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Uncoupling translocation from translation:
Implications for transport of proteins
across the membrane of the endoplasmic reticulum

by

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UNCOUPLING TRANSLOCATION FROM TRANSLATION: IMPLICATIONS FOR TRANSPORT OF PROTEINS ACROSS THE MEMBRANE OF THE ENDOPLASMIC RETICULUM

by Eve Perara

The obligate coupling of translocation to translation is a characteristic feature of the localization of proteins to the endoplasmic reticulum (ER). This feature of translocation has hindered the elucidation of mechanism since translocation of a polypeptide could only be studied during the brief period of time and under the specific conditions required for its translation. Cell-free translation systems which can be programmed with exogenous mRNAs and supplemented with microsomal membranes derived from the rough endoplasmic reticulum to reconstitute translocation in vitro have been used extensively to identify and characterize molecular components involved in the translocation process. We have used molecular genetics techniques to manipulate expression plasmids which encode altered proteins whose expression in such cell-free systems or in vivo has provided insight into the mechanism of translocation. By engineering an amino terminal signal sequence to an internal position we have shown that a signal sequence can direct translocation of both flanking protein domains both in cell-free translocation systems and in intact cells. The translocation of the (previously synthesized) amino terminal domain by the internal signal sequence demonstrated that translocation of a given protein domain need not be coupled to the elongation of that domain and suggested that translocation could be uncoupled from ongoing protein synthesis experimentally. Dissociation of translocation across the ER membrane from elongation in vitro and in intact cells was achieved by generating polypeptide chains whose translation was not terminated and which remained associated as peptidyl

tRNAs to the synthesizing ribosome. Such polypeptides were translocated independent of chain elongation providing an energy source was available and providing they remained associated with the ribosome, suggesting a role for the ribosome in translocation, distinct from its role in protein synthesis. A polypeptide was engineered which was translocated in vitro following termination and release from the ribosome, indicating that the ribosome may not be necessary for the process of translocation per se. Thus, a role for the ribosome in maintaining a translocation competent state and/or as a ligand for membrane recognition is suggested. This role appears to be essential physiologically since translocation following release from the ribosome was not seen in vivo.

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HISTORICAL BACKGROUND AND INTRODUCTION

The localization of proteins to specialized membrane-bound compartments and the resultant segregation of biochemical functions is a characteristic feature of eukaryotic cells. Thus, the mitochondria are the exclusive site of oxidative phosphorylation because F_1 ATPase, cytochrome oxidase, and other enzymes are specifically localized to the mitochondrial membranes. Likewise, oxidative detoxification occurs in the endoplasmic reticulum, degradation of endocytosed proteins in the lysosomes, and so on, as a consequence of protein targeting and localization. While the delimiting lipid bilayer(s) of subcellular compartments serves to maintain specific proteins within the organelles, it poses a distinct barrier to the initial correct segregation of these proteins. Since the synthesis of all but a few proteins (those encoded by mitochondrial or chloroplast DNA) occurs in the cytoplasm, mechanisms must exist by which proteins are efficiently and accurately sequestered into their specific membranous compartments. The subject of this dissertation is the transport of proteins across the endoplasmic reticulum (ER) membrane. This is the first step in the sorting of constituent proteins of the ER as well as proteins destined for the plasma membrane, Golgi complex, lysosome, or the exterior of the cell.

HISTORICAL BACKGROUND

In pioneering work on the secretory pathway, Palade and co-workers found that nascent secretory proteins were associated with the ER membrane via the large ribosomal subunit of the synthesizing polysomes (Sabatini et al., 1966; Palade, 1975) and that the mature polypeptides were released to the lumen of the ER (Redman et al., 1966) while cytoplasmic proteins were synthesized on free polysomes (Redman, 1969). These observations raised the question of how mRNAs encoding secretory proteins could be specifically selected to be translated on ER-bound ribosomes. Early hypotheses included models in which the specificity was proposed to reside in the

translating ribosome, in untranslated regions of mRNA, or in the nascent polypeptide chain itself.

An answer to this question emerged following the development of cell-free translation systems from crude cytosolic extracts which could be programmed with purified mRNAs. It was found that the primary cell-free translation products of (secretory) immunoglubulin light chains differed from authentic light chain by an amino-terminal extension that was not observed in translation products of nonsecretory proteins (Milstein et al., 1972). This additional peptide segment was proposed to be involved in the segregation of secretory proteins to the ER lumen (Milstein et al., 1972). This idea was confirmed by experiments in which cell-free translation reactions were supplemented with microsomal membranes derived from the rough ER to reconstitute translocation. It was found that secretory proteins were synthesized with amino-terminal sequences not present either in secretory products in vivo or in vitro products which were localized to the microsomal lumen, suggesting that the precursor protein was processed to the mature form by an enzymatic activity of the membranes (Blobel and Dobberstein, 1975 a, b). Moreover, since ribosomal subunits from free polysomes were used to translate the secretory protein (IgG light chain) which was segregated within microsomal membranes which had been stripped of ribosomes (Blobel and Dobberstein, 1975b), it was established that the information for translocation of a protein across the ER membrane is encoded in the mRNA itself, and not in the translation apparatus (e.g. the ribosomes).

The concept of "vectorial discharge" of the nascent polypeptide across the membrane was established by the work of Redman and Sabatini (1966), which demonstrated that puromycin-released nascent (incomplete) polypeptide chains were localized to the lumen of vesicles isolated from the rough ER. This idea was

confirmed by the finding that nascent polypeptides emerging from the ribosome were protected from proteases by the microsomal membrane (Sabatini and Blobel, 1970). In addition, a number of posttranslational modifications of proteins known to occur in the luminal space of the ER, such as cleavage of signal sequences by signal peptidase (Blobel and Dobberstein, 1975a; Miyata and Akazawa, 1982), transfer of core oligosaccharides from lipid-linked intermediates to asparagine residues (Lingappa et al., 1978a; Glabe et al., 1980), and intrachain disulfide bond formation (Bergman and Kuehl, 1979), have been shown to be carried out on nascent polypeptides as they traverse the ER membrane. Completed secretory protein precursors are incapable of being translocated (Blobel and Dobberstein, 1975b; Lane et al., 1979) and, in fact, there seems to exist a brief period early in a protein's elongation during which translocation can be initiated (Rothman and Lodish, 1977). Thus a picture has emerged of obligate coupling of the transport of secretory proteins to their biosynthesis, i.e., translocation of a given protein occurs concomitant with translation. This feature of protein translocation across the ER membrane distinguishes it from the transport of proteins to other organelles (Schatz and Butow, 1983) and from the export of bacterial proteins (Randall, 1983).

