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Mize, Nancy K.

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ARTIFICIAL TRANSMEMBRANE PROTEINS: CONSTRUCTION, SYNTHESIS AND INSERTION INTO THE ENDOPLASMIC RETICULUM MEMBRANE

by

NANCY K. MIZE

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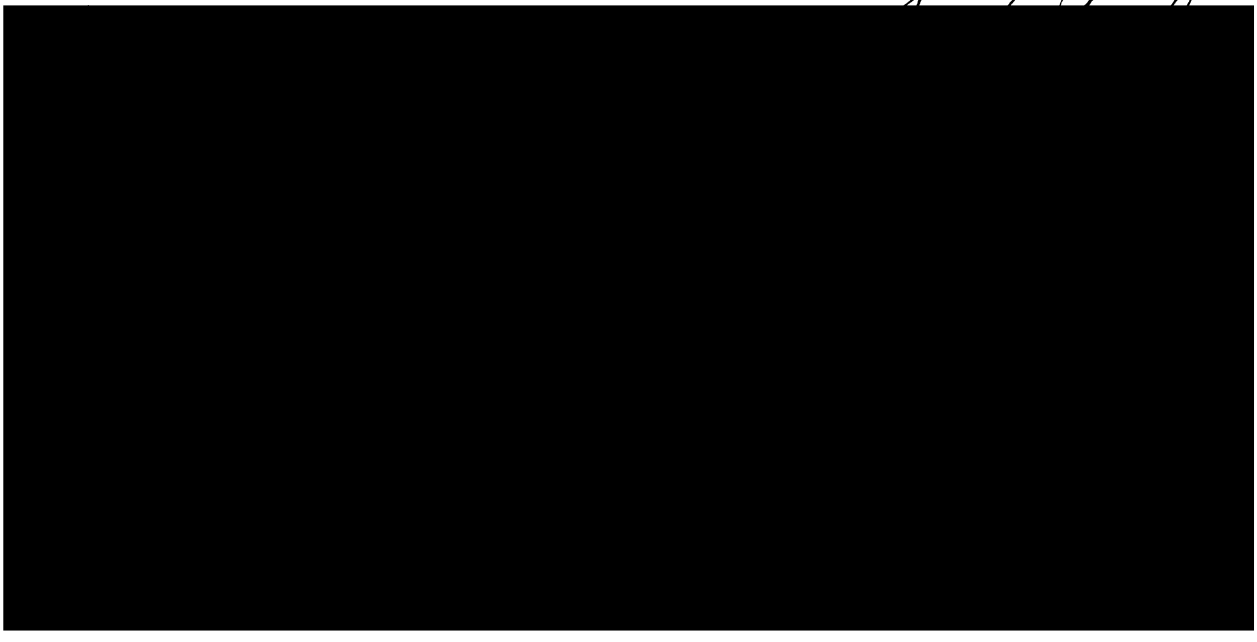

ARTIFICIAL PROTEINS: ANALYSIS OF MECHANISMS AND RULES FOR MEMBRANE INSERTION

ABSTRACT

Nancy K. Mize

In order to examine the mechanisms and rules of membrane insertion of simple transmembrane proteins, I have constructed several artificial proteins at the DNA level. With an *in vitro* transcription-translation system utilizing microsomal membranes, I have used these constructed, chimeric proteins to demonstrate that a hydrophobic region, previously defined as a stop transfer sequence, is capable of functioning as an uncleaved signal peptide to direct protein insertion into the ER membrane. I have demonstrated that this membrane insertion is dependent on Signal Recognition Particle (SRP) as are typical, cleaved, amino-terminal signal peptides. The major difference between the action of the signal peptide and the stop transfer sequence is that after insertion and cleavage of the signal peptide, a soluble secretory protein is generated, but after insertion of the stop transfer sequence, a transmembrane protein is generated.

Using other chimeric transmembrane proteins either with or without an amino terminal signal peptide, I have also demonstrated that the same transmembrane protein segment can span the membrane in opposite directions. The insertion of these artificial proteins via interaction with SRP indicates that the protein lacking a signal peptide initially inserts its hydrophobic

(stop transfer) region into the bilayer as a loop unlike a stop transfer sequence which inserts in an extended conformation.

This unexpected signal-like activity of the stop transfer sequence and the reversal of a transmembrane protein's assymetry have implications for models of membrane assembly of simple and complex proteins which are discussed here.

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Chapter 1

TRANSMEMBRANE PROTEINS: SYNTHESIS AND INSERTION INTO THE ENDOPLASMIC RETICULUM MEMBRANE

BACKGROUND

In 1975, George Palade described a model for movement of newly synthesized secretory proteins between cell compartments. Using radioautography adapted to electron microscopy and comparing these results with those from cell fractionation and an *in vitro* slice system, Palade and his colleagues (Caro and Palade, 1964; Redman et al., 1966; Castle et al., 1972) were able to trace radioactive proteins from their site of synthesis to secretion from the pancreatic exocrine cell. They found that pancreatic secretory proteins move from the endoplasmic reticulum (ER) via the golgi complex to the plasma membrane. The first step of the secretory pathway described by Palade (1975) is entry into the ER.

The Signal Hypothesis/Signal Sequence

The signal to move secretory proteins from the cytoplasm into the ER and the secretory pathway was first uncovered with *in vitro* protein synthesis experiments. In 1972, Milstein et al. presented evidence that immunoglobulin light chain mRNA translated *in vitro* yields a product that is larger than the *in vivo* protein. In 1975, using an *in vitro* assay with added microsomal membranes, Blobel and Dobberstein showed further evidence that processing to the mature, *in vivo* form requires a functional ribosome-membrane attachment, and that the processing activity is localized to the membranes. This extension of the Signal Hypothesis they presented (Blobel and Dobberstein, 1975b) suggests that the amino terminal 1500 molecular weight cleaved

portion is a "signal" for translating ribosomes to attach to the microsomal membrane where a temporary proteinaceous pore in the membrane is formed in conjunction with the ribosomal pore through which the nascent chain is extruding. Thus, proteins destined to cross the microsomal membrane contain in their mRNA sequence, an initial region coding for this signal sequence which in some way acts to coalesce the postulated temporary membrane pore with the ribosome and allow the nascent chain to extrude into the ER lumen.

The signal sequence, as defined by Blobel (1980), is a discrete segment of the nascent polypeptide chain which contains information for the translocation of the protein across a membrane; the information is addressed to specific integral membrane proteins which recognize the signal. A typical ER signal sequence, as defined by von Heijne (1985), is located at the amino terminus of the protein; it is approximately 15-30 amino acids long, and is cleaved by signal peptidase in the ER lumen. It has a hydrophobic region approximately 15 amino acids in length preceded by a short charged stretch and followed by 5 or more polar amino acid residues which include the cleavage site. There is no apparent consensus sequence defining a signal, but the predominant characteristic of an ER signal sequence appears to be a hydrophobic stretch of amino acids (Watson, 1984) which is recognized by specific cellular components.

Some of the membrane associated proteins which play a role in the targeting and translocation of the signal sequence have been characterized (Walter et al., 1984; Walter, 1987) and have led to the model shown in Figure 1. In 1980 and 1981, Walter and Blobel characterized the first of these membrane associated proteins, signal recognition protein (SRP), later renamed signal recognition particle
because it was found to contain a 7S RNA. SRP is composed of 6 different polypeptides and 1 small RNA molecule. The functions of SRP in protein targeting, elongation and translocation have been characterized by Siegel and Walter (1988). At the ER membrane, SRP interacts with SRP receptor, or docking protein (Gilmore et al., 1982; Meyer, et al., 1982) which provides for targeting to the ER membrane. Recently, a signal sequence receptor (SSR) has been localized as an ER membrane protein (Wiedmann et al., 1987). SSR receives the signal sequence presumably after the ribosome has lodged on the ER membrane. SRP and SRP receptor (or docking protein) are then released to recycle to target other newly emerging signal sequences.

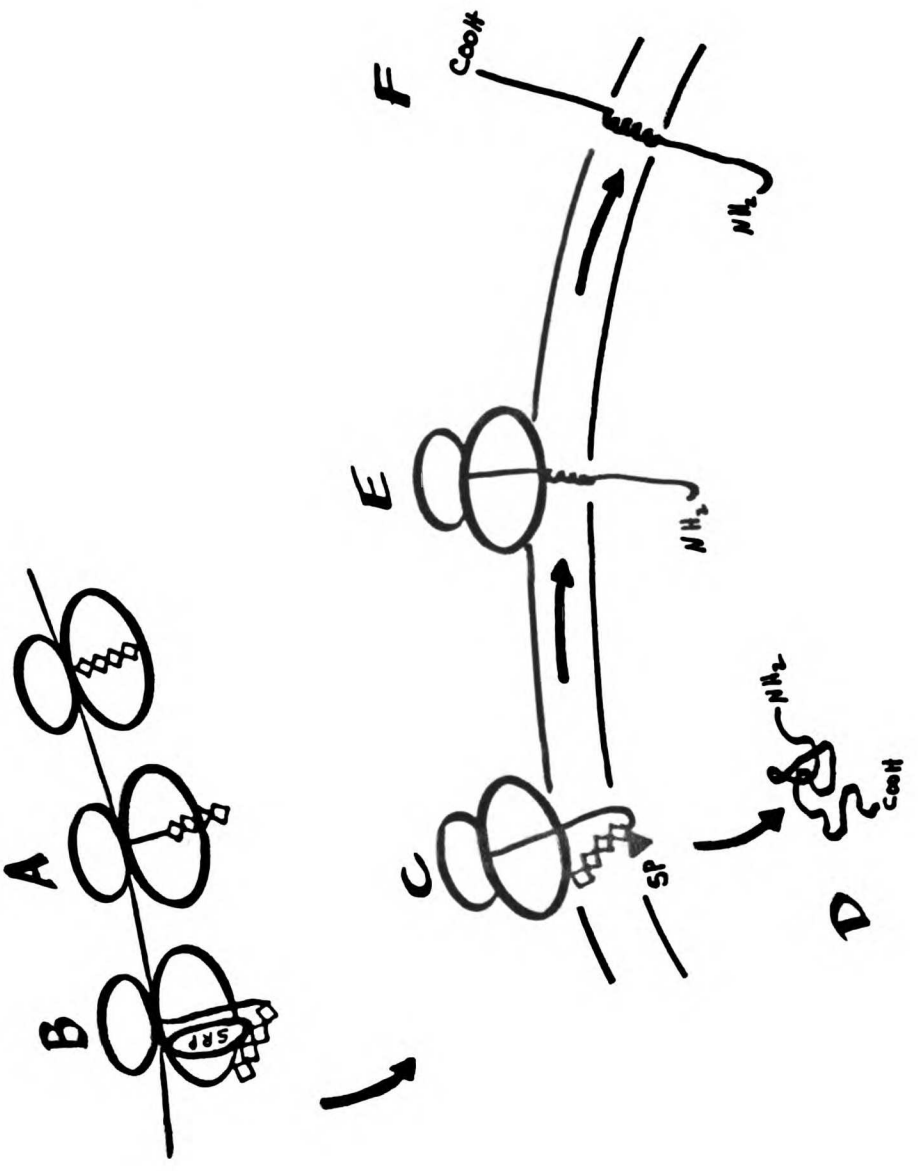
The ER signal sequence, the ribosome, SRP, SRP receptor and SSR all function in targeting nascent chains into the ER membrane. The mechanisms by which the nascent chain moves from the ribosome across the ER membrane are still unknown. Two models have emerged to explain movement of newly synthesized proteins across the ER membrane: One model suggests that movement of nascent peptide chains across the membrane involves direct

interaction of the nascent peptide with the lipid bilayer. This model predicts that the movement of the protein from the cytoplasmic side into the lipid bilayer and out again into the lumen of the ER is thermodynamically favored (Engelman and Steitz, 1981; Wickner and Lodish, 1985). The alternative model predicts a protein-protein interaction in a receptor mediated fashion for the movement of newly synthesized proteins across the ER membrane. This model suggests a proteinaceous pore continuous with the channel in the ribosome through which the nascent chain is extruded into the ER lumen (Blobel and Dobberstein, 1975; Blobel, 1980).

Stop Transfer/Membrane Anchor

Unlike secretory proteins, membrane proteins are not completely extruded into the ER lumen, but span the membrane with a stretch of hydrophobic amino acids which serve as a membrane anchor or stop transfer (Yost et al., 1983; Davis and Model, 1985; Davis et al., 1985; Adams and Rose, 1985). Membrane proteins with an internal stop transfer sequence and an amino-terminal signal peptide, are presumed to be targeted to the ER membrane in the same fashion as secretory proteins (Fig. 1A,B,C): as the amino terminal signal peptide emerges from the ribosome, SRP binds the signal and targets the complex to the ER membrane. As with secretory proteins, SRP binds SRP receptor and the signal sequence binds SSR in the membrane. Unlike secretory proteins, membrane proteins are not completely extruded into the ER lumen. Instead a hydrophobic stretch, or stop transfer sequence,

Figure 1. Model for SRP dependent targeting and membrane insertion (adapted from Walter et al. 1984). When an ER signal sequence, represented by diamonds, emerges from the ribosome (A), the signal sequence is then available to interact with SRP (B). SRP functions to target the nascent chain-SRP-ribosome complex to the ER membrane (C), where it forms a functional ribosome-membrane junction. Signal peptidase (SP), located on the lumenal side of the ER membrane, cleaves off the signal peptide and secretory proteins are translocated across and extruded into the lumen of the ER (D). However in the case of membrane proteins, sometime after emergence of the signal peptide, a hydrophobic stop transfer region of amino acids, represented by a squiggle, emerges from the ribosome and interacts with the ER membrane to stop the transfer of the rest of the nascent chain across the membrane (E), and a transmembrane protein is generated (F).


A
B
SRP
C
SP
D
NH2
COOH
E
NH2
F
COOH
NH2

emerges from the ribosome and somehow engages with the membrane (whether with lipid, SSR, or some other component of the postulated proteinaceous tunnel is not known) and halts the transfer of the remainder of the protein across the ER membrane (Fig. 1E,F) (Blobel, 1980; Sabatini et al., 1982). Thus a typical transmembrane protein, with an amino-terminal signal peptide, inserts its newly generated amino terminus into the lumen of the ER and its carboxy terminus is left in the cytoplasm (Fig. 1F). This asymmetry is achieved by the action of the signal peptide with SRP and SSR to begin translocation across the membrane and the action of the stop transfer to stop the transfer of the carboxy end of the protein across the membrane as it emerges from the ribosome.

However, there are several other variations of simple membrane proteins which span the membrane one time (Table I). Several identified membrane proteins do not have an amino terminal signal peptide; these proteins insert either the amino or carboxy terminus across the membrane. In this case the membrane spanning region of the protein acts as both a signal and an anchor, or signal/anchor (Chapter 2 of this work, Speiss & Lodish, 1986; Zerial et al., 1987).

Membrane insertion. Analysis of mechanisms and rules for insertion of simple membrane proteins: the goal of this investigation.

Simple membrane proteins with an amino terminal signal peptide and a stop transfer sequence assemble in the ER membrane with the amino terminus in the ER lumen after signal peptide cleavage (Gething and Sambrook, 1982; Yost et al., 1983; Guan and Rose, 1984; Doyle et al., 1986). However, many membrane proteins do not have an amino-terminal signal peptide to direct their membrane insertion (see Table I), and many proteins are more complex and span the membrane several times (Braell and Lodish,1981; Friedlander and Blobel, 1985; Mueckler and Lodish, 1986).

