolytically processed. Since it ends up in the intermembrane space, one may assume that it only crosses the outer membrane.
3. Transport of proteins to the matrix, inner membrane, and intermembrane space — this, the largest group of proteins, crosses (at least in part) both the outer and inner mitochondrial membrane. They appear to have targeting signals at their N-terminus. All these proteins appear to require a protease-sensitive receptor on the surface of the mitochondria, an electrochemical potential difference across the inner membrane, and usually a matrix-located protease. Some proteins will cross the inner membrane completely and therefore become constituents of the matrix. Others will get stuck in the inner membrane and become components of it. Further sorting may occur if a mem-

brane-bound protein is cleaved by a protease located in the intermembrane space so that parts of these proteins would become constituents of this compartment. It has been suggested that junctions between the inner and outer membrane may be the sites of protein import.[11,344] The polypeptides would thereby cross both membranes simultaneously rather than one after the other. Direct evidence for this assumption has been provided recently by the demonstration that the precursor of the β-subunit of the F_1-ATPase can be trapped in an intermediate state where the N-terminus is already accessible to the matrix protease while the majority of the polypeptide chain is still located outside the mitochondrium. Energy is only required for the transmembrane transposition, not for the import of the major domain of the protein.[325a]

IV. FINAL REMARKS

It is obvious that great progress in the understanding of intracellular protein transport has been made during the last decade. In particular, we are approaching a detailed knowledge of the translocation of secretory proteins across the ER membrane. The discoveries of SRP and its receptor (docking protein) have provided important proof for predictions of the signal hypothesis. It is likely that other constituents of the transport machinery will be discovered during the next few years, eventually leading to the in vitro reconstitution of the translocation process by use of purified components. Hopefully, we shall also be able to elucidate the molecular mechanism by which a protein traverses a membrane, a question, which appears most challenging at present. New assay systems have to be developed in order to study the steps beyond the initiation of translocation. Chemical cross-linking with the nascent polypeptide chain and the search for mutants in membrane components involved in the process will probably be of great significance toward this goal.

The knowledge on membrane proteins clearly lags behind that of secreted proteins as indicated by the speculative nature of the models and the many open questions. In particular, the structure of complex membrane proteins is insufficiently known and the mechanism of their membrane incorporation has not yet been studied in depth. It is likely that DNA-recombinant technology and kinetic studies in vitro using purified components of the translocation apparatus and drugs or antibodies interfering at certain stages of the process will greatly contribute to the progress in the field. Such methods will allow the testing of the importance of a given sequence for the topology of a membrane protein.

It appears of general importance that protein transport across the RER membrane, the cytoplasmic membrane in bacteria, and the mitochondrial membrane(s), although distinct in several aspects, share similarities. The transported proteins appear to have signal peptides usually located at the N-terminus and cleaved off after their transfer across the membrane. Receptors recognizing the signal peptides also appear to be a general feature. One may speculate that in all cases where translocation of proteins across membranes occurs (as opposed to imbedding), there is a receptor-mediated transient formation of an aqueous tunnel in the membrane through which the polypeptide is transported, as suggested by the amphipatic tunnel hypothesis put forward in Section II.G. Such a model could also explain processes excluded from the present review, such as the import of toxins into cells where one of the subunits interacts with the membrane while the other penetrates into the interior.[345]

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