Assembly of integral transmembrane proteins into the ER membrane shares the above features of secretory protein translocation. They are synthesized on membrane bound polysomes (Morrison and Lodish, 1975), possess amino terminal cleaved signal sequences (Lingappa et al., 1978a) and compete with nascent secretory proteins for membrane-associated components involved in translocation (Lingappa et al., 1978a), suggesting that their assembly in the membrane occurs via the same mechanism as secretory protein translocation. Therefore, models for protein translocation account not only for complete translocation of secretory proteins but

also the partial translocation and membrane integration of integral transmembrane proteins.

The Signal Hypothesis

The above observations led to the formulation of the "signal hypothesis" (Blobel and Dobberstein, 1975b; Blobel, 1980; for review see Walter et al., 1984). According to the signal hypothesis, synthesis of secretory (and transmembrane) proteins begins on free ribosomes in the cytoplasm. Upon emergence of the signal sequence from the large subunit of the synthesizing ribosome, polysomes are targeted to the membrane of the ER where the nascent chain is translocated across the membrane concomitant with its synthesis. The signal sequence is proposed to facilitate translocation of the nascent chain through interactions with a series of receptors both in the cytoplasm and in the ER membrane. Transport of the protein across the membrane is proposed to occur via a proteinaceous pore or tunnel in the bilayer whose activation and assembly is facilitated by the signal sequence. Information for termination of translocation (e.g. in the case of transmembrane protein biogenesis), encoded also in a discrete segment of the polypeptide chain which is distinct from the signal sequence (termed "stop transfer sequence"), also is predicted to act via particular receptors in the membrane.

Signal sequences

Most secretory proteins and many transmembrane proteins are synthesized as precursors with transient amino-terminal signal sequences as are bacterial exported proteins. Compilations of known signal sequnces show no primary sequence homology, but reveal structural features that appear to be conserved (Watson, 1984; von Heijne, 1985). Signal sequences typically range from 15 to 30 amino acid residues in length. The extreme amino terminus usually carries a net positive charge and is

followed by a very hydrophobic core of variable length (at least six consecutive hydrophobic or uncharged amino acids). Often small side chain amino acids, such as glycine or alanine, occur at positions -1 and -3 of the cleavage site at the extreme carboxy terminus (von Heijne and Blomberg, 1979; von Heijne, 1984). Bacterial and eukaryotic signal sequences are virtually indistinguishable from one another structurally (von Heijne, 1985). In fact, eukaryotic proteins can be secreted and processed by bacteria (Talmadge et al., 1980 a, b) and, likewise, prokaryotic proteins are correctly segregated and processed when expressed in eukaryotic cell-free systems (Müller et al., 1982) or intact cells (Wiedmann, et al., 1984). Mutational analysis shows that point mutations and small deletions in a number of prokaryotic signal sequences prevent or diminish their secretion from *E. coli* (reviewed in Silhavy, et al., 1983). A systematic analysis of signal sequence structure and function in eukaryotic systems remains to be done.

Signal sequences usually occur at the extreme amino termini of most secretory and many integral transmembrane proteins and are cleaved from the nascent polypeptide before translation is complete. There are at least two exceptions to this rule: (1) Internal and uncleaved signal sequences have been described recently for a number of transmembrane proteins (Bos et al., 1984; Friedlander and Blobel, 1985; Eble et al., 1986; Lipp and Dobberstein, 1986; Spiess and Lodish, 1986; Zerial, et al., 1986) (2) The secretory protein ovalbumin, does not have a cleaved signal sequence (Palmiter et al., 1978) but has the functional equivalent (Lingappa et al., 1978b), the location of which remains controversial (Lingappa et al., 1979; Meek et al., 1982; Braell and Lodish, 1982; Tabe et al., 1984).

Research of the past decade has focused largely on the identification of components involved in translocation. This work has relied heavily on cell-free translation systems in which the components for proteins synthesis can be

fractionated from wheat germ extracts or reticulocyte lysates and the translocation process reconstituted by the addition of rough microsome derived from canine pancreas.

Signal Recognition Particle

When translation of a secretory protein is carried out in the presence of microsomal membranes, the mature protein is translocated to the vesicle lumen. Extraction of microsomal membranes with high salt renders the membranes incompetent for translocation. Signal recognition particle (SRP) activity was first recognized by the observation that the high salt wash of microsomal membranes restored translocation activity to rough microsomes which had been extracted with high salt (Warren and Dobberstein, 1978). The SRP molecule has since been purified to homogeneity from canine pancreas rough microsomes (Walter and Blobel, 1980), and its role in translocation has been studied in detail (Walter and Blobel, 1981 a, b; Walter et al., 1981). SRP has been shown to be required for translocation of secretory (Walter and Blobel, 1980; Stoffel, et al., 1981), lysosomal (Erickson, et al., 1983) and integral transmembrane proteins, both those with cleaved, amino terminal signals (Anderson, et al., 1982) and those with internal, uncleaved signal sequences (Lipp and Dobberstein, 1986; Spiess and Lodish, 1986; Zerial, et al., 1986). Subcellular fractionation demonstrates a roughly equal distribution of SRP between a membrane-associated and cytoplasmic (ribosome-associated or free) state (Walter and Blobel, 1983b). It's role appears to be that of a cytoplasmic "adaptor" for signal-bearing polysomes, targeting them to the ER membrane.