Topogenic models for membrane insertion

Friedlander and Blobel (1985) proposed that bovine opsin achieved its transmembrane asymmetry by alternating signal and stop transfer sequences. However, Audigier et al. (1987) in Blobel's laboratory has shown that 5 of the 6 transmembrane segments of opsin investigated maintained both signal and stop transfer activity (signal/anchor activity). All of the isolated segments were at least partially extractable with alkali, indicating that the stop transfer function is not absolute. In each case they found that the amino-terminal 34 residues were translocated across the microsomal membrane *in vitro*, indicating that the

Table 1. Comparison of the membrane orientation of several simple single-spanning membrane proteins. Group I: transmembrane proteins which have an amino-terminal cleaved signal peptide and translocate the amino terminus across the membrane (out of the cytoplasm). Group II: proteins which have no cleavable signal and translocate the carboxy terminus across the membrane. Group III: proteins which have no cleavable signal and translocate the amino terminus across the membrane.

TABLE I: VARIATIONS OF SIMPLE TRANSMEMBRANE PROTEINS

Protein	Which terminus is translocated?	Cleaved signal peptide?	Reference
Group I:			
VSV Glycoprotein	N	+	Rose & Gallione, 1981
Hemagglutinin	N	+	Gething & Sambrook, 1982
Membrane IgM heavy chain	N	+	J. Rogers et al., 1980
LDL receptor	N	+	Russell et al., 1984
Group II:			
Transferrin Receptor	C	-	Zerial et al., 1987
Asialoglycoprotein Receptor	C	-	Speiss & Lodish, 1986
Paramyxovirus HN	C	-	Hiebert et al., 1985
Mouse Invariant chain	C	-	Lipp & Dobberstein, 1986
Influenza neuraminidase	C	-	Bos et al., 1984; Markoff et al., 1984
Group III:			
M_2 protein of Influenza A	N	-	Hull et al., 1988
Influenza B virus NB protein	N	-	Williams & Lamb, 1986
V-erbB glycoprotein 74	N	-	Hayman et al., 1983
Sindbis virus PE_2 protein	N	-	Bonatti and Blobel, 1979

transmembrane segments themselves do not solely determine membrane asymmetry, and that the domains flanking the transmembrane segments may have an effect on membrane asymmetry.

Unfolding models for membrane insertion

More recently, dynamic properties of the domains flanking hydrophobic regions have been proposed to affect translocation (Eilers and Schatz, 1986; Randall and Hardy, 1986; Rothman and Kornberg, 1986; Fitts et al., 1987; Crooke et al., 1988). Eilers and Schatz (1986) show that binding to a specific ligand has been shown to inhibit transfer of a protein across the mitochondrial membrane, and from these studies they suggest that partial unfolding is required for transfer of the protein across mitochondrial membranes post-translationally. Crooke et al. (1988) demonstrate that a "trigger factor" is required to keep ProOmpA in a translocation-competent state which is a more protease sensitive state, suggesting partial protein unfolding is required for transfer across the membrane. Randall and Hardy (1986) correlate a competence for transfer across the membrane with a lack of tertiary structure by changes in the sensitivity of maltose-binding protein to protease. All these studies suggest that partial unfolding is required for post-translational transfer of secretory proteins across the bilayer. ER translocation of some parts of transmembrane proteins occurs post-translationally and may also require a partially unfolded state, but this has not yet been studied. The membrane assembly of simple and complex

membrane proteins may be directed by combinations of signal and stop transfer topogenic sequences in combination with some as yet unknown properties of domains flanking these topogenic sequences.

In this work I have chosen to investigate the insertion of very simple membrane proteins in order to uncover some general rules and mechanisms by which simple and more complicated proteins assemble in the membrane. My approach has been to utilize an *in vitro* system which lends itself well for the combined investigation of protein synthesis and insertion into microsomal membranes and to construct, using DNA, various artificial proteins which exploit the isolated functions of the signal and stop transfer domains. I have sought to understand the role of the stop transfer domain in its interaction with SRP and with the ER membrane (Chapter 2) and I have uncovered some of the rules governing the asymmetric insertion of simple transmembrane proteins (Chapter 3).

Chapter 2

A STOP TRANSFER SEQUENCE FUNCTIONS AS A SIGNAL FOR NASCENT CHAIN TRANSLOCATION ACROSS THE ENDOPLASMIC RETICULUM MEMBRANE.

Summary

In concert with a signal peptide, a stop transfer sequence functions to stop the transfer of the carboxy terminus of a protein across the membrane of the endoplasmic reticulum during synthesis; this generates a transmembrane protein (Yost et al., 1983; Guan and Rose, 1984). Here, we demonstrate that the stop transfer sequence, derived from the transmembrane segment of IgM heavy chain, is capable of functioning as a signal for membrane insertion and partial translocation of a protein into the endoplasmic reticulum lumen. By manipulation at the DNA level, we have constructed an artificial protein without a typical signal peptide but with a stop transfer sequence located approximately 140 amino acid residues from the amino terminus of the protein and 110 residues from the carboxy terminus of the protein. The stop transfer mediated membrane insertion of this protein is dependent on signal recognition particle (SRP) as are typical amino terminal cleaved signal peptides. But, unlike typical signal peptides, the stop transfer sequence is not cleaved by signal peptidase and is not located at the amino terminus of the protein. Like an internal signal peptide, the internal stop transfer sequence directs the insertion of either the amino or carboxy terminal flanking domains. The functions of signal peptides and stop transfer sequences have been previously defined (for reviews see Blobel, 1980, Sabatini et al., 1982; Wickner and Lodish, 1985; Garoff, 1985). This unexpected discovery that a stop transfer sequence can act as a signal to direct membrane insertion of

flanking domains influences current models and concepts on the mechanisms of membrane assembly of simple and complex membrane proteins.

Introduction

A major process in eucaryotic cells is the sorting of proteins from the cytoplasm into the secretory pathway. This process begins at the endoplasmic reticulum (ER), where proteins destined for the secretory pathway are first inserted into and across the bilayer separating the cytoplasmic compartment from the extracytoplasmic compartments. Nascent proteins are directed to the ER membrane by a signal peptide that interacts wtih signal recognition particle (SRP) (Walter et al., 1981; Walter and Lingappa, 1986).

This process begins in the same way for a transmembrane protein destined for the secretory pathway: an amino terminal signal peptide emerges from the ribosome, SRP interacts with the signal peptide to target the ribosome/nascent chain/SRP complex to the ER membrane. However, once the ribosome-membrane junction has been established and the nascent chain has begun to extrude into the ER lumen, a hydrophobic membrane spanning segment or stop transfer sequence emerges from the ribosome and enters the membrane bilayer. This stop transfer sequence functions to stop the transfer of the rest of the nascent chain across the bilayer. In this chain of events the signal peptide is required to begin the translocation of the nascent chain across the membrane before the

stop transfer can function to stop the transfer of the carboxy terminus of the protein across the membrane. This partial translocation across the membrane generates a transmembrane protein.

Here, we uncover a new function for the stop transfer sequence. By using molecular biology to construct new artificial proteins at the DNA level, we are able to analyze the function of the stop transfer sequence in the absence of a preceding signal sequence. This combination of constructed plasmid DNA with an *in vitro* transcription/ translation system and the co-translational addition of microsomal membranes yields a powerful approach for the study of specific domains of secretory and transmembrane proteins and the roles of these domains in membrane assembly (reviewed by Garoff, 1985).

Previously Yost et al. (1983) in our laboratory, used the transmembrane segment of IgM heavy chain to demonstrate the capability of this segment in the presence of a preceding signal peptide to act as a stop transfer. Here we use this artificial protein behind a more efficient promoter and in the presence and absence of an amino terminal signal peptide to demonstrate the unexpected capacity of the stop transfer sequence to function as a signal for insertion into the ER membrane. Like a typical signal peptide, the stop transfer sequence has a substantial stretch of hydrophobic amino acid residues with charges on either side. Our results indicate that the stop transfer mediated membrane

insertion is dependent on SRP and that the stop transfer is also capable of directing the insertion of either flanking domain of this artificial protein. The similarities of function of the signal peptide and the stop transfer sequence suggest that the mechanisms of initiation and termination of translocation may involve some or all of the same membrane associated components.

Experimental Procedures

Materials

Restriction enzymes, SP6 RNA polymerase, T4 DNA ligase, calf intestinal phosphatase, endoglycosidase H, Triton X-100, and Klenow were from Boehringer Mannheim (Houston, TX) and New England BioLabs (Beverly, MA). RNAase inhibitor was from Promega Biotec (Madison, WI); Staphylococcal protein A Sepharose, from Pharmacia (Piscataway, NJ). Rabbit anti human globin sera was from Cappel (Cochranville, PA), and rabbit anti bovine prolactin, from United States Biochemical Corp. (Cleveland, OH). Rabbit anti E. Coli lactamase antisera was a gift from Dr. Chung Nan Chang. Proteinase K was from Merck (FRG), 35-S Methionine from New England Nuclear (Boston, MA), NEM, from Calbiochem Bering Corp. (CA).

Plasmid Constructions

Expression plasmids: pSLMG, pLMG, pGMP and pGSP. These artificial proteins were constructed in vitro by DNA reconstruction of existing plasmids, pG6 (Yost et al., 1983) and pGSP (previously termed pSPgGP1; Perara and Lingappa, 1985). pG6 was modified

by EcoR1/BstE11 elimination of 50 bp, followed by insertion of an Nco1 linker (GCCATGGC), leaving 150 bp coding for the carboxy terminus of membrane IgM heavy chain. The entire coding region was engineered behind the SP6 promoter. Thus, this pG6-derived construct, pSLMG, includes coding regions for lactamase with its signal sequence (182 codons), for the transmembrane region of IgM (M segment, 50 codons), and for chimpanzee α-globin (143 codons). pLMG was constructed by truncation of the globin of pSLMG at BstE11 and cleavage in the SP64 polylinker at Xba1 with religation and subcloning, followed by partial digestion with Ssp1 in the presence of ethidium bromide, Bal 31 digestion, repair with Klenow fragment, and religation. DNA sequencing, using the dideoxy method (Sanger et al., 1977), confirmed the deletion of the initial methionine and 14 subsequent codons of the signal sequence. Mobilities of the encoded translation products on SDS-PAGE, and immunoreactivity with lactamase and globin antibodies, are consistent with initiation of synthesis at amino acid number 66 (methionine), which is the next AUG in the coding region of lactamase. Thus pLMG encodes amino acids 66-182 of lactamase (without its signal sequence), the 50 amino acid M segment, and the first 110 codons of globin ending at BstE11.

pGMP was constructed from pGSP by insertion of the M segment at the BstE11/Nco1 site of pGSP preceding the coding region for the prolactin signal. Subcloning was followed with deletion of the prolactin signal by cleavage at the EcoR1 site at the distal end of the M segment and at the Pvu11 site 50 codons beyond the amino

terminus of mature prolactin. Religation yielded pGMP, which encodes the first 110 amino acids of chimpanzee α-globin, including a 7 amino acid artificial glycosylation site at the BssH11 site of globin, the 50 amino acid M segment, and the C-terminal 150 codons of mature prolactin. The parent plasmid, pGSP, encodes the same 117 amino acids of globin (with the glycosylation site), fused to the signal of prolactin (30 codons) and all 199 codons of mature prolactin. The coding regions of the four constructs are diagrammed in Figures 1 and 5.

Transcription-Linked Translation

Plasmids were transcribed with SP6 polymerase *in vitro* in 10 microliter volumes; 2 microliters of the mRNA transcription mix (without further purification) was subsequently translated in 10 microliter volumes with rabbit reticulocyte lysate in the absence or presence of canine pancreatic microsomal membranes as described previously (Perara and Lingappa, 1985). Translations in wheat germ extract for the SRP experiments in Figure 8 were carried out exactly as described by Erickson and Blobel, 1983. Microsomal membranes were prepared as described by Walter and Blobel, 1983. Translation reactions were incubated at 24 C for 60 minutes. KRMs (potassium washed membranes) and SRP were prepared as described by Siegel and Walter, 1985.

Posttranslational Assays

Protease protection experiments and immunoprecipitations were performed as described previously (Perara and Lingappa, 1985).

Products were analyzed by SDS-PAGE, fluorography and autoradiography. Densitometry was performed on an LKB 2222 Ultrakscan XL densitometer. The values given are adjusted for methionine content (see figure legends for details).

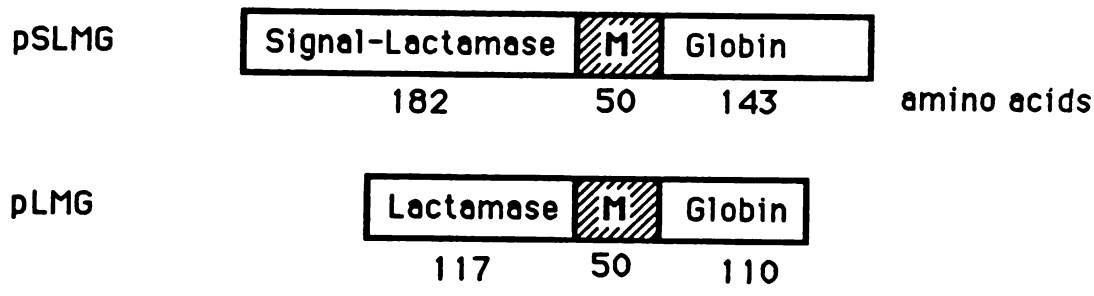
Results

To investigate membrane assembly of proteins during their synthesis, we have dissected and reconstructed the functional domains of proteins. Using this approach we have further characterized the stop transfer domain and the role it plays in membrane insertion and translocation. Protein fusions were made at the DNA level and used to program an *in vitro* transcription-translation system. Microsomal membranes were added to test for stop transfer and signal functions of these protein fusions.

Expression Plasmids, SLMG and LMG

We have shown previously (Yost et al., 1983), that an artificial protein inserts in microsomal membranes as directed by an amino terminal signal peptide and that translocation stops as the stop transfer segment enters the bilayer region to create a transmembrane protein. To elucidate more precisely the role of the stop transfer in this process of membrane assembly, we have used the SP6 promoter and polylinker to construct plasmids coding for two artificial proteins, SLMG and LMG, diagramed in Figure 1. pSLMG encodes S, the 23 amino acid signal peptide of lactamase; followed by 159 amino acids of mature lactamase, L; M, the carboxy 50 amino acids of IgM heavy chain (which includes

Figure 1. Expression plasmids pSLMG and pLMG. Coding regions for SLMG and LMG are inserted behind the SP6 promoter. pSLMG encodes a cleavable signal peptide (S) followed by beta lactamase (L), the carboxy-terminal 50 amino acids including the transmembrane region of IgM heavy chain (M), and globin (G). pLMG was constructed by deletion of the last 33 codons of globin and Bal 31 deletion of the first methionine codon of lactamase. Thus, pLMG does not encode the signal peptide and translation of its mRNA begins at the next available methionine, 66 codons downstream, such that the first 66 amino acids of LMG are missing. (For details, see text)

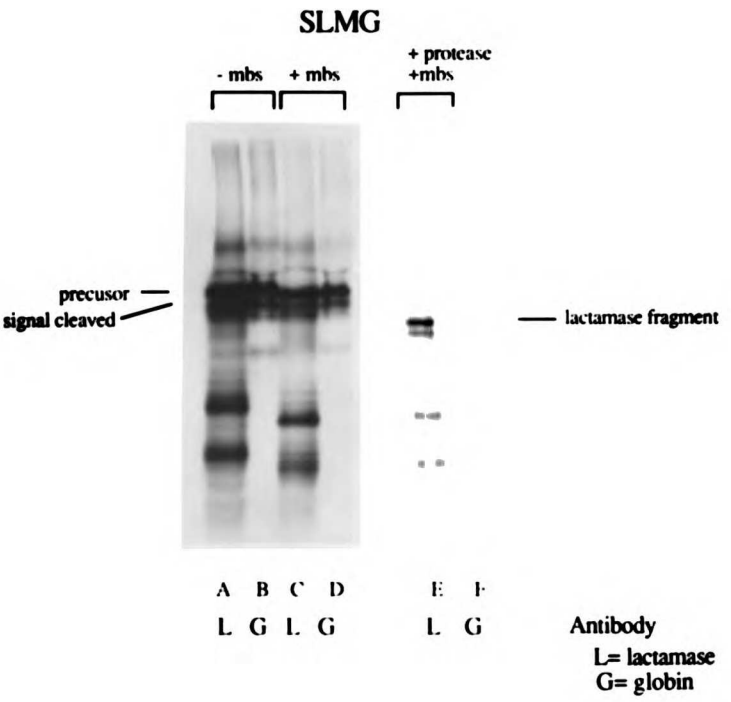

pSLMG
Signal-Lactamase
M
Globin
182
50
143
amino acids
pLMG
Lactamase
M
Globin
117
50
110

the approximately 23 amino acid membrane spanning region and stop transfer function); and G, 143 amino acids composing chimpanzee α-globin. pLMG encodes the same protein domains as pSLMG except that pLMG lacks the last 33 codons of globin; also pLMG does not include the amino-terminal signal peptide coding region and the first 43 codons of mature lactamase, as the initial AUG has been eliminated by Bal 31 digestion so that protein synthesis begins at the first available AUG of pLMG, 66 codons downstream from that in SLMG. Both SLMG and LMG coding domains were inserted downstream from the SP6 promoter and transcribed in the presence of GpppG cap (see Experimental Procedures for details).