Structure

SRP is a ribonucleoprotein complex composed of six nonidentical polypeptides: a 19 kDa and a 54 kDa monomer, and two heterodimers, one

of a 9 and a 14 kDa polypeptides and the other composed of a 68 and a 72 kDa polypeptides (Siegel and Walter, 1985) plus one molecule of the small cytoplasmic 7SL RNA (Walter and Blobel, 1982). The 7SL RNA has been proposed to function as a scaffolding, along which the polypeptide subunits assemble (Walter and Blobel, 1983a; Siegel and Walter, 1985; Andrews et al., 1985,1987).

Signal Sequence Recognition

While SRP was shown to bind translationally inactive ribosomes, the emergence of a signal sequence from the large ribosomal subunit results in an increase in the affinity of SRP for the ribosome by as much as four to five orders of magnitude (Walter et al., 1981). SRP binds the signal sequence directly. This was suggested first by the finding that incorporation of the amino acid analog, β-hydroxyleucine, into the leucine-rich signal sequence of preprolactin abolished SRP-signal sequence interactions (Walter et al., 1981). Incorporation of a photoactivatable cross-linking amino acid analog into the signal sequence of nascent preprolactin has allowed cross-linking to the 54 kDa subunit of SRP, providing direct evidence for signal sequence-SRP binding (Kurzchalia et al., 1986; Krieg et al., 1986). This binding is reversible and appears to occur only in the context of the synthesizing ribosome (Krieg, et al., 1986; Wiedmann, et al., 1987a).

Elongation Arrest

In wheat germ cell-free translation systems the binding of SRP results in specific translational arrest of secretory proteins (Walter and Blobel, 1981b) which is released by the addition of microsomal membranes (Walter and Blobel, 1981b; see below). This elongation arrest function has been mapped to the 9/14 kDa heterodimer and to 7SL RNA sequences homologous to Alu DNA. Preparation of SRPs lacking either of these domains yields a particle which can recognize signal

sequences and facilitate translocation across salt-treated (SRP-free) microsomal membranes yet lacks the elongation arrest activity (Siegel and Walter, 1985, 1986). These partially reconstituted particles are active in promoting protein translocation, but only during a brief window of time early in nascent chain growth. Thus it appears that the role of SRP arrest may be to extend the window of time during which the nascent polypeptide is in a translocation competent state.

The mechanism by which SRP arrests translation is currently unclear. Analysis of the structure and domain function of the SRP molecule presents an interesting possibility. The dimensions of the SRP molecule are such that the particle could span from the site at which the signal sequence emerges from the large ribosomal subunit to the elongation site between the two ribosomal subunits (Andrews, et al., 1985). The 54 kDa subunit binds to signal sequences directly (Kurzchalia et al., 1986; Krieg, et al., 1986); it has been suggested that the 9/14 kDa subunit and/or the 7SL RNA of SRP (domains shown to be necessary for SRP-arrest, Siegel and Walter, 1985, 1986) may also bind to the ribosome, hindering subsequent binding of aminoacyl-tRNAs and continued protein synthesis.

The physiological significance of the elongation arrest activity of SRP is unclear since not all signal sequence-bearing proteins experience a tight elongation arrest by SRP (Anderson, et al., 1983), nor is strict elongation arrest by canine SRP observed in some mammalian cell-free systems (Meyer, 1985). However, a kinetic delay in chain elongation is observed specifically for signal sequence-bearing proteins in a fractionated mammalian translation system supplemented with purified canine SRP (P. Walter, personal communication). This is consistent with the idea that an important role of SRP may be to extend the window of time during which the nascent secretory protein is in a "translocation competent state."

Signal Recognition Particle Receptor

The SRP receptor (also termed docking protein, Meyer et al., 1982a) is an ER membrane protein localized to the cytoplasmic face of the membrane (Meyer et al., 1982b) and has been purified from dog pancreas rough microsomes using SRP affinity chromatography (Gilmore et al., 1982 a,b). A 60 kDa cytoplasmic domain of SRP receptor can be cleaved from the membrane by proteases and added back to reconstitute translocation activity (Walter et al., 1979; Meyer and Dobberstein, 1980 a,b), but apart from the membrane it is inactive (Gilmore et al., 1982). SRP receptor has recently been shown to consist of two subunits, the previously identified 69 kDa polypeptide (now termed α subunit) and a 30 kDa β subunit (Tajima et al., 1986).

Release of Elongation Arrest

The release of SRP-induced elongation arrest of secretory proteins by microsomal membranes is a function of the SRP receptor (Gilmore et al., 1982a) and appears to occur via a direct interaction between SRP and SRP receptor (Gilmore et al., 1982b; Gilmore and Blobel,1983). Attachment of the arrested translation complex to the ER membrane is mediated by SRP-receptor and is accompanied by displacement of SRP from both the ribosome (Gilmore and Blobel, 1983) and the signal sequence (Wiedmann, et al., 1987a). A domain of the 69 kDa α subunit consisting of clusters of predominantly basic mixed charge residues and which resembles nucleic acid binding proteins has been suggested to bind to SRP, possibly via the 7SL RNA (Lauffer et al., 1985).

Targeting

The SRP receptor plays a critical role in facilitating translocation beyond that of releasing SRP-induced elongation arrest. Partially reconstituted SRPs which do not arrest nascent chain elongation but are capable of facilitating translocation

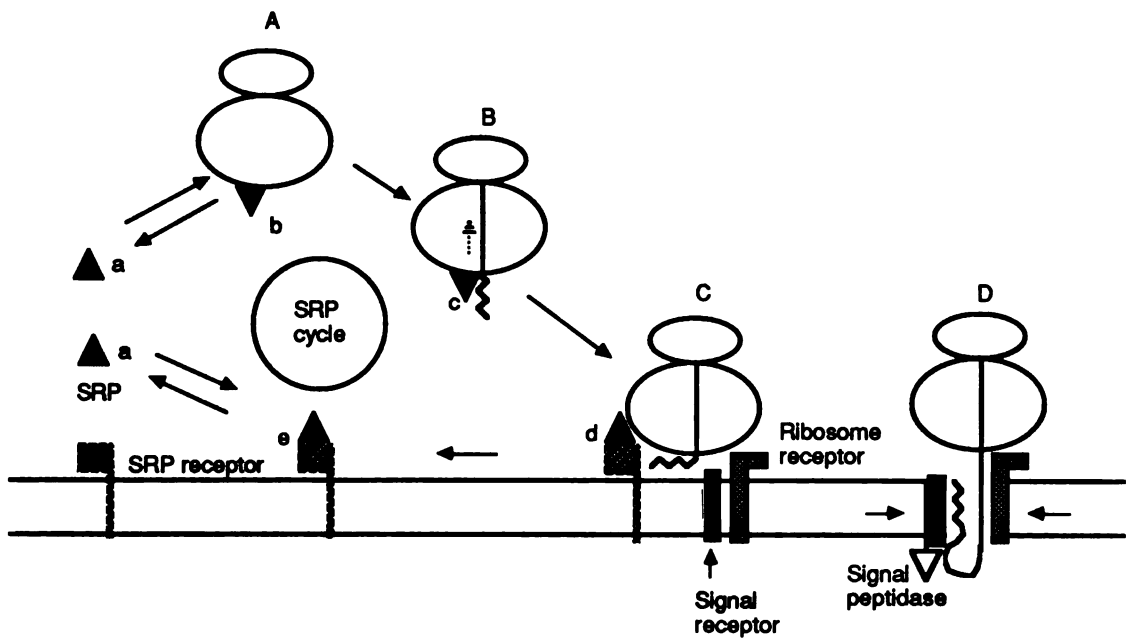
require SRP receptor (Siegel and Walter, 1985), suggesting an essential role for the receptor in targeting the ribosome-nascent chain complex to the microsomal membrane.

Quantitation of SRP and SRP receptor in pancreatic cells indicates that both are present in substoichiometric amounts relative to membrane-bound ribosomes (Walter and Blobel, 1980; Gilmore et al., 1982b) and, thus, that SRP receptor is not involved directly in the process of translocation. Rather, it appears that the nascent chain-ribosome-SRP-SRP receptor interaction is transient and that the role of SRP and its receptor is primarily to target the nascent secretory polypeptide to the ER membrane.

SRP Cycle

Initial targeting events as they are understood currently have led to the model of the SRP cycle depicted in Figure 1-1 (for review, see Walter et al., 1984). Upon emergence of a signal sequence from ribosomes synthesizing secretory proteins in the cytoplasm, SRP binds the signal sequence directly, interrupting chain elongation and perhaps maintaining the nascent chain-ribosome complex in a translocation competent conformation. The affinity of SRP for its receptor on the cytoplasmic face of the ER membrane targets the SRP-ribosome-nascent chain complex to that membrane system. Following interaction with its receptor in the ER membrane, SRP loses its affinity for the signal sequence-bearing ribosome and releases the complex, perhaps to other receptors in the membrane (see below). Translation resumes and translocation across the membrane occurs. It is not known whether SRP is involved in initiating the translocation event or whether its only role is to target the nascent chain to the correct location.

Figure 1-1. Model of signal recognition particle (SRP) cycle for targeting nascent secretory and transmembrane proteins to the ER membrane (adapted from Walter et al., 1984). Soluble SRP (a) exists in equilibrium with a membrane-bound form, presumably bound to SRP receptor (e), and a ribosome-bound form (b). On translation of mRNA encoding a signal sequence for targeting to the ER membrane (zigzag lines), the affinity of SRP for the translating ribosome is enhanced (represented by dashed arrow, B) and SRP binds to the signal sequence directly (c), effecting elongation arrest (B-C). On interaction with ER membranes, elongation arrest is released and SRP and SRP-receptor are free to be recycled (SRP cycle, a-e), the synthesizing ribosome interacts with other transmembrane proteins, leading to formation of a functional ribosome-membrane junction, translation resumes, and translocation across the membrane occurs.


A
B
C
D
a
b
c
d
e
SRP
SRP cycle
SRP receptor
Ribosome receptor
Signal receptor
Signal peptidase

Ribosome Binding Proteins

Binding of polysomes synthesizing secretory proteins to the ER membrane has long been thought to play an important role in the vectorial transport of nascent chains across the membrane (Redman and Sabatini, 1966). Such ribosomes bind to the ER membrane both via their nascent chains (Gilmore and Blobel, 1985) and directly via their large subunits through a salt-labile interaction (Adelman et al., 1973). Binding is saturable and sensitive to proteases (Hortsch et al., 1986). Formation of a "functional" ribosome-membrane junction is required for translocation across microsomal membranes (Gilmore and Blobel, 1985) and requires the participation of GTP (Connolly and Gilmore, 1986).

The ribophorins (I and II), two integral membrane glycoproteins which are present in rough microsomes but absent from smooth membranes (Kreibich et al., 1978a), have been suggested as ribosome receptor(s). Several indirect lines of evidence support this idea, including cofractionation of ribosomes and ribophorins following either detergent solubilization (Kreibich et al., 1978a) or protein cross-linking treatment (Kreibich et al., 1978b) of rough microsomes. Also, a good stoichiometry exists between the number of ribophorins and the ribosome binding capacity of rough microsomes (Marcantonio et al., 1984). However, controlled proteolysis of rough microsomes suggests that ribophorins may not mediate functional ribosome binding directly since ribosome binding activity of rough microsomes is lost following protease treatment to which ribophorins appear resistant (Hortsch et al., 1986). In addition, translocation of some secretory proteins may occur in vitro across smooth microsomes which lack ribophorins (Bielinska et al., 1979). Thus, the putative ribosome binding proteins of the ER membrane remain to be identified.