In vitro Synthesis and Membrane Insertion of SLMG and LMG

Both pSLMG and pLMG were translated in reticulocyte lysate and subsequently immunoprecipitated with lactamase and globin antibodies. In the absence of microsomal membranes, transcribed and translated pSLMG yields a 40 kd product immunoreactive with both lactamase and globin antibodies with the predicted electrophoretic mobility for its amino acid content (Fig. 2 A,B). On addition of microsomal membranes during the translation reaction, SLMG demonstrated a slightly faster mobility (equivalent to a loss of approximately 2 kd) that is attributable to cleavage of its 23 amino acid signal peptide (Fig. 2 C,D). As an additional indicator of membrane insertion, we added proteinase K post-translationally. That part of a transmembrane protein inserted into or across the bilayer will be protected from protease

Figure 2. Transmembrane insertion. pSLMG was transcribed with SP6 polymerase and subsequently translated in reticulocyte lysate in the presence or absence of microsomal membranes. Proteinase K was added post-translationally (lanes E and F) to digest those portions of peptides not protected by the membranes. Products were subsequently immunoprecipitated with globin or lactamase antisera. mbs, microsomal membranes

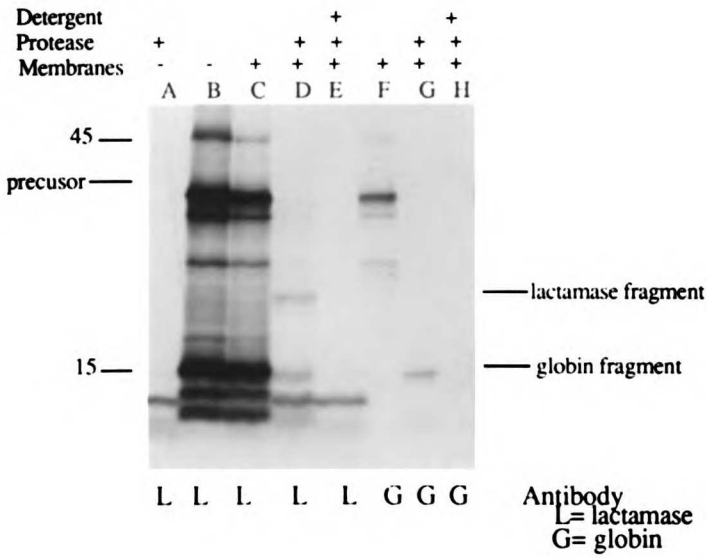

SLMG
- mbs
+ mbs
+ protease
+mbs
precusor
signal cleaved
lactamase fragment
A B C D E F
L G L G L G
Antibody
L= lactamase
G= globin

(Morimoto et al., 1983). We found partial protection of SLMG, indicative of transmembrane orientation. In Figure 2E a lactamase immunoreactive fragment is shown, indicating that the lactamase domain of SLMG has been translocated into the microsomal lumen; however, no globin immunoreactive domain is visible (Fig. 2F), indicating as expected that the globin domain on the carboxy terminal end of the protein is left cytoplasmically disposed and accessible to protease. This is consistent with the previous findings of Yost et al. (1983) for a transmembrane orientation with the amino terminus translocated into the microsomal lumen. As controls, addition of detergent or addition of membranes post-translationally to SLMG or LMG translation reactions resulted in non-protected, protease sensitive peptides.

Similarly, transcription and translation of pLMG yields a transmembrane protein. On addition of microsomal membranes there is no shift in electrophoretic mobility, as there is no signal peptide to be cleaved (Fig. 3B, C and F). However, on post-translational addition of protease, both lactamase and globin immunoreactive fragments are visible (Fig. 3D, G). And both of the protected fragment sizes were slightly larger than expected for the lactamase or globin domains alone, presumably due to protection of an attached remnant of the M segment. Protease protection and specific immunoreactivity of these domains with either globin or lactamase antisera is indicative of membrane insertion and translocation of either the carboxy or the amino flanking domain, respectively. In control experiments, no

Figure 3. Transmembrane insertion. pLMG was transcribed with SP6 polymerase and subsequently translated in reticulocyte lysate in the presence or absence of microsomal membranes. Proteinase K was added post-translationally to digest those portions of peptides not protected by the membranes. Products were subsequently immunoprecipitated with globin or lactamase antisera.

LMG


Detergent
Protease
Membranes
A B C D E F G H
45
precusor
15
lactamase fragment
globin fragment
L L L L L G G G
Antibody
L= lactamase
G= globin

fragments were protected from protease in the absence of membranes (Fig. 3A), or in the presence of post-translationally added microsomal membranes (data not shown), or in the presence of nonionic detergent added post-translationally to disrupt the membrane bilayer (Fig. 3E, H). These results suggest that LMG which lacks a signal peptide, and whose only hydrophobic region is the M segment or stop transfer sequence, is capable of inserting in the membrane to create a transmembrane protein. Figure 4 shows densitometry analysis of protease protection of the carboxy and amino terminal domains of SLMG and LMG. Collectively, the data here suggest that membrane insertion of LMG, unlike SLMG, occurs in either direction, i.e., either the carboxy or amino terminus of LMG may insert in the microsomal lumen to create a transmembrane protein of either orientation.

Expression Plasmids, GMP and GSP

In order to test the stop transfer/signal function in the context of a different chimeric protein and to compare this internal stop transfer sequence with an internal signal sequence, we looked at 2 other protein fusions, GMP and GSP (Fig. 5). pGSP encodes G, 110 codons of chimpanzee α-globin plus 7 codons of an artificial glycosylation site followed by all of preprolactin (199 codons), including the signal sequence of prolactin (30 codons) (pSPgGP1, Perara and Lingappa, 1985). pGMP encodes: G, the same globin domain with the same artificial glycosylation site; M, 50 codons

Figure 4. Densitometry analysis of fragments of SLMG and LMG protected from protease. Densitometry analysis of the data in figure 3 are shown here as bar graphs. % Protease protection for lactamase (L) is calculated as the density (absorbance as determined by the LKB 2222 densitometer) of the lactamase immunoreactive fragment protected from protease divided by the density of the lactamase immunoreactive precursor (LMG) times 100% and adjusted for methionine content. For example, %protease protected L= L/LMG x 100% x 9/6 methionines; %protease protected G= G/LMG x 100% x 9/3 methionines. *In vitro* translations were performed in the presence of ^{35}S methionine. The methionine content of LMG is 9 per LMG molecule: G=3 methionines; L=6 methionines; M=0 methionines. After insertion into the microsomal membrane the signal peptide is cleaved, therefore its methionine (1) is not used in these calculations.

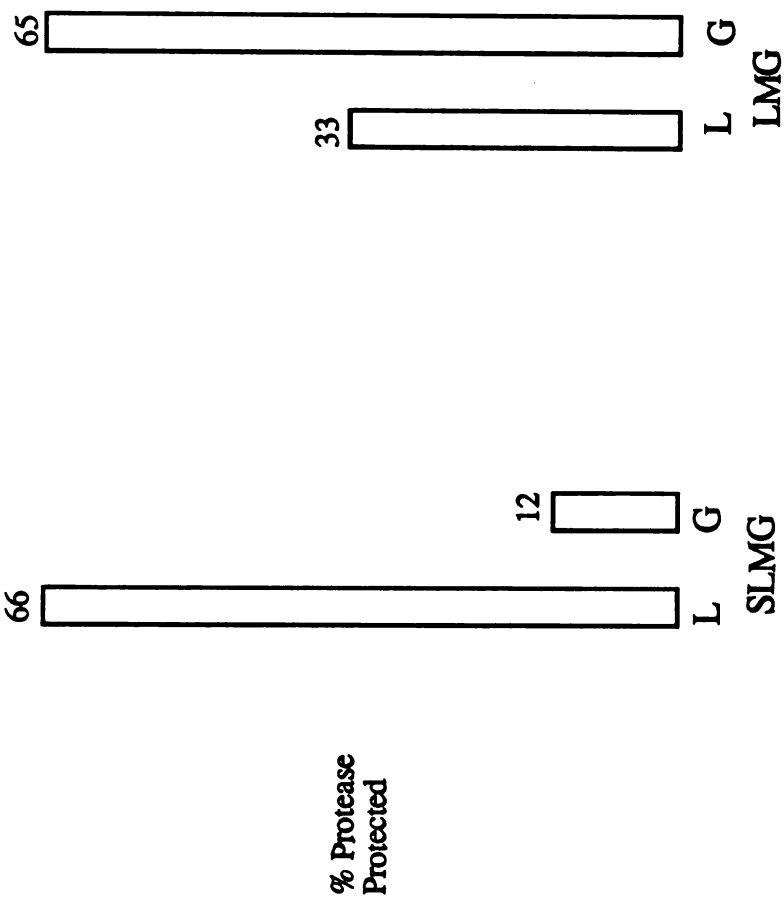
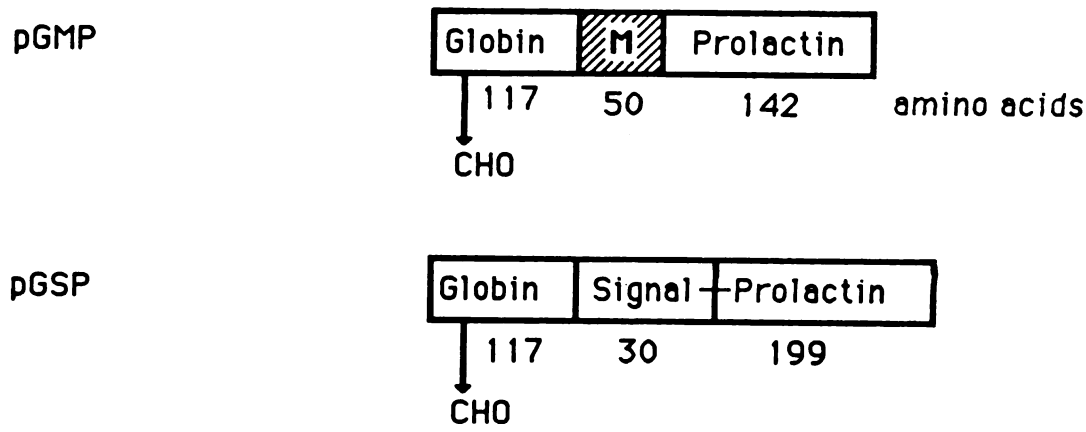

% Protease Protected
66
12
L
G
SLMG
65
33
L
G
LMG

Figure 5. Expression plasmids pGMP and pGSP. Coding regions for GMP and GSP are inserted behind the SP6 promoter. pGMP encodes 110 residues from the amino terminus of chimpanzee α-globin with a 7 amino acid artificial glycosylation site inserted near the amino terminus (in the BsshII site of globin), 50 residues of the carboxy end of IgM heavy chain including the transmembrane region, and 142 residues of the carboxy end of prolactin without its signal sequence. pGSP encodes the same 117 residues of globin with the artificial glycosylation site, and all 199 residues of prolactin with its 30 amino acid signal peptide.


pGMP
Globin
M
Prolactin
117
50
142
amino acids
CHO
pGSP
Globin
Signal
Prolactin
117
30
199
CHO

of the extreme carboxy terminus of IgM heavy chain; and P, 150 codons of the extreme carboxy terminus of mature bovine prolactin (which does not include the signal peptide). The postulated membrane-spanning region of M encompasses 23 of the 50 amino acid M segment and contains the putative stop transfer function. It was suspected that the hydrophobic membrane-spanning region, or stop transfer sequence of GMP, was initiating membrane insertion because the prolactin and globin domains alone have been shown incapable of directing insertion into the membrane (Lingappa et al., 1984; R. Rothman et al., 1988). Thus, pGMP contains an internal stop transfer sequence within the M segment, and pGSP has an internal signal sequence, S, with its native cleavage site intact.

In vitro Synthesis and Membrane Insertion of GSP and GMP

We have previously shown that the signal peptide of prolactin continues to function as a signal for membrane insertion when it is located in an internal position in GSP (Perara and Lingappa, 1985). Here we compare the internal stop transfer sequence in GMP with the signal of prolactin, constructed internally in GSP, for similarities in signal activity.

mRNAs encoding GMP and GSP were transcribed *in vitro* with SP6 polymerase. Transcription reactions were then added to reticulocyte lysate for translation in the presence and absence of membranes. Transcription and translation of pGMP resulted in a 35 kD protein as predicted for its amino acid content. Unlike an

internal signal peptide, GMP does not cleave on addition of microsomal membranes (Fig. 6C, D), but instead generates a transmembrane protein. Like LMG described above, GMP is able to translocate flanking domains across the ER bilayer as determined by protected fragments generated in the presence of proteinase K (Fig. 6F). When the globin domain is translocated, the 7 amino acid artificial glycosylation site is available for addition of sugars and as evidenced here, a small fraction of GMP is glycosylated (Fig. 6C, D). This is an additional indicator of membrane insertion. These data suggest that GMP like LMG is capable of membrane insertion in the absence of an amino terminal signal peptide.

Unlike GMP, addition of membranes co-translationally results in cleavage of the GSP peptide generating prolactin and globin fragments (Fig. 6H). These fragments are generated by the action of signal peptidase in the ER lumen and the fragments are protected from proteinase K added post-translationally (Fig. 6I), indicative of translocation across the microsomal membranes.

Both GSP and GMP translocate either domain flanking the signal or stop transfer region, although the prolactin domain following the signal activity is more efficiently translocated (see Fig. 7). It remains to be determined whether one flanking domain inserts for one GMP molecule and the other flanking domain inserts for another molecule, if some fraction of the GMP molecules may insert both flanking domains as a loop, or if flanking domains

Figure 6. Stop transfer and signal sequence mediated translocation. pGMP and pGSP were transcribed with SP6 polymerase and subsequently translated in reticulocyte lysate in the presence or absence of microsomal membranes. Proteinase K was added post-translationally to digest those portions of polypeptides not protected by the membranes. Products were subsequently immunoprecipitated with globin or prolactin antisera and subjected to polyacrylamide gel electrophoresis. Molecular weight indicators are 45 and 15 kD. mb, microsomal membranes.

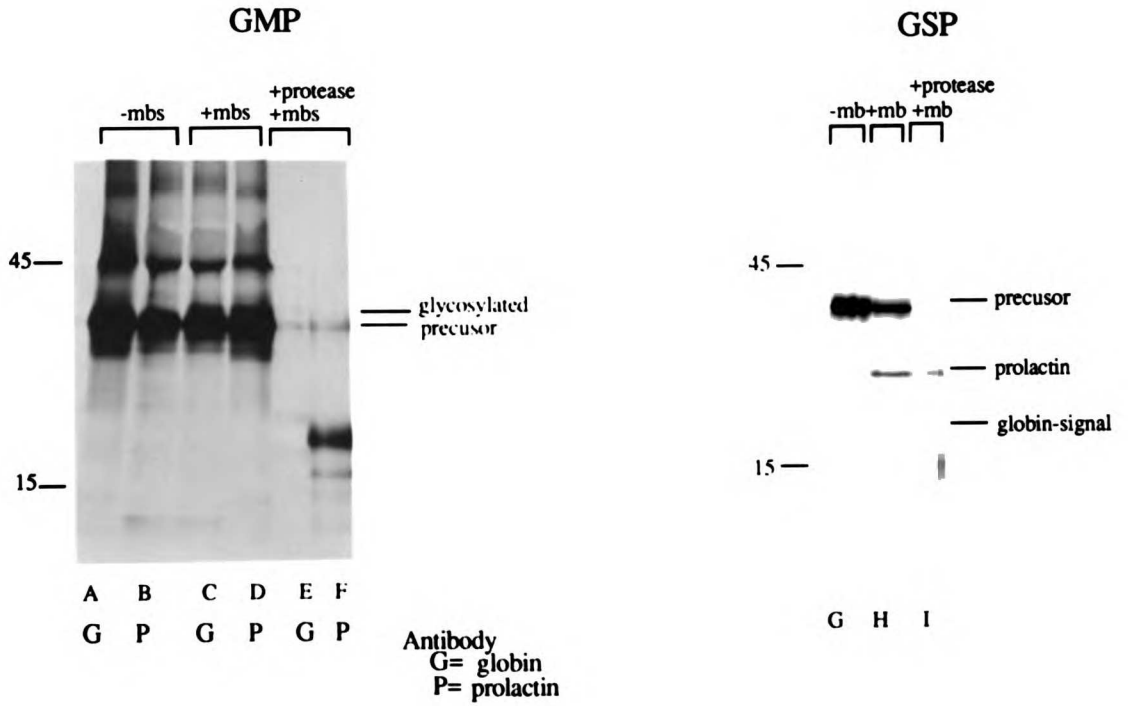
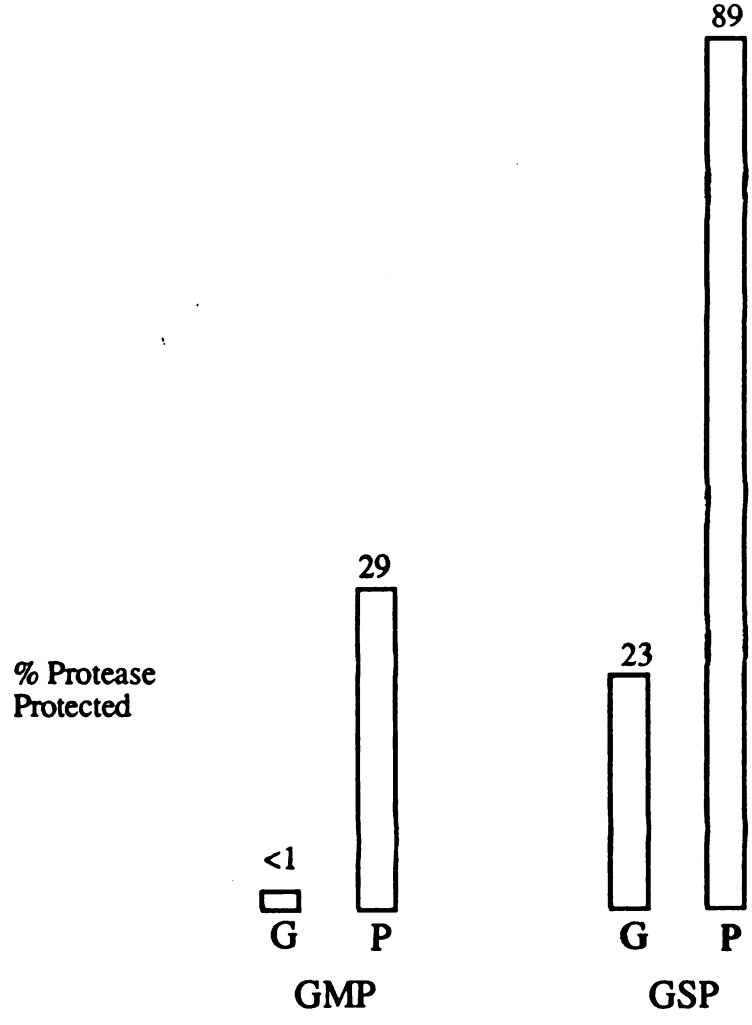

GMP
-mbs
+mbs
+protease
+mbs
45
15
glycosylated
precusor
A B C D E F
G P G P G P
Antibody
G= globin
P= prolactin
GSP
-mb
+mb
+protease
+mb
45
15
precusor
prolactin
globin-signal
G H I

Figure 7. Densitometry analysis of the protease protected fragments of GMP and GSP. Densitometry analysis of the data in figure 6 are shown here as bar graphs. % Protease protection for prolactin (P) is calculated as the density (absorbance as determined by the LKB 2222 densitometer) of the prolactin immunoreactive fragment protected from protease, divided by the density of the precursor (GMP) times 100% and adjusted for methionine content. For example, %protease protected P= P/GMP x 100% x 7/4 methionines; %protease protected G= G/GMP x 100% x 7/3 methionines. *In vitro* translations were performed in the presence of ^{35}S methionine. The methionine content of GMP is 7 per GMP molecule: G=3 methionines; P=4 methionines; M=0 methionines. The methionine content of GSP is 10: GS fragment= G +S = 2+1 =3 methionines; P= 7 methionines.


% Protease Protected
<1
29
23
89
G
P
G
P
GMP
GSP

may insert in the ER lumen and subsequently slip back out into the cytoplasm as has been suggested for other molecules (Garcia et al., 1988).

Membrane insertion of GMP is abolished when N-ethyl maleimide (NEM) treated salt-washed membranes are used in this assay (data not shown). NEM is a sulfhydryl alkylating agent which has been shown to inhibit translocation in microsomal membranes (Gilmore et al.,1982). This suggests that SRP receptor is required for membrane insertion of GMP.

SRP dependence of stop transfer mediated membrane insertion

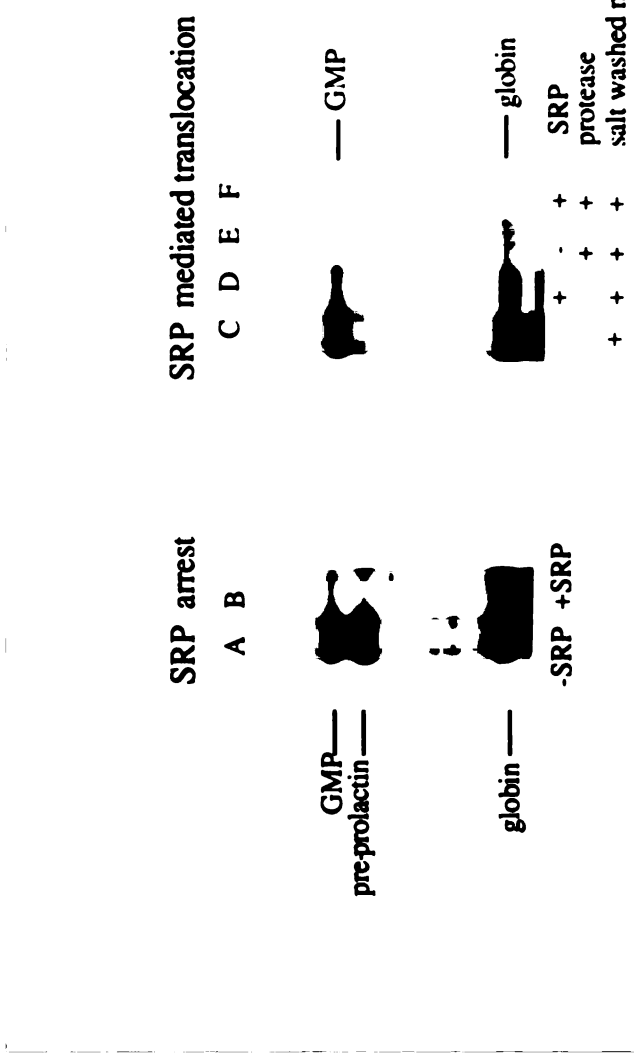
The data presented here indicate that the stop transfer segment of GMP and LMG derived from IgM heavy chain can function as a signal for membrane insertion in our *in vitro* system. Secretory proteins with amino terminal signal peptides have been shown to be dependent on SRP for membrane insertion *in vitro* (Walter et al., 1981; Walter and Lingappa, review, 1986). We wished to determine if this stop transfer/signal activity in GMP was dependent on SRP. To test this, we investigated two indicators of SRP dependence: elongation arrest by SRP in the absence of membranes, and SRP dependent membrane insertion.

As before, SP6 plasmids were transcribed *in vitro* and transcription mix was added to translation reactions. In these SRP experiments translation was carried out in wheat germ lysate, which is deficient in SRP activity, in the presence or absence of

salt washed membranes. SRP was then exogenously added to some of the translation reactions as shown in Figure 8. In addition to mRNA coding for GMP, prolactin and globin mRNAs were also added to the translation reaction as controls. Globin is a cytoplasmic protein, not normally directed to the membrane, and therefore its synthesis or elongation is not arrested by SRP (Walter et al., 1981a) as is found here (Fig. 8 A,B). Preprolactin is a secretory protein with an amino terminal signal peptide and its synthesis has been shown to be arrested by SRP (Walter et al., 1981a). This is also the case here (Fig. 8 A,B). Like preprolactin which contains a signal peptide, synthesis of GMP which contains a stop transfer sequence is arrested by SRP as shown in Figure 8 A,B. We observed in these experiments that preprolactin required 15 nM SRP and GMP required 150 nM SRP for arrest of synthesis (data not shown). The elongation arrest of preprolactin and GMP, but not of globin, suggests that GMP contains a region, presumably the hydrophobic stop transfer sequence, which interacts with SRP in a manner similar to that of the signal sequence of preprolactin.

The second panel of Figure 8 shows evidence for SRP dependent membrane insertion. Here GMP is compared with globin which is cytoplasmic and does not enter the membrane. When a cytoplasmic protein, e.g. globin, does not enter the microsomal membrane, addition of protease externally in this *in vitro* system will completely digest the protein as seen here in Figure 8F. If a protein has been inserted in the microsomal membranes in a

Figure 8. Membrane insertion of GMP is dependent on signal recognition particle (SRP). Lanes A and B show SRP arrest of protein synthesis of GMP and pre-prolactin. Globin and prolactin are transcribed and translated with GMP as internal controls. Lanes C,D,E, and F demonstrate SRP dependent membrane insertion of GMP. Proteinase K was used at .1 mg/ml.


SRP arrest
A B
GMP
pre-prolactin
globin
-SRP +SRP
SRP mediated translocation
C D E F
GMP
globin
SRP
protease
salt washed membranes
+ - + +
+ + + +
+ + + +

transmembrane fashion, addition of protease externally will generate a smaller fragment which has been partially protected by the membrane as seen in Figure 8F for GMP. Compare this with Figure 8E in which no SRP was present and no fragment was generated, indicating that in the absence of SRP, no membrane insertion has occurred. These data suggest that GMP, a transmembrane protein, requires SRP for its membrane insertion.

Discussion

In 1983, the transmembrane segment of IgM heavy chain was first shown to function as a stop transfer to halt the translocation of an artificial protein across microsomal membranes *in vitro* (Yost et al., 1983). Here we present evidence that this stop transfer sequence can function as a signal for membrane insertion in the absence of a preceding signal sequence, that this membrane insertion is dependent on SRP, and that the stop transfer sequence inserts either flanking domain into the ER lumen.

There are several unexplained discrepancies in the results presented here and those presented previously (Yost et al. 1983) concerning the function of the stop transfer sequence in the absence of a preceding signal sequence. The results presented by Yost et al. show that the transmembrane stop transfer segment is not capable as acting as a signal for membrane insertion of globin; these results may differ from the data here due to an extra 17 amino acids preceding the M region in their construction. However, the data show here that the M region may be preceded

by either lactamase or globin domains and membrane insertion still occurs. Although most of the translation experiments presented here utilized reticulocyte lysate, for the SRP experiments (and others not presented here) we used wheat germ lysate, and in the two translation systems there was no difference in stop transfer function. However, the discrepancies from the previous work may be due to a more quantitative analysis of the data afforded here by the SP6 polymerase system. R. E. Rothman et al. (1988) from our group have also reported data for stop transfer sequence signal function in agreement with those presented here.

Both GSP and GMP preferentially insert the protein domain following the signal activity (Fig. 7), however, GSP is much more efficiently translocated. A major difference between the two molecules is the signal peptide cleavage site in GSP. The action of signal peptidase on GSP may enhance the translocation efficiency of GSP.

As an addendum to the signal hypothesis (Blobel and Dobberstein, 1975), the data here indicate that at least two types of protein segments contain information which can serve to target proteins to the ER membrane: signal peptides and stop transfer (or membrane spanning) regions. The key for targeting nascent chains to the ER membrane is interaction with SRP. In the case of an amino terminal signal peptide, SRP interaction is proposed to occur as soon as the signal peptide emerges from the translating

ribosome, i.e. after synthesis of about 70 amino acids of the nascent chain (Gilmore and Blobel, 1985). In the case of a protein without an amino terminal signal peptide, but with an internal membrane spanning region like LMG and GMP described here, SRP interacts with the nascent chain after the amino terminal end of the protein has been synthesized and partially extruded from the ribosome into the cytoplasm. In its natural state, i.e. following a signal peptide, a stop transfer sequence does not have the opportunity to interact with SRP because it emerges from a ribosome which has already been targeted to and attached to the membrane by the preceding signal peptide. We have uncovered the latent activity of the stop transfer sequence by removing the signal peptide and allowing nascent chain synthesis to occur on a functionally free ribosome in the cytoplasm. Thus when the stop transfer sequence emerges from the ribosome synthesizing LMG or GMP, it is not yet tied to the membrane and is free to interact with SRP to be targeted to the membrane in a similar manner as targeting of a signal peptide.

The current model for membrane insertion of transmembrane proteins without a cleavable signal sequence postulates that the membrane spanning signal/anchor region of the protein is responsible for targeting, insertion, and stop transfer or anchor activity (Speiss and Lodish, 1986). Models for stop transfer activity (Yost et al., 1983; Blobel, 1980; Friedlander and Blobel, 1985), postulated that some membrane spanning regions function

only to stop the transfer of the rest of the protein across the membrane and that stop transfer sequences could be distinguished from signal/anchor regions. In light of the findings presented here, this distinction becomes less plausible.

Friedlander and Blobel (1985) propose alternating signal and stop transfer membrane spanning segments for the seven transmembrane segments of bovine opsin. In view of the latent activity of a stop transfer to act as a signal, this alternating signal and stop transfer model may depend on the availability of these segments to interact with SRP. Currently, it is not known whether SRP is required for subsequent insertion of hydrophobic segments following the first signal region.