Signal Sequence Receptor

In addition to ribosome binding sites, an independent signal sequence receptor in the ER membrane has also been proposed and a candidate recently identified. The existence of a signal sequence receptor was suggested by the observations that posttranslational binding of signal-bearing proteins to rough microsomal membranes was specific, saturable, and protease sensitive (Prehn et al., 1980, 1981). In addition, a nascent, SRP-arrested polypeptide was shown to bind to microsomal membranes in an SRP-receptor-dependent manner and to remain associated with the membrane even after extraction of the ribosome with puromycin and high salt. The polypeptide was extractable with protein denaturants such as urea or alkaline pH (Gilmore and Blobel, 1985).

Following disengagement of SRP and its receptor, the signal sequence of preprolactin has been shown to interact with a ~35 kDa integral membrane glycoprotein of the ER (Wiedmann et al., 1987). This signal sequence receptor was identified through a series of experiments utilizing an amino acid analog containing a photoactive group which was incorporated into signal sequences in cell-free translation reactions. The role of this putative receptor in translocation remains to be determined. It may mediate direct signal sequence:lipid interactions, induce or activate a proteinaceous channel in the membrane (Blobel, 1980) or be a component of such a channel.

Signal Peptidase

Signal peptidase is an integral membrane protein presumed to act on the luminal side of the ER membrane. Since cleavage of signal sequences occurs only on translocated secretory proteins unless the microsomal membrane has been solubilized (Jackson and Blobel, 1977). Because signal peptidase removes signal

sequences from nascent polypeptides as they cross the ER membrane (Blobel and Dobberstein, 1975b; Miyata and Akazawa, 1982) it has been suggested to be associated with a complex of proteins that are involved in other aspects of translocation and which may form a channel in the membrane (Blobel, 1980; Evans et al., 1986). Signal peptidase has been purified from canine pancreas rough microsomes as a relatively abundant complex of four to six polypeptides (Evans et al., 1986). Bacterial leader peptidase I, which can accurately cleave eukaryotic signal sequences (Watts et al., 1983), exists as a single polypeptide (Wolfe et al., 1982). It is believed that eukaryotic signal peptidase is very similar to this bacterial enzyme since bacterial secretory proteins can also be processed accurately by canine rough microsomes (Müller et al., 1982). By analogy, it is thought that eukaryotic signal peptidase also exists as one polypeptide and that the additional copurifying proteins may be involved in other translocation-related processes. The observation that this complex exists in roughly stoichiometric amounts relative to membrane-bound ribosomes lead to speculation that it may form a core around which still other membrane proteins assemble to form a translocation apparatus in the membrane (Evans et al., 1986).

INTRODUCTION

Tremendous progress has been made in identifying molecular components involved in signal recognition and targeting to the ER membrane. However, the mechanism by which proteins are transported across the bilayer remains obscure. Do signal sequences facilitate translocation by activating a catalytic mechanism or through direct interactions with the membrane? Is the energy to drive translocation derived from the thermodynamics of protein-lipid interactions, from protein synthesis, or does energy-requiring proteinaceous machinery in the membrane actively move the protein across the bilayer? A major obstacle to the elucidation of mechanism has been the obligate coupling of a protein's translocation to its

synthesis. Thus, transport events can be analyzed only during the narrow window of time and under the particular conditions required for protein synthesis.

I have approached the questions regarding the mechanism of translocation by using molecular genetic techniques to create or manipulate substrates for translocation (i.e. the polypeptide to be translocated). By studying the translocation of these engineered substrates insights into the translocation process can be gained. This approach has been used previously to demonstrate that a signal sequence coding region engineered at the amino terminus is sufficient to direct translocation of a normally cytoplasmic protein across microsomal membranes in vitro and in vivo (Lingappa et al., 1984; Simon et al., 1987). Similarly, engineering a "stop transfer" sequence into a previously secreted protein has been shown to direct transmembrane integration (Yost, et al., 1983).

The work presented here may be viewed as a progression towards the complete dissociation of translocation across the ER membrane from protein synthesis. Chapters 2 and 3 describe the translocation (in vitro and in vivo, respectively) of a fusion protein with an internalized signal sequence of a secretory protein. The observation that translocation of the (previously synthesized) amino terminal domain can be directed by the internal signal sequence demonstrated that translocation of this domain did not occur concomitant with its synthesis and suggested that translocation could be experimentally uncoupled from elongation. Chapter 4 describes such a dissociation, achieved by cell-free expression of a truncated cDNA which lacks a termination codon, and points to a role for the ribosome in translocation which is distinct from its role in protein synthesis. Translocation of a completed and released polypeptide is demonstrated in Chapter 5 and elongation independent translocation of both ribosome-dependent and ribosome independent substrates is assayed in intact cells.

Chapter 2

A FORMER AMINO TERMINAL SIGNAL SEQUENCE ENGINEERED TO AN INTERNAL LOCATION DIRECTS TRANSLOCATION OF BOTH FLANKING PROTEIN DOMAINS

Introduction

It is generally accepted that signal sequences play a critical role in the translocation process. However, it is unclear whether they facilitate translocation via direct interactions with the lipid bilayer of the membrane or by interactions with other proteins in the ER membrane. Other fundamental aspects of the translocation process are equally obscure. To what extent is signal sequence function constrained by the nature of the chain being translocated and the location of the signal sequence within that chain? What role, if any, does signal sequence cleavage play in the process of translocation? What is the fate of the cleaved signal peptide? Why are secretory proteins completely translocated across the bilayer, whereas integral membrane proteins only partially translocated?

While most secretory proteins possess cleaved, amino-terminal signal sequences, a number of transmembrane proteins possess internal, uncleaved signal sequences (Bos et al., 1984; Friedlander and Blobel, 1985; Spiess and Lodish, 1986). In addition, the secretory protein, ovalbumin, contains an uncleaved signal sequence (Palmiter et al., 1978) which has been proposed to be internal (Lingappa et al., 1979). To determine whether a previously amino terminal signal sequence can function from an internal position in a protein, we engineered the signal sequence of bovine preprolactin between the coding regions for globin and prolactin, and then engineered an N-linked glycosylation site into the globin coding region. The results presented here have implications for a variety of issues in protein translocation.