From the results presented here, it appears that membrane spanning segments which follow a signal peptide, act as stop transfer sequences as shown previously, but a stop transfer sequence without a preceding signal peptide is capable of acting as a signal for ER membrane insertion in an SRP dependent manner. This demonstrates that one hydrophobic membrane spanning segment may have different functional roles for membrane assembly depending on its context within the nascent chain, including availability for SRP interaction. The elucidation of the mechanisms by which complex proteins are assembled in the ER membrane, and the role of SRP and other factors in this process remain to be explored.

Chapter 3

REVERSAL OF TRANSMEMBRANE PROTEIN ASYMMETRY WITH A SIGNAL PEPTIDE *IN VITRO*

Summary

By DNA construction, we have generated two proteins which differ only by the presence or absence of a 23 amino acid signal peptide which becomes cleaved by signal peptidase upon translocation across the ER membrane. Cleavage of the signal peptide generates a protein which is identical in amino acid composition to the protein without a signal peptide, but which preferentially spans the membrane in an opposite transmembrane orientation. The same hydrophobic region with its flanking positive and negative charges spans the membrane in opposite orientations in these two transmembrane proteins. With a preceding, cleaved signal peptide, the hydrophobic transmembrane region functions as a stop transfer and inserts linearly across the bilayer, but without the preceding signal peptide, the transmembrane region interacts with SRP to function as a signal/anchor and inserts into the bilayer in a looped conformation yielding the opposite transmembrane polarity. The implications of these findings for assembly of proteins into the endoplasmic reticulum membrane are discussed.

Introduction

Membrane topology of a protein is determined by information contained within the nascent polypeptide chain. For secretory and plasma membrane proteins, this information is decoded at the endoplasmic reticulum (ER) membrane (Wiedmann et al., 1987; Walter, 1987), the initial site of membrane insertion.

Two models have emerged to account for interaction of the nascent chain with the ER membrane to generate an integral membrane protein: One hypothesis (Blobel, 1980; Friedlander and Blobel, 1987) proposes that discrete "topogenic sequences" within nascent polypeptides, e.g. signal, insertion, and stop transfer sequences, interact with the proteinaceous machinery in the membrane to direct protein assembly in a receptor mediated fashion. This model proposes a proteinaceous channel in the membrane continuous with the cleft in the ribosome through which the nascent chain emerges into the ER lumen. The alternative model suggests direct interaction of the nascent chain with the lipid bilayer in a protein-lipid contact which is thermodynamically favored for transfer of the protein across the membrane and into the ER lumen (Engelman and Steitz, 1981). Wickner (1979) proposes "spontaneous insertion domains" formed by the nascent chain that interact with the bilayer to direct the integration of proteins into the ER membrane. The molecular mechanisms of insertion and generation of topology of nascent membrane proteins remain to be elucidated.

Some of the proteins involved in the translocation process have been characterized: When the signal peptide emerges from the ribosome, it interacts with signal recognition particle (SRP) (Walter and Blobel, 1981b). The SRP/nascent chain/ribosome complex is targeted to the ER membrane where SRP binds SRP receptor (or docking protein) (Meyer et al., 1982; Gilmore et al., 1982). The

signal peptide then is handed over to the signal sequence receptor (SSR) in the ER membrane (Wiedmann et al., 1987; Walter, 1987) and the nascent chain is translocated across the bilayer by some unknown mechanism. Signal peptidase, located on the lumenal side of the ER membrane, cleaves the signal peptide and thus, the newly generated amino terminus of the mature protein is located in the ER lumen (Blobel and Dobberstein, 1975). A secretory protein is completely extruded across the membrane and into the lumen. Extrusion of a transmembrane protein into the ER lumen is interrupted when a stop transfer emerges from the ribosome and halts the further transfer of the protein across the membrane (Yost et al., 1983; Rose and Guan, 1984; Reviews: Blobel, 1980; Sabatini et al., 1982). The partially extruded protein spans the bilayer in a transmembrane fashion.

Some transmembrane regions have been shown not only to anchor the protein in a transmembrane fashion as a stop transfer, but also to act as an uncleaved signal peptide to target the protein to the membrane, i.e. signal/anchor domains (Spiess and Lodish, 1986; Mize et al., 1986; Zerial et al.,1987; Sivasubramanian and Nayak, 1987). In general two types of hydrophobic domains have been shown to target proteins from the cytoplasm to the ER membrane: signal peptides and signal/anchor sequences.

Both signal peptides and signal/anchor sequences contain hydrophobic domains about 15 to 25 amino acid residues long, however, the signal and signal/anchor sequences are not directly

interchangeable. When a signal peptide is located in an internal position like a signal/anchor sequence, the signal peptide is recognized and cleaved by signal peptidase, and no transmembrane protein is generated, instead the mature protein is completely translocated into the ER lumen in two parts (Perara and Lingappa, 1985). Conversely when a signal/anchor or stop transfer sequence is located at the amino terminus of a protein, the protein is inserted and anchored in the membrane (Sivasubramanian and Nayak, 1987; Sakaguchi et al., 1987; R.E. Rothman et al., 1988).

Simple transmembrane proteins span the membrane once with either the amino or carboxy terminus translocated into the ER lumen. Those simple transmembrane proteins with the carboxy terminus located in the ER lumen generally have no cleaved signal peptide (e.g., transferrin receptor, Zerial et al., 1987; asialoglycoprotein receptor, Speiss and Lodish, 1986; neuraminidase, Bos et al., 1984). Those with the amino terminus in the ER lumen, and the carboxy terminus left on the cytoplasmic side of the bilayer, have been described as type I membrane proteins (Garoff, 1985). Many proteins with their amino terminus in the ER lumen have two hydrophobic domains: a trans-membrane stop transfer sequence and an amino-terminal cleaved signal peptide (e.g., VSV G protein, Rose and Gallione, 1981; Hemagglutinin, Gething and Sambrook, 1982; membrane IgM heavy chain, Rogers et al., 1980; LDL receptor, Russell et al., 1984). Some proteins have no amino terminal signal peptide, but are able

to transfer their amino terminus across the membrane (e.g., M_2 protein of influenza A, Hull et al., 1988; influenza B virus NB protein, Williams and Lamb, 1986; V-erbB glycoprotein 74, Hayman et al., 1983). If one hydrophobic transmembrane region is sufficient to insert and anchor a protein in the membrane, why do some simple membrane proteins contain a second topogenic signal at the amino terminus? We show evidence here that the amino-terminal cleaved signal peptide insures a lumenal disposition of the amino terminus of a transmembrane protein.

Previously, we have shown that a signal peptide on the amino terminus of an artificial transmembrane protein directs insertion of the amino terminus of the protein in the ER lumen (Yost et al., 1983; Mize et al., 1986). We have also shown that without a signal peptide, the stop transfer region of the protein is capable of acting as a signal/anchor to insert either the carboxy or amino terminus into the ER lumen (Mize et al., 1986).

Here we show that a fusion protein containing an internal stop transfer sequence, but lacking a signal peptide, preferentially spans the membrane with its carboxy terminus translocated inside the microsomal lumen and amino terminus left on the cytoplasmic side of the membrane. When a signal peptide is added to this protein, the preferred orientation is reversed. These results suggest that in the case of a simple single spanning, trans-membrane protein, the amino-terminal signal peptide plays a critical role in determining protein orientation with respect to the

membrane. We discuss how the signal peptide may influence protein folding or other factors that may be involved in translocation of the nascent chain across the ER membrane and how the transmembrane signal/anchor domain takes on a different, looped conformation for membrane insertion in the absence of a preceding signal.

Experimental Procedures

Materials

Restriction enzymes, SP6 RNA polymerase, T4 DNA ligase, calf intestinal phosphatase, endoglycosidase H, Triton X-100, and Klenow were from Boehringer Mannheim and New England BioLabs. RNAase inhibitor was from Promega Biotec; Staphylococcal protein A Sepharose, from Pharmacia. Rabbit anti human globin sera was from Cappel, and rabbit anti bovine prolactin, from United States Biochemical Corp. Proteinase K was from Merck, ^{35}S Methionine from New England Nuclear.

Plasmid Constructions

Expression plasmids, GMP and SGMP. These artificial membrane proteins were constructed *in vitro* by DNA recombination. pGMP codes for exactly the same fusion protein as pSGMP, except GMP is lacking the amino-terminal 23 amino acid signal peptide derived from β-lactamase. Construction of pGMP was described previously (Mize et al., 1986). Construction of pSGMP was accomplished by excision of the HindIII segment from GMP and insertion of a

HindIII segment from pSPG1 (Perara et al., 1986) which includes the 23 amino acid signal peptide from β-lactamase followed by globin. Both pGMP and pSGMP were cloned behind the SP6 promoter; the regions coding for protein are shown in Figure 1. The circles represent the seven amino acid residue synthetic glycosylation site inserted by DNA recombination into the BsshII site of globin.

S: 23 amino acid signal peptide of β-lactamase; **G**: amino-terminal 110 amino acid residues of chimpanzee α-globin including a 7 amino acid synthetic glycosylation site [AHNGSGS]; **M**: carboxy terminal 50 residues of Immunoglobulin M heavy chain including 22 residues amino terminal to the transmembrane segment, 25 residues composing the transmembrane segment, and the carboxy 3 residues, lysine-valine-lysine, which are cytoplasmically disposed in the native heavy chain (Rogers et al., 1980); **P**: carboxy terminal 143 residues of mature bovine prolactin.

In Vitro Transcription and Translation

Plasmids were transcribed with SP6 polymerase *in vitro*; the mRNA transcription mix was subsequently translated in rabbit reticulocyte lysate in the absence or presence of canine pancreatic microsomal membranes as described previously (Perara and Lingappa, 1985). Microsomal membranes were prepared as described (Walter and Blobel, 1983). Translation reactions were incubated at 24 C for 60 minutes.

Posttranslational Assays

Endoglycosidase H digestion used to determine core glycosylation, protease protection experiments, and immunoprecipations were performed exactly as described previously with the same antisera (Perara and Lingappa, 1985). Products were analyzed by SDS-PAGE, fluorgraphy and autoradiography.

pH 11.5 Carbonate treatment of microsomal membranes releases proteins not integrated into the lipid bilayer including peripheral and secretory proteins (Fujiki, 1982). After translation in the presence of microsomal membranes, membranes were separated from free particulate by centrifugation through a .5 M sucrose cushion and the pellet resuspended in .1 M Na2CO3, pH 11.5 for 30 minutes on ice. Centrifugation at 30 psi in a Beckman airfuge for 30 minutes was used to separate the membrane pellet from the solubilized components. The pellet was resolubilized in an equal amount of .1 M Na2CO3, and pellet and supernatant were immunoprecipitated as above with prolactin antisera and analyzed by SDS-PAGE, fluorography and autoradiography.

Densitometry was performed on an LKB 2222 Ultrascan XL densitometer as described previously (Perara and Lingappa, 1985). For densitometry graphs, the values given are adjusted for methionine content. The globin domain contains 3 methionines, the prolactin domain, 4, the M segment, none, and the signal peptide, 1 methionine. GMP thus contains 7 methionines and SGMP after cleavage of the signal (on addition of membranes) also

contains 7 methionines. Bands plus membranes are compared to bands plus membranes and protease, both immunoprecipitated with the same antibody. Adjustments are made for methionine content, e.g., % P protected from protease = P/GMP x 7/4 methionines x 100%, and G= G/GMP x 7/3, where P is the prolactin domain protected from protease; G, globin domain protected from protease; GMP, precusor domain without protease, plus membranes (P, G, GMP and SGMP are represented by the area under the curve from the densitometry reading printout).

Results

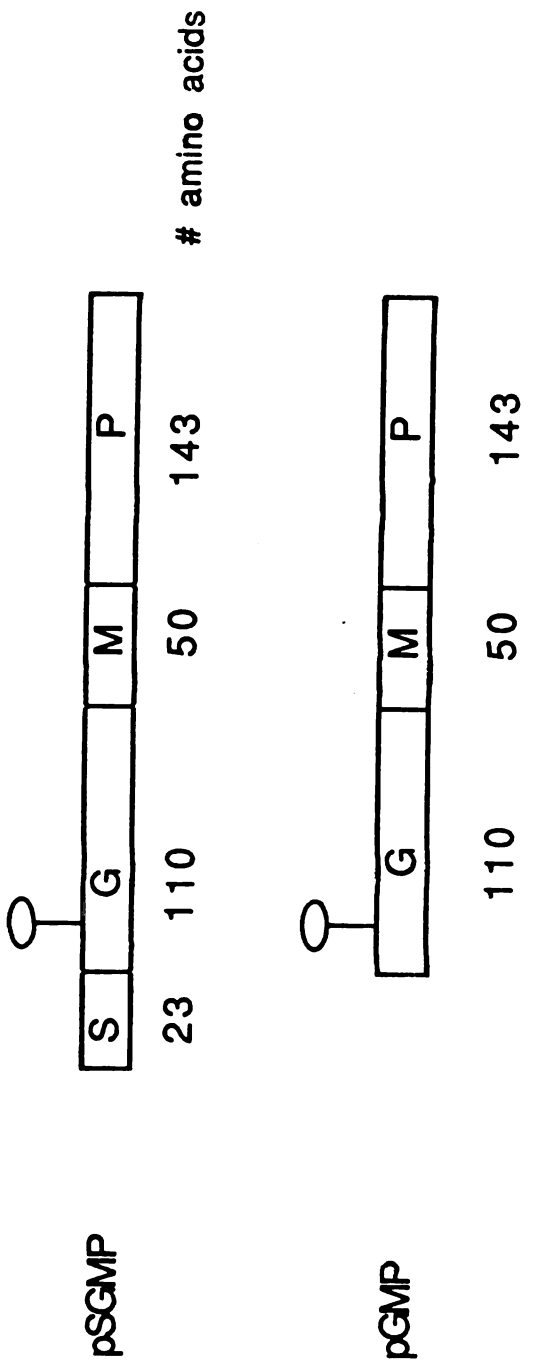
We have combined passenger protein segments known to be compatible for transfer across the membrane with hydrophobic protein segments which are capable of initiating transfer across the membrane in an SRP dependent manner. We use these protein fusions to test the functions of the signal peptide and the stop transfer domains for polarity of membrane insertion. Plasmids coding for these artificial transmembrane proteins were transcribed *in vitro*, and the resultant mRNA was translated with reticulocyte lysate in the presence or absence of microsomal membranes. The radioactively labelled proteins were then assayed for transmembrane orientation in this cell-free system. The specificity of insertion across the membrane was assayed by signal peptide cleavage, the presence or absence of glycosylation of the amino terminal ends of the proteins, and by sensitivity to externally added proteases and subsequent immunoprecipitation with amino and carboxy domain specific antibodies.

Expression plasmids, GMP and SGMP

The passenger components used in these protein fusions, globin, G, and prolactin, P, are known to be compatible for transfer across the membrane when preceded by a signal peptide, and the hydrophobic "topogenic" components, signal and stop transfer domains, are capable of initiating transfer across the membrane in an SRP dependent manner (Lingappa 1984; Mize et. al, 1986; R.E. Rothman, 1988).

The two fusion protein plasmids utilized in this study, pGMP and pSGMP (Fig. 1), each contain the same passenger components, G and P, and the same transmembrane segment, M, derived from Immunoglobulin M heavy chain. This transmembrane segment, M, has previously been demonstrated to function as a stop transfer (Yost et al., 1983) and as a signal for membrane insertion (Mize et al., 1986). SGMP, but not GMP has an amino-terminal signal peptide, S, from β-lactamase; both proteins contain a synthetic N-linked glycosylation site 21 amino acid residues downstream from the initial methionine of the globin domain. Both plasmids were inserted behind the SP6 promoter and transcribed in the presence of GpppG cap (see Experimental Procedures for details).

Figure 1. Expression plasmids, SGMP and GMP. pGMP codes for exactly the same fusion protein as pSGMP, except GMP is lacking the amino terminal 23 amino acid signal peptide. Both SGMP and GMP coding regions are inserted behind the SP6 promoter; the protein coding regions are shown here. The circles represent artificial glycosylation sites in the globin domains. See Experimental Procedures for details of construction.


pSGMP
S
G
M
P
23
110
50
143
amino acids
pGMP
G
M
P
110
50
143

In vitro synthesis and glycosylation

Both pSGMP and pGMP were translated in the reticulocyte lysate system with and without added microsomal membranes and subsequently immunoprecipitated with prolactin antibody and treated with or without endoglycosidase H (Fig. 2). In the absence of membranes, transcribed and translated pSGMP yields an approximately 38 Kd species (Fig. 2A) as predicted for its amino acid content. On addition of membranes, SGMP has a slightly higher apparent molecular weight which is attributable to cleavage of its 23 amino acid signal peptide and addition of oligosaccharides at its N-glycosylation site, indicating that the amino-terminal globin domain has been inserted into the microsomal lumen (Fig. 2B, see also Fig. 4, lanes 2 and 6). In Fig. 2C, the SGMP band shifts down due to removal of oligosaccharides by endoglycosidase H.

pGMP yields a slightly smaller product than pSGMP due to the absence of a 23 amino acid signal (Fig. 2D). As expected, after cleavage of the signal peptide and removal of the oligosaccharides, SGMP has the same mobility as GMP. GMP is not efficiently glycosylated as demonstrated by a lack of change in electrophoretic mobility after translation in the presence of microsomal membranes (Fig. 2E; see also Fig. 4, lanes 10 and 14). After treatment with endoglycosidase H, SGMP, but not GMP, shifts to a lower apparent molecular weight due to removal of oligosaccharides (Fig. 2, compare C and F). Figure 3 indicates the extent of glycosylation of SGMP and GMP molecules by

Figure 2. *In vitro* synthesis and glycosylation. The DNA was transcribed *in vitro*, and the resultant mRNA was translated in rabbit reticulocyte lysate in the absence (lanes A,D) or the presence (lanes B,C and E,F) of canine pancreatic microsomal membranes. After translation, all samples were immunoprecipitated with antibodies to bovine prolactin and subsequently samples in lanes C and F were treated with endoglycosidase H (Endo H) to cleave attached oligosaccharides. Immunoprecipitated translation products were analyzed by SDS-PAGE and fluorography. RMs, rough microsomal membranes.

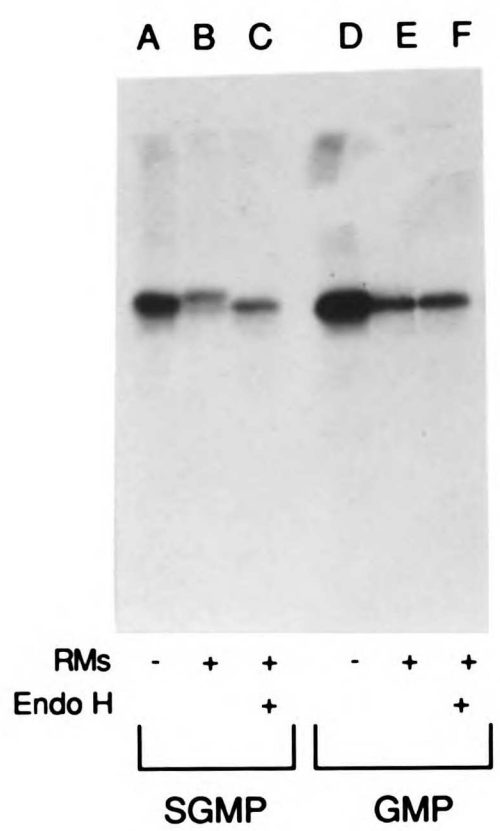
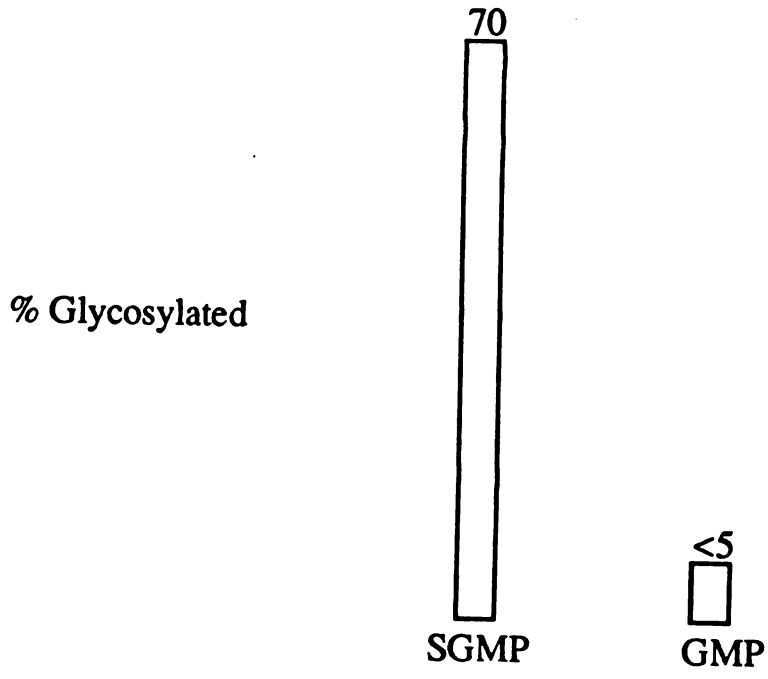

A B C D E F
RMs - + + - + +
Endo H + +
SGMP
GMP

Figure 3. Densitometry analysis of addition of oligosaccharides. Lanes 6 and14 of Figure 4 showing SGMP and GMP glycosylation were analyzed (using a shorter film exposure) with an LKB 2222 Ultrascan XL laser densitometer. Glycosylated band (upper of the two bands) was calculated as a percentage of glycosylated plus nonglycosylated bands in either lane 6 (SGMP) or 14 (GMP). Similar results were obtained from analysis of Figure 2, lanes B and E.


% Glycosylated
70
<5
SGMP
GMP

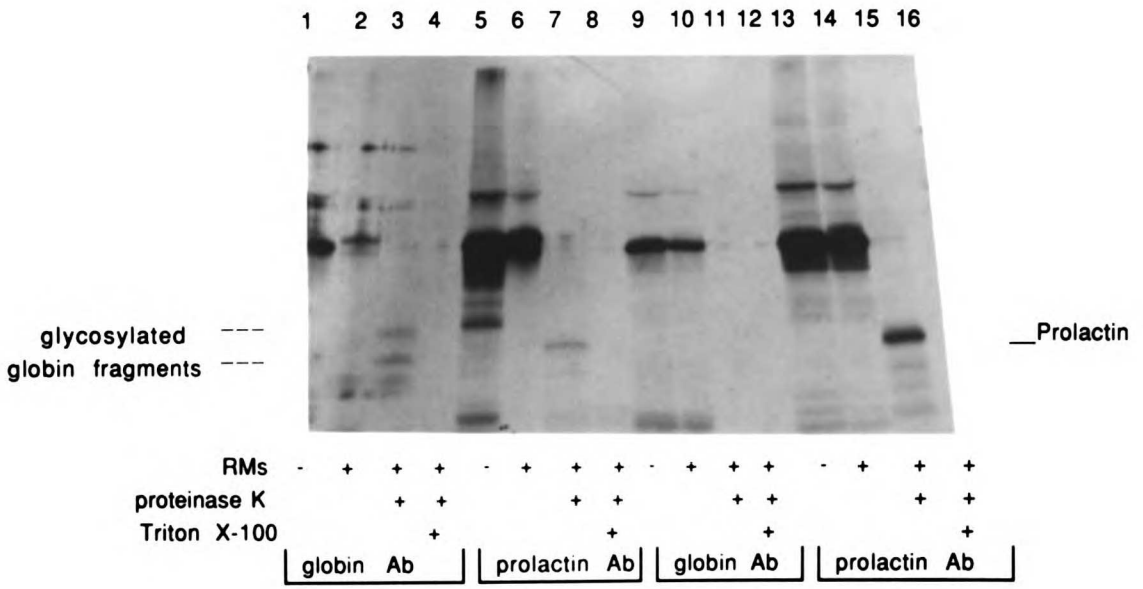
densitometry analysis of the glycosylated vs. non-glycosylated fragments in Figure 4 (lanes 6 and 14 at a shorter exposure). 70% glycosylation is approximately the maximum glycosylation detectable in this *in vitro* system (Walter and Blobel, 1983). These data indicate that SGMP molecules insert their amino termini into the lumen of the microsomal membranes, as observed by addition of oligosaccharides and cleavage of the signal peptide and that GMP molecules do not insert their amino termini.

In vitro synthesis and proteolysis

As an additional test of orientation, proteinase K was added post translationally to digest the cytoplasmically exposed domains of these artificial proteins (Fig. 4). Those protein domains translocated across the microsomal membrane are protected from externally added protease (Walter and Blobel, 1983; Morimoto et al., 1983). Detergent is added in control samples to disrupt the bilayer and thus disrupt the protection from protease which was provided by the membrane (Fig.4, lanes 4,8,12,16).

The data in figure 4 show that the globin domain on the amino end of the SGMP protein is translocated across the membrane into the lumen of the microsome where it is protected from protease (Fig.4, lane 3), but accessible to signal peptidase and addition of oligosaccharides (Fig.4, lane 2). These protease protection experiments with globin antisera (Fig. 4, lanes 1-4) confirm the glycosylation results of Fig 2 which used prolactin antisera.

Figure 4. *In vitro* synthesis and proteolysis. Both proteins, SGMP and GMP, were synthesized in the reticulocyte cell-free system in the absence or presence of microsomal membranes with and without added proteinase K (0.2 mg/ml) or Triton X-100 detergent (1%). Products were immunoprecipitated with either anti-human hemoglobin (globin Ab) or with anti-bovine prolactin (prolactin Ab) and analyzed with SDS-PAGE, flurography and autoradiography. RMs, rough microsomal membranes.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
glycosylated
globin fragments
Prolactin
RMs
proteinase K
Triton X-100
globin Ab
prolactin Ab
globin Ab
prolactin Ab
SGMP
GMP

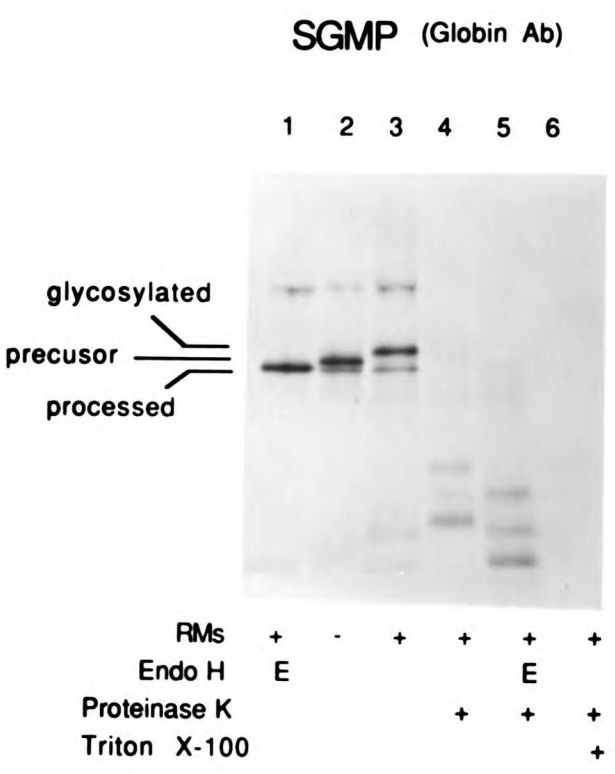
The protected prolactin fragments in lane 7 of Fig. 4 indicate that SGMP is also capable of inserting its carboxy terminus into the microsomal lumen, but at a much reduced efficiency (see Fig. 5, and compare lane 7 with lane 15, Fig. 4). It was suspected that the amino signal peptide of SGMP may occasionally be missed for targeting to the membrane. And in this case the second topogenic signal, the transmembrane or M segment, occasionally may function as it does for GMP and thus insert the carboxy end of the molecule such that the prolactin domain is protected from externally added protease. However, in this case one would expect to see some proportion of uncleaved precursor in the absence of protease, and this is not detectable in our system. Alternatively, the signal peptide may initially enter the membrane, be cleaved and then released back in the cytoplasm as has been shown with Hepatitis B virus precore protein (Garcia et al., 1988). At this point globin will be emerging from the ribosome in much the same fashion as the GMP molecule which lacks a signal peptide (Fig. 4, lanes 9-16). Translocation may be reinitiated by the transmembrane segment and membrane insertion could occur in the same fashion as for GMP as described below. This may account for the prolactin protected fragments of SGMP observed (Fig. 4, lane 7).

When similar protease experiments are carried out with GMP molecules which lack an amino-terminal signal peptide, the prolactin domains (which reside on the carboxy termini) are well

protected (Fig. 4, lane 15; also see Fig. 5) as determined by immunoprecipitation with prolactin antibody after proteolysis. The amino terminus of GMP is very poorly glycosylated (see lanes 10 and 14, Fig. 4). Protection of the amino globin domains can only be detected on long exposures; these data agree with the glycosylation results of Fig. 2 which used prolactin antisera. These data are analyzed quantitatively by densitometry analysis as described below.

Figure 5 shows transcribed and translated SGMP after addition of proteinase K and endoglycosidase H with globin antisera immunoprecipitation. This allows us to examine more closely the amino-terminal globin domain of SGMP. Figure 5, lane 4, shows that as before the globin domain is protected from protease. It was expected that some of the bands in lane 4 represented glycosylated globin fragments, so endoglycosidase H was added to remove oligosaccharides (Fig. 5, lane 5). As shown in figure 5, there appear to be different size globin fragments protected from protease and at least two fragments are glycosylated as assayed by a shift down on the gel with addition of endoglycosidase H. It is not known what these bands represent with respect to the extent of translocation of the amino-terminal globin domain of SGMP molecules. The inserted domain may be fully or partially translocated across the ER membrane, or alternatively, the proteinase K may be fully or partially digesting the proteins some of which may still be attached to ribosomes.

Figure 5. Protease protection and glycosylation of the amino-terminal globin domain of SGMP. SGMP was synthesized in reticulocyte lysate in the absence or presence of microsomal membranes with and without added proteinase K (0.2 mg/ml). Endoglycosidase H was added to proteolyzed and nonproteolyzed products (lanes 1 and 5) to determine the addition of sugars to the amino-terminal globin domain. All products were immunoprecipitated with anti human hemoglobin (Globin Ab). Processed indicates the nonglycosylated, cleaved signal peptide form; precursor indicates nonglycosylated, noncleaved form; and the glycosylated polypeptide has also had sugars added and its signal peptide cleaved. RMs, rough microsomal membranes; E or Endo H, endoglycosidase H.