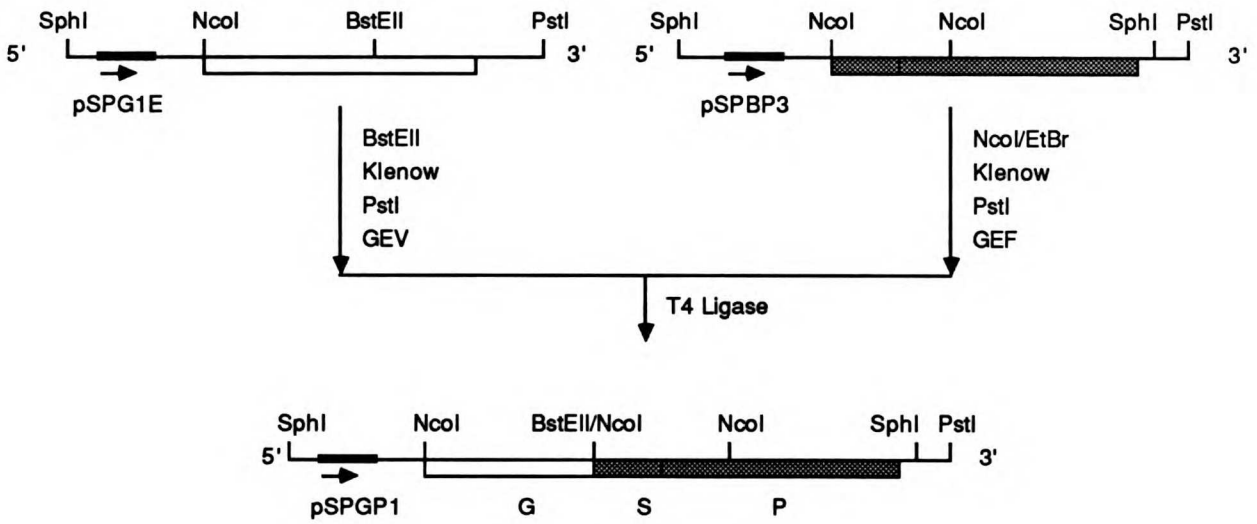
Results

Fig. 2-1 shows the scheme by which the entire coding region for bovine preprolactin was inserted, in frame, 109 codons downstream from the initiation codon of a chimpanzee α-globin cDNA clone previously engineered behind the SP6 promoter (pSPG1E). Putative positive colonies were selected for ampicillin resistance and screened for the presence of restriction fragments of predicted sizes for the globin, signal sequence, and prolactin coding domains, as well as the SP6 promoter. These characteristic restriction fragments from both parent plasmids, pSPG1E and pSPBP3, and from the new construction, pSPGP1, are displayed in Fig. 2-1 B.

Upon expression in a transcription-coupled rabbit reticulocyte lysate cell-free translation system, pSPGP1 encoded a fusion protein of ~32 kD with both globin and prolactin immunoreactivity (Fig. 2-2, lanes A-D) called preGSP. When translation reactions were supplemented with microsomal membranes two additional translation products, not present in the absence of membranes, were seen after electrophoresis on polyacrylamide gels in sodium dodecyl sulfate (SDS PAGE) with subsequent autoradiography (Fig. 2-2, lanes E-G). One of these bands, termed P1, was found to be anti-prolactin but not anti-globin immunoreactive and to co-migrate with authentic mature bovine prolactin (Fig. 2-2, lane F). The other product, termed GS1, was anti-globin but not anti-prolactin immunoreactive, and migrated with an apparent molecular weight slightly greater than that of authentic full-length globin (14 kD, Fig. 2 lane E). When membranes were added after completion of protein synthesis with further incubation, neither of these bands were generated (data not shown). The difference in the relative intensities of the bands in the autoradiographs is due to the methionine distribution in the [^{35}S]methionine-labeled, newly synthesized proteins. Upon processing, of the eleven methionines in preGSP, P1 contains seven and GS1 only four.

Figure 2-1. Construction scheme and restriction map of pSPGP1 fusion plasmid. (A) Construction scheme for pSPGP1, in which the entire coding region of bovine preprolactin was inserted into an SP6 plasmid containing chimpanzee α-globin cDNA (pSPG1E) such that the signal sequence of the resulting hybrid fusion protein is located 109 amino acids from the amino terminus (see Chapter 7 for details). Only intervening sequences between the SphI and PstI sites of the respective (and otherwise identical) vectors are shown. Relevant restriction sites of pSPGP1 and parent plasmids, pSPG1E and pSPBP3 are noted above the line, with the corresponding translation product indicated underneath. Chimpanzee α-globin coding regions are indicated by white bars and preprolactin by stippled bars. The heavy black lines indicate the SP6 promoter, and arrows on the plasmid diagrams indicate the direction of transcription. The white bar of pSPGP1 represents the 109 amino acid globin domain (G) and the stippled bar the 229 amino acids of preprolactin (P). The 30 amino acid signal sequence of prolactin is also indicated (S). (B) Restriction endonuclease analysis of pSPBP3, pSPGP1, and pSPG1E. Plasmid DNA (5 μg) was digested in a volume of 10 μl with NcoI and SphI. Samples were prepared and electrophoresed on a 5% polyacrylamide gel in Tris-Borate-EDTA and stained with ethidium bromide. Lane 1, pSPBP3; lane 2, pSPGP1; lane 3, pSPG1E; lane 4, pBR322 digested with HinfI as size markers, indicated in kilobases.

A.


SphI
NcoI
BstEII
PstI
5'
3'
pSPG1E
SphI
NcoI
NcoI
SphI
PstI
5'
3'
pSPBP3
BstEII
Klenow
PstI
GEV
NcoI/EtBr
Klenow
PstI
GEF
T4 Ligase
SphI
NcoI
BstEII/NcoI
NcoI
SphI
PstI
5'
3'
pSPGP1
G
S
P

B.