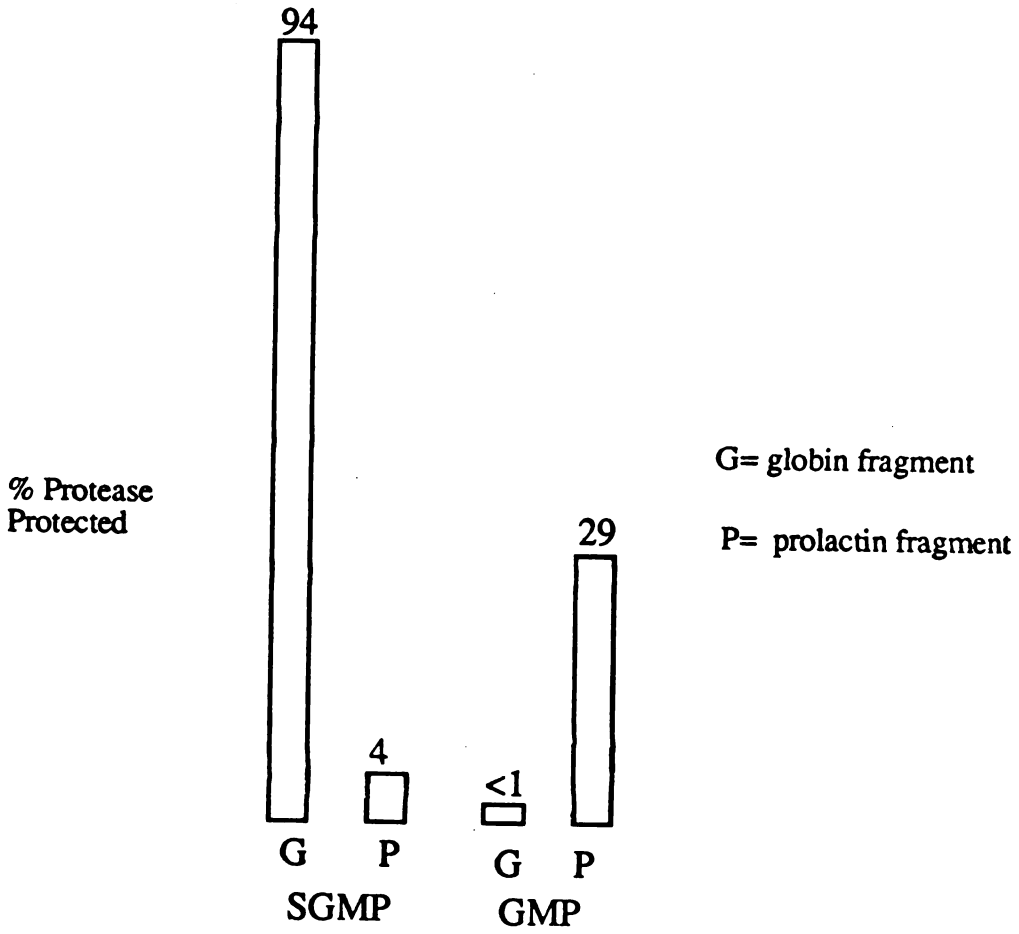

SGMP (Globin Ab)
1 2 3 4 5 6
glycosylated
precusor
processed
RMs + - + + + +
Endo H E E
Proteinase K + + +
Triton X-100 +

Densitometric analysis of glycosylation and protease protection

Densitometric analysis of Figure 4 (shown in Figure 3) gives a quantitative indication of the extent of glycosylation of GMP and SGMP which each contain the synthetic glycosylation site in the globin domain near the amino terminus. Figure 3 shows that SGMP is much more efficiently glycosylated than GMP, indicating that the amino terminus of SGMP is preferentially located in the microsomal lumen.

Analysis of the proteolyzed fragments in Figure 4 (see Experimental Procedures for calculations) by densitometry and adjusting for methionine content gives a comparison of the amino and carboxy termini protected from externally added protease (shown in Figure 6). The prolactin at the carboxy end of GMP is 7 times better protected than the prolactin at the carboxy end of SGMP. And the globin at the amino end of SGMP is very efficiently protected, while the protected globin fragments of GMP can only be detected by long exposures. Taken together with the glycosylation results, these protease protection data suggest that GMP preferentially translocates its carboxy terminus across the microsomal membrane and SGMP translocates its amino terminus across the membrane in a reversal of transmembrane polarity.

Figure 6. Densitometry analysis of fragments of SGMP and GMP protected from protease. The data in figure 4 are shown here as bar graphs. % Protease protection for prolactin (P) is calculated as the density (absorbance as determined by the LKB 2222 densitometer) of the prolactin immunoreactive fragment protected from protease divided by the density of the prolactin immunoreactive precursor (GMP) times 100% and adjusted for methionine content. For example, % protease protected P = P/GMP x 100% x 7/4 methionines; % protease protected G = G/GMP x 100% x 7/3 methionines. *In vitro* translations were performed in the presence of 35S methionine, therefore the bands representing the smaller, proteolyzed fragments have less methionines and appear less dense. The methionine content of GMP is 7 per GMP molecule: G = 3 methionines; P = 4 methionines; M = 0 methionines. After insertion into the microsomal membrane, the signal peptide is cleaved, therefore its methionine (1) is not used in the calculations for SGMP protease protection.

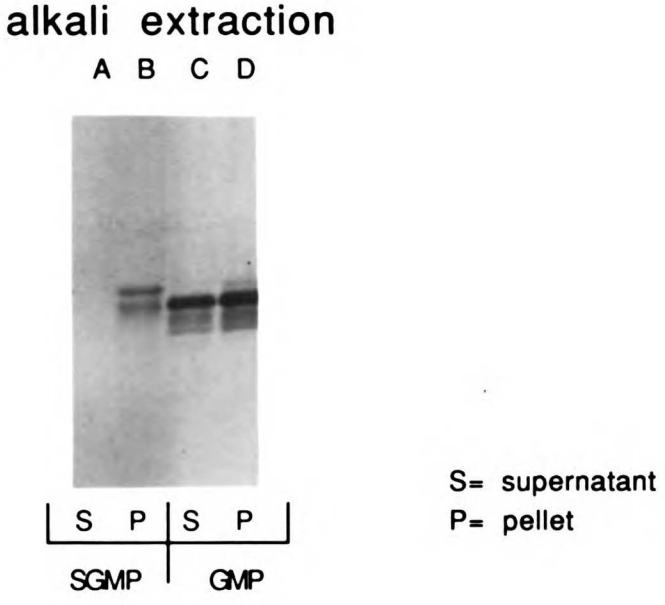

% Protease Protected
94
4
<1
29
G
P
G
P
SGMP
GMP
G= globin fragment
P= prolactin fragment

Membrane integration

Incubation of microsomal membranes post translationally with sodium carbonate at alkaline pH releases those proteins which are not well integrated with the lipid bilayer (Fujiki, 1982). Subsequent centrifugation yields a pellet which contains the disrupted microsomal membranes containing integral membrane proteins. First, microsomal membranes were centrifuged through a sucrose cushion to remove those molecules not associating with the membrane. Then carbonate extraction was carried out on the sucrose pellet. GMP molecules are more easily extracted from the membrane than SGMP molecules (Fig. 7). SGMP molecules pellet completely with the membranes (Fig. 7 B), but GMP molecules are approximately 40% carbonate extractable and released into the supernatant (Fig. 7 C), although both of these proteins are partially resistant to externally added protease (Fig. 4), indicative of a transmembrane orientation.

This is similar to the findings of Audigier et al. (1987), for the dissected transmembrane segments of bovine opsin. They suggested that the alkali extractability and protease resistance of the opsin constructs might be due to the retention of the transmembrane proteins in a water accessible channel within the bilayer as has been shown for chains in the process of translocation (Gilmore and Blobel, 1985). It is interesting to note that in the study presented here, both SGMP and GMP utilize the same transmembrane segment with the same flanking domains;

Figure 7. Membrane integration. Translations were performed in reticulocyte lysate in the presence of microsomal membranes. Membranes were isolated by centrifugation through a sucrose cushion and subsequently resuspended in .1 M sodium carbonate buffer (pH 11.5) which releases non-integrated proteins (Fujiki et al., 1982). After incubation in sodium carbonate and centrifugation, pellet, P and supernatant, S, fractions were characterized by SDS-PAGE and fluorography. SGMP shows glycosylated and nonglycosylated forms integrated in the membrane (lane B).


alkali extraction
A B C D
S P S P
SGMP GMP
S= supernatant
P= pellet

after signal cleavage, the overall hydrophobicities are the same, yet they are extracted from the membrane with carbonate quite differently. It appears in this case that simple hydrophobicity is not the determining factor for the difference of membrane integration. Another possibility for the differential carbonate extractibility of SGMP and GMP may be the simple reversal of the transmembrane segment in the bilayer. This may in some way create a less stable interaction with the lipid, perhaps by folding of the flanking domains in different cellular compartments, or charges on either side of the bilayer (Cutler et al., 1986).

Figure 8 shows the amino acid sequence derived from the extreme carboxy terminus of IgM heavy chain and used for the M region of GMP and SGMP in these studies. The region proposed to span the membrane in the native protein is underlined. It is interesting to note that the 3 carboxy amino acid residues (lysine-valine-lysine), which are cytoplasmically disposed in the native molecule, have two positive charges. von Heijne (1986) has postulated that positively charged residues, e.g. lysine and arginine, next to the transmembrane region might serve to retain that section of a protein in the cytoplasm. This is the case for transmembrane orientation of the M region of SGMP, but not for GMP. Cutler et al. (1986) found that changing positively-charged cytoplasmic residues of Semliki Forest Virus E2 protein to no charge or to negative charges did not change the transmembrane topology, but that the mutant proteins are 3 to 4 times more extractable with

Figure 8. Amino acid sequence of the M segment of SGMP and GMP (J. Rogers et al., 1980). The membrane spanning region and flanking regions from IgM heavy chain used in these constructions is shown here. The hydrophobic membrane spanning region is underlined and the charged amino acid residues are shown.

Membrane and Membrane-flanking Regions of μ_m Chain

--- Thr Glu$^-$ Arg$^+$ Thr Val Asp$^-$ Lys$^+$ Ser Thr Glu$^-$ Gly Glu$^-$ Val Asn Ala Glu$^-$ Glu$^-$

Glu$^-$ Gly Phe Glu$^-$ Asn Leu Trp Thr Thr Ala Ser Thr Phe Ile Val Leu Phe Leu

Leu Ser Leu Phe Tyr Ser Thr Thr Val Thr Leu Phe Lys$^+$ Val Lys$^+$

J. Rogers et al., Cell 20: 303 (1980)

alkali. This is in agreement with the increased alkali extractability of GMP which with the reversed transmembrane segment has more than 6 negative charges on the cytoplasmic side of the membrane, unlike SGMP which retains the 2 positively charged residues on the cytoplasmic side of the transmembrane segment (like the transmembrane segment in its native state in IgM heavy chain) and is resistant to alkali extraction. The role of charges in generation and maintenance of protein transmembrane topology is presently unknown.

Discussion

Taken together, these data suggest that GMP preferentially translocates its carboxy terminus and the same molecule with an amino cleaved signal peptide, SGMP, translocates its amino terminus across the microsomal membrane. The same transmembrane segment, M, spans the membrane with opposite polarity in these two proteins.

These results indicate that it is not the transmembrane segment which determines the transmembrane orientation of membrane proteins. This has also been concluded in the investigation of five of the bovine opsin transmembrane segments: Audigier et al. (1987) concluded that the amino terminus was translocated independently of the transmembrane segment used in various constructions, and further proposed that the amino terminus itself may be the determinant for flipping of the amino terminus into the microsomal lumen. In this case of bovine opsin

the amino-terminal flanking domain was translocated without an amino-terminal signal peptide. These results suggest that domains outside of the transmembrane region may be important for determination of transmembrane protein asymmetry.

SGMP and GMP span the membrane with opposite polarity, but consist of the same transmembrane segment and the same flanking domains. The difference is the signal peptide of SGMP versus no signal peptide in GMP. The signal peptide serves to target SGMP to the membrane before the globin domain emerges from the ribosome. According to current models for ER membrane insertion (Walter, 1987), after targeting of the SRP/signal peptide/ribosome complex to the membrane, the translating ribosome forms a ribosome-membrane junction. Through this junction, globin (G) of SGMP would be extruded as it is synthesized and emerges from the ribosome. Therefore, in the case of SGMP with the amino-terminal signal peptide, the globin domain would not be allowed to fold into a globular structure in the cytoplasm before transfer across the membrane, but would be contained in the ribosome and extruded into the ER lumen through the ribosome-membrane junction as it is synthesized.

However, in the case of GMP, the translating ribosome is not targeted to the membrane until the transmembrane region of the M segment emerges from the ribosome and interacts with SRP to be targeted to the membrane. In this case, when the hydrophobic transmembrane region of GMP emerges from the ribosome, 110

amino acids of globin and 21 amino acids of the M domain have already been synthesized and extruded from the ribosome in the cytoplasm. The data here show that the prolactin domain which follows the transmembrane signal activity is preferentially transfered across the membrane. The amino terminal domain (131 residues) of GMP does not preferentially transfer across the bilayer as determined by glycosylation and protease protection data (Figures 2,3,4,5). Several groups have proposed that a nascent chain must be in a partially unfolded state for transfer across the bilayer (Wickner, 1979; Randall and Hardy, 1986; Eilers and Schatz, 1986). The 131 residue amino-terminal domain of GMP may be thermodynamically unfavored for transfer across the membrane due to folding, charges or hydrophobicity, or to some unknown interaction with cytoplasmic components.

The initial insertion of a signal peptide has been postulated to occur in a loop conformation (Engelman and Steitz, 1981) which would leave the amino terminus of the signal peptide on the cytoplasmic side of the membrane. It seems likely that insertion of a protein with an internal, uncleaved transmembrane region may also insert initially in a loop conformation which could leave either the amino or carboxy terminus of the protein on the cytoplasmic side of the membrane. If either the amino or carboxy terminus is attached to some component in the cytoplasm or folded unfavorably, the other terminus may be free to translocate across the membrane. This type of loop or hairpin insertion for transmembrane proteins has been suggested by Spiess and Lodish

(1986) and by Lipp and Dobberstein (1986). We expect that GMP inserts in the membrane in this way.

When the transmembrane segment, M, is preceded by a cleaved signal peptide as in SGMP, the signal peptide targets the translating ribosome to the membrane where a postulated ribosome-membrane junction is formed through which the nascent chain is extruded as it is synthesized. The hydrophobic, transmembrane segment functions as a stop transfer. In this case, the transmembrane segment, M of SGMP, extrudes from the ribosome already engaged with the membrane and is not physically available to interact with SRP in the cytoplasm or to form a loop before interaction with the membrane. Therefore, in the case of SGMP which has a preceding signal peptide, the transmembrane segment is inserted into the membrane in an extended (not looped) conformation and acts as a stop transfer to halt the transfer of the carboxy terminus across the membrane, and its potential signal function is not utilized. We predict that SGMP and GMP accommodate the same transmembrane segment, but membrane insertion of the transmembrane (M) segment occurs either in an extended or loop conformation functioning as a stop transfer or signal/anchor depending on the presence or absence of an amino-terminal signal peptide.

Membrane proteins which span the membrane more than once may be expected to involve more subtle and complex interactions with adjacent transmembrane or flanking regions,

with SRP, or with components as yet unidentified. We have demonstrated here that more than the transmembrane region of a protein is required to determine protein topology. Some combination of signal peptides and membrane spanning segments with influences of their flanking domains (including nascent chain folding, distribution of charges, hydrophobicity, amphipathicity, etc. that may yield a thermodynamically favored or unfavored transfer across the bilayer) probably interact to determine membrane protein assembly.

Chapter 4

CONCLUSIONS AND FUTURE DIRECTIONS

Construction of chimeric proteins by manipulation of cDNA, coupled with *in vitro* transcription and translation assays have allowed us to test the mechanisms of membrane insertion *in vitro*. From biochemical analysis of these chimeric proteins co-translationally inserted into microsomal membranes we found that 1) a stop transfer sequence of a protein can function as a signal for membrane insertion, and 2) transmembrane protein asymmetry can be reversed with a signal peptide.