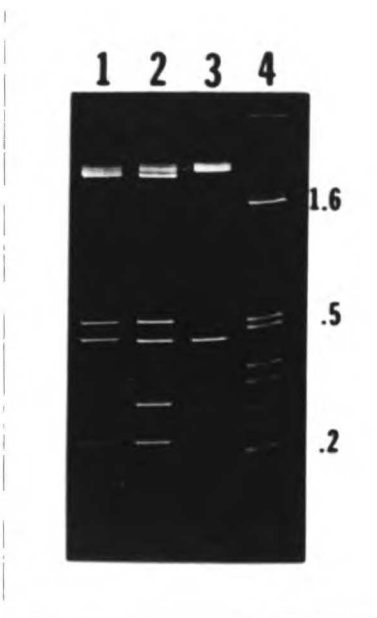

1
2
3
4
1.6
.5
.2

Figure 2-2. In vitro translation products of pSPGP1. Plasmid DNA was transcribed by SP6 RNA polymerase in a volume of 10 µl and a 2 µl sample translated in rabbit reticulocyte lysate as described. After 1 h at 23°C, aliquots (0.5 µl) were immunoprecipitated with either rabbit anti-human hemoglobin (lane A), rabbit anti-ovine prolactin (lane B), or normal rabbit serum (lane D). One aliquot was not immunoprecipitated but applied directly to SDS PAGE (lane C). Translations were simultaneously performed as above except that dog pancreas rough microsomes were present at a concentration of 5 A_{280} U/ml. Samples were prepared as before. Lane E, anti-globin immunoprecipitate; lane F, anti-prolactin immunoprecipitate; lane G, total translation products; lane H, nonimmune serum immunoprecipitate. Large arrowheads pointing downward indicate pre GSP and the arrowheads pointing upward show the cleavage products, GS1 and P1 in lanes E and F, respectively. Positions of authentic mature prolactin and globin (~14 kDa) are indicated as P and G, respectively, in the center lane where molecular weight markers are also indicated in kilodaltons. Note: the low molecular weight band in lanes C and G (total translation products) that runs at the same apparent molecular weight as the globin marker, G, is rabbit globin synthesized from endogenous mRNA in the rabbit reticulocyte lysate. The different relative intensities of this band in lanes C and G are due to different exposure times for lanes A-D and lanes E-H. Lanes E-H required a longer exposure time because of the inhibition of protein synthesis by the addition of dog pancreas rough microsomes. Samples were prepared and separated by SDS PAGE and bands viewed by autoradiography.

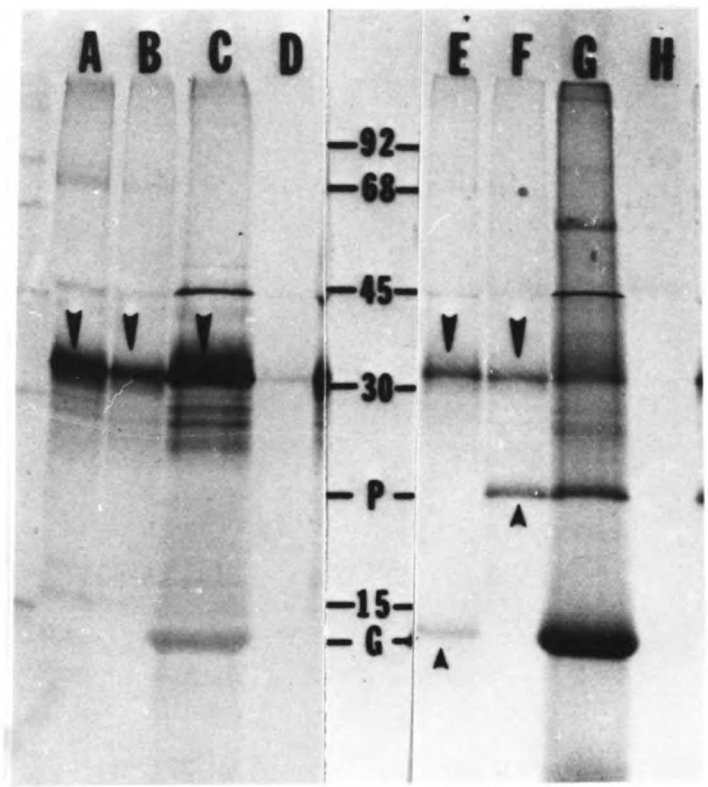

A
B
C
D
E
F
G
H
92
68
45
30
P
15
G

Fig. 2-3 demonstrates that when membranes were present during translation, GS1 and P1 were generated in a 1:1 ratio and that the percentage processing of both products increased correspondingly with membrane concentration. These data indicate that GS1 and P1 are generated from a common (nascent) precursor and that the processing activity is associated with the microsomes.

Our interpretation of these results is that the prolactin signal sequence, now localized internally, is still functional as evidenced by accessibility of the signal sequence cleavage site to signal peptidase, a lumenally disposed enzyme of the endoplasmic reticulum (Jackson and Blobel, 1977). The products of this cleavage are authentic prolactin (P1) and globin with the prolactin signal sequence attached at its carboxy terminus (GS1).To determine which of these products were completely translocated across the membranes, we used two different experimental approaches. First, posttranslational proteolysis with proteinase K was used to localize the precursor and the two cleavage products. Any polypeptide that is completely translocated across the bilayer will be resistant to proteolysis unless the integrity of the membrane is abolished by the addition of nonionic detergents (Blobel and Dobberstein, 1975b). If a protein spans the membrane, its molecular weight will be reduced by digestion of the cytoplasmically disposed domains (Katz, et al., 1977; Lingappa, et al., 1978a). A protein localized completely outside of the membrane vesicles will be totally digested by the added protease (Blobel and Dobberstein, 1975a, b). As can be seen in Fig. 2-4, the precursor, pre GSP, is completely degraded (lanes A, B, G, H). However, some chains of GS1 (23%; lane H) and almost all chains of P1 (89%; lane G) were protected from proteolysis (bands were quantified by scanning densitometry as described in Chapter 7). When nonionic detergent was included with the protease to disrupt the protecting lipid bilayer, all protection from proteolysis was abolished (lanes I and J).