Analysis of stop transfer sequence function

In chapter 2, I show that a hydrophobic transmembrane region, previously defined as a stop transfer sequence (Yost et al., 1983), functions like a signal peptide to direct membrane insertion of an artificial membrane protein. This insertion is shown to occur in a Signal Recognition Particle (SRP) dependent manner and generates a transmembrane protein. I demonstrate that a stop transfer sequence differs from a signal peptide in that the stop transfer has no cleavage site for signal peptidase and is therefore retained in the mature protein, but the signal peptide is cleaved by signal peptidase; the stop transfer sequence serves to generate a transmembrane protein, while the signal peptide serves to generate a soluble, secretory protein in the endoplasmic reticulum (ER) lumen.

Analysis of asymmetric insertion of simple transmembrane proteins

In Chapter 3, I show that an artificial transmembrane protein inserts either its amino or carboxy terminus into the ER lumen *in vitro* depending on the presence or absence of a signal peptide. By DNA construction, we were able to generate two proteins which differ only by the presence or absence of a 23 amino acid signal peptide which becomes cleaved by signal peptidase upon translocation across the ER membrane. Cleavage of the signal peptide generates a protein which is identical in amino acid composition to the protein without a signal peptide, but which preferentially spans the membrane in an opposite transmembrane orientation.

Implications for mechanisms of membrane insertion: loop versus linear

Insertion of the signal peptide as a loop

The Blobel and Dobberstein signal sequence hypothesis (1975a) for membrane insertion suggested that the initial insertion of nascent chains occurs in a vectorial fashion with the most amino-terminal residue inserting first. More recent models have suggested that an amino-terminal signal peptide inserts into the membrane as a loop, leaving the amino terminus of the signal peptide in the cytoplasmic compartment with the ribosome from which it emerged (Inouye et al., 1977; Engelman and Steitz, 1981; Sabatini et al., 1982; Inouye et al., 1986). Evidence for this comes from 1) analysis of mutations at the proposed β bend near the

cleavage site of the signal peptide, correlated with changes in translocation of the nascent chain (Inouye et al., 1986), and from 2) thermodyamic predictions of the secondary structure of the signal peptide before and after insertion (Engelman and Steitz, 1981).

Insertion of the signal/anchor as a loop

By using artificial proteins, I show in chapter 3 that a signal/anchor region inserts initially as a loop when it is not preceded by a cleaved signal peptide. The insertion of signal/anchor regions resembles that of signal peptides: it is SRP and SRP receptor dependent. This work and that from other laboratories (Spiess and Lodish, 1986; Lipp and Dobberstein, 1986; Kuhn, 1987) indicate that insertion of an internal signal/anchor domain also occurs in a loop or hairpin conformation as shown in Figure 1. Transmembrane proteins that leave their amino terminus in the cytoplasm, e.g. transferrin receptor, asialoglycoprotein receptor, etc., require a loop conformation for their co-translational insertion into the bilayer: the amino terminus is left in the cytoplasm and the carboxy terminus is tied up in the ribosome, so the middle region is left to insert in the membrane before translation is completed. This concurs with the interaction of SRP with the internal signal/anchor region and targeting of this internal region to the membrane for insertion.

Figure 1. Signal/anchor function: membrane insertion of GMP. In the absence of an amino-terminal signal peptide, the globin domain of the nascent chain is synthesized before the transmembrane (signal/anchor) region emerges and interacts with signal recognition particle (SRP). Therefore, after synthesis of globin, the SRP/nascent chain/ribosome complex is targeted to the endoplasmic reticulum membrane where SRP binds SRP receptor (or docking protein) and releases the transmembrane signal/anchor region. The signal/anchor region is shown here inserting in a loop conformation. After targeting and insertion of the looped nascent chain in the membrane, the carboxy end of the protein is synthesized and extruded into the endoplasmic reticulum lumen.

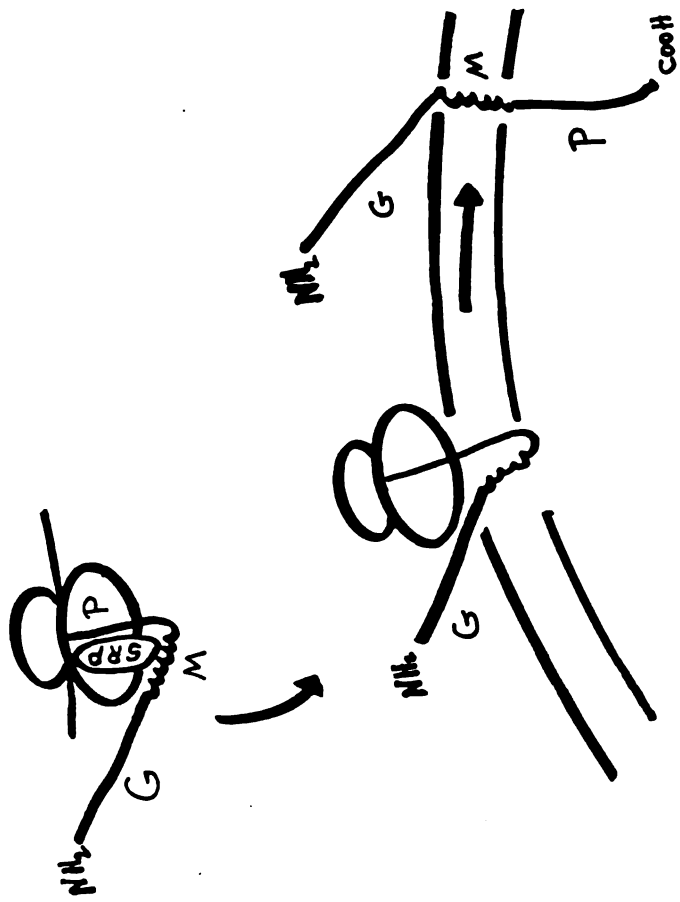
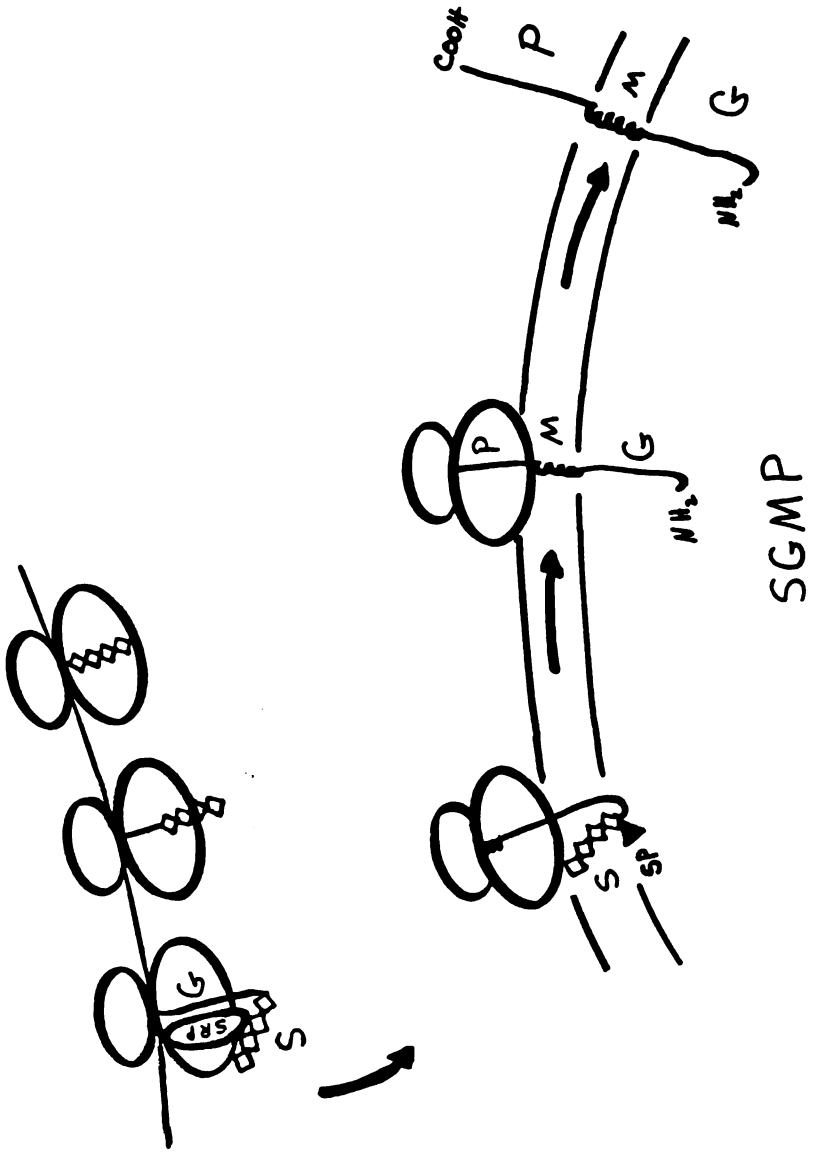

P
SRP
M
G
NH2
M
COOH
P
G
NH2
GMP

Figure 2. Stop transfer function: membrane insertion of SGMP. When the signal peptide (represented here by diamonds) emerges from the ribosome, it interacts with SRP and the SRP/nascent chain/ribosome complex is targeted to the membrane where SRP is released. Upon insertion into the endoplasmic reticulum lumen, the signal peptide is cleaved by signal peptidase, generating a new amino terminus of the mature protein in the lumen. The nascent chain continues to grow and the transmembrane region emerges from the ribosome and into the membrane where it acts as a stop transfer to stop the transfer of the carboxy end of the protein across the membrane.


P
M
G
SRP
S
SP
SGMP

Insertion of the stop transfer sequence linearly

However, when the signal/anchor region is preceded by a signal peptide (SLMG, Chapter 2; SGMP, Chapter 3), it is not accessible to interact with SRP because it is extruded linearly from the ribosome which has already been attached to the membrane by the amino terminal signal peptide as shown in Figure 2. In this position the M region acts as a stop transfer and not a signal. In chapter 3, we show that the same transmembrane segment of a protein may function as a signal/anchor or as a stop transfer depending on whether it is allowed to interact with SRP and form a loop or inserts into the membrane linearly and that this insertion depends on the presence or absence of a preceding signal peptide. The presence of preceding hydrophobic regions thus affects the insertion of adjacent hydrophobic domains.

Implications for models of membrane assembly

Insertion sequence models

Several groups have suggested that hydrophobic insertion sequences, e.g. signal peptides, signal/anchor, and stop transfer domains are responsible for generation of transmembrane protein topology (Blobel, 1980; Engelman and Steitz, 1981; Friedlander and Blobel, 1985). Friedlander and Blobel (1985) propose that a multi-spanning membrane protein is inserted by a series of signal and stop transfer domains in alternating order and that combinations of insertion sequences could account for topologies of various membrane proteins. Engelman and Steitz (1981) predict that the positions of the hydrophobic and polar helices of

inserted helical hairpins may determine which domains are translocated and which helices remain in the membrane. Of the two sides of the helical hairpin inserted into the membrane, they propose that a hydrophobic helix will remain in the membrane, and a polar helix will be transferred across. They suggest that the helical hairpin model accounts for the generation of all topologies of simple and complex membrane proteins. When the nascent chain first inserts in the membrane, the helical hairpin model predicts an α helix on one side of the inserted hairpin and a β structure on the other. The more polar β structure is predicted to slip through the membrane and the more hydrophobic α helix to remain in the lipid bilayer. Construction and translation of artificial proteins could be used to test this model. SRP may also function in this model mechanism to hold the nascent chain in a loop conformation for membrane insertion, and SSR may hold the α helical domain in the membrane as SSR has been shown to interact with a signal peptide (Wiedmann et al., 1987). More studies are needed to substantiate these interactions.

Role of the signal peptide for insertion of the amino terminus

If one hydrophobic region, i.e. signal/anchor, is sufficient to insert and anchor a transmembrane protein in the ER membrane, why do some proteins also have an amino-terminal cleaved signal peptide? I propose in Chapter 3, that by formation of a postulated ribosome-membrane junction, the signal peptide allows for transfer of the amino terminus of the nascent chain from the ribosome and across the bilayer without the opportunity to

interact with cytoplasmic components or to fold into a conformation unfavorable for translocation. A signal peptide on the amino terminus of a transmembrane protein favors a topology with the amino terminus in the ER lumen (Rogers et al., 1980; Rose and Gallione, 1981; Gething and Sambrook, 1982; Russell et al., 1984).

Role of the signal/anchor and flanking domains for membrane assembly

The results I present here in Chapter 3 and those of other groups (Audigier et al., 1986; Zerial et al., 1987; Hull et al., 1988) point to an important role of the domains flanking transmembrane regions in defining protein membrane asymmetry. In the absence of an amino terminal signal peptide, either flanking domain of a simple transmembrane protein may insert in the membrane. The characteristics of protein domains which allow them to pass through the membrane, or to interact in a less transient way with the bilayer, have been difficult to sort out to date and remain a perplexing problem. Folding, hydrophobicity, charges, amphipathicity or a combination of these as defined by the thermodynamics of transfer across the membrane may determine the final asymmetry of a protein in the bilayer. Alternatively, interaction with as yet unknown cytoplasmic or membrane components may also influence asymmetry.

Role of the stop transfer for membrane assembly

What is a signal or signal/anchor, and what is a stop transfer? All 3 of these "topogenic" domains are hydrophobic and capable of interacting with SRP (Anderson et al., 1982; also see Chapter 2, Figure 8). If an ER signal or signal/anchor is defined by its interaction with SRP, then a stop transfer may be defined by its lack of interaction with SRP. With a preceding signal peptide, an internal hydrophobic domain emerges from the ribosome across the membrane without the opportunity to interact with SRP or other cytoplasmic components and functions as a stop transfer. We have shown that this same hydrophobic domain may interact with SRP and function as a signal/anchor in the absence of a preceding cleaved signal peptide (Chapter 2). Thus, the evidence presented in this work suggests that the difference between stop transfer and signal/anchor sequences is positional and may be dependent on interaction with SRP or other cytoplasmic components.

Walter (1987) suggests that after emerging from the ribosome, signal peptides first interact with SRP and then are handed off to signal sequence receptor (SSR) in the membrane. Interaction of prolactin signal peptide with the integral membrane SSR has been demonstrated by Wiedmann et al. (1987) by cross-linking experiments. The role SSR in the insertion of transmembrane proteins has yet to be explored. More elegant reconstitution experiments may make this investigation possible in the future.

The alternating signal and stop transfer model proposed by Friedlander and Blobel (1985) has been revised (Audigier et al., 1987) by the finding that 5 of the 6 tested transmembrane regions of bovine opsin can act as signals when tested out of the context of the complete opsin molecule. Our finding that a stop transfer region may act either as a signal/anchor or a stop transfer depending on adjacent topogenic signals, requires that the model for membrane insertion by alternating signal and stop transfer regions be modified to adapt for influences of preceding hydrophobic regions on stop transfer/signal function. Spiess and Handschin (1988) unsuccessfully attempted to separate signal from stop transfer activity by dissection of the signal/anchor domain of asialoglycoprotein receptor. We suggest that these two domains are inseparable, and that it is the position within the protein which determines the stop transfer or signal function of the membrane spanning region.

Membrane assembly of multi-spanning membrane proteins

One of many important problems still unsolved with respect to multi-spanning membrane proteins is whether SRP is required for insertion of only the first signal domain or whether SRP is required also for insertion of each following membrane spanning domain after the protein has already been targeted to the membrane. With a secretory protein, prolactin, Siegel and Walter (1988) have shown that each nascent chain on a polysome requires an SRP molecule for its membrane insertion, but the question of SRP dependence of the subsequent hydrophobic

domains on one nascent chain remains unsolved. Also the role of SSR for the insertion of simple and more complex membrane proteins remains untested awaiting successful reconstitution of protein translocation from dissected components of the membrane and cytoplasm. From these types of further analysis of insertion of simple, single- and double-membrane spanning proteins, it should be possible to uncover more of the rules and mechanisms governing membrane protein insertion and attempt to apply these rules to the assembly of more complex multi-spanning membrane proteins.

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