Figure 2-3. Co-translational conversion of nascent pre GSP to GS1 and P1 as a function of membrane concentration. Translation of pSPGP1 was carried out in rabbit reticulocyte lysate (as described in Chapter 7) in the presence of varying membrane concentrations (0, 1.25, 2.5, or 5.0 A_{280} U/ml). Aliquots (5 µl) were immunoprecipitated with either rabbit anti-human hemoglobin serum (solid black circles) or rabbit anti-ovine prolactin (open circles). After SDS PAGE, bands were viewed by autoradiography and quantitated by scanning densitometry as described in Chapter 7. Data is expressed as a percent of total specific immunoreactive chains that have been processed.

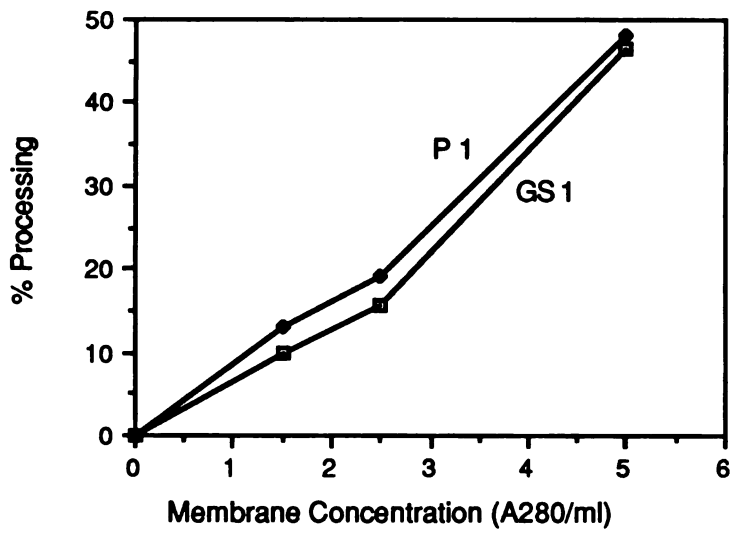
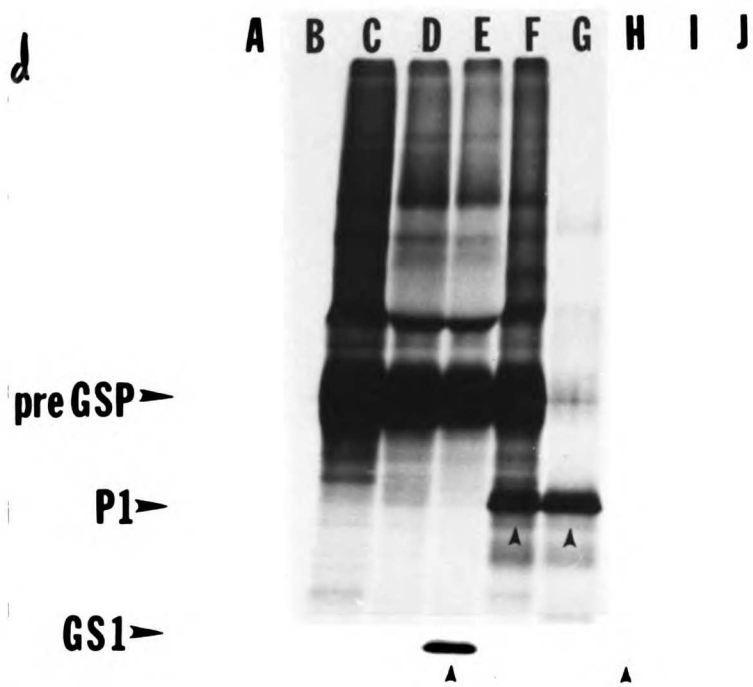

50
40
30
20
10
0
% Processing
P 1
GS 1
0
1
2
3
4
5
6
Membrane Concentration (A280/ml)

Figure 2-4. Transmembrane translocation of globin-prolactin fusion protein. Plasmid DNA from pSPGP1 was transcribed and translated in rabbit reticulocyte lysate. Some translation reaction mixtures included dog pancreas membranes at 2.5 A_{280} U/ml (+ mb, lanes E-J). After translation some aliquots (5 µl) were treated for 1 h at 0°C with proteinase K at 0.1 mg/ml (+ protease, lanes A, B, and G-J), in some cases in the presence of 1% Nikkol to disrupt the membranes (+ det., lanes I and J). See Chapter 7 for details. Samples were all diluted and immunoprecipitated with either anti-globin (G, lanes B, D, E, H, and J) or anti-prolactin serum (P, lanes A, C, F, G, and I), separated by SDS PAGE and viewed by autoradiography.


d
A B C D E F G H I J
pre GSP
P1
GS1

To rule out the possibility that the relatively poor protection of GS1 was due to a slightly increased intrinsic protease resistance of the molecule relative to preGSP, rather than to translocation across a protecting lipid bilayer, we investigated the kinetics of proteloysis, We varied both the time of protease digestion (from 15 to 90 min) and the protease concentration (from 0.1 to 0.4 mg/ml final concentration of proteinase K). At all time points and at all protease concentrations virtually none of the preGSP chains but the same percentage of GS1 chains were protected (<1% and ~20%, respectively, as determined by scanning densitometry; data not shown). Our interpretation of these data is that the now internal prolactin signal sequence is able to direct not only the subsequently synthesized prolactin domain across the microsomal membrane, but also the already completed globin domain, albeit with lower efficiency.

To further test our interpretation we engineered a glycosylation site into the globin-coding region of pSPGP1. This artificial glycosylation site inserted into the BssHII site (~20 amino acids from the amino terminus) of globin results in translation-coupled core glycosylation of the globin domain when an amino terminal signal sequence is present to direct the nascent globin chains into the ER lumen (Perara and Lingappa, unpublished observations). Fig. 2-5 illustrates the scheme by which the coding region for one of these glycosylated globin constructs (pSPSG1) was first modified by deletion of the signal sequence coding region to generate pSPSG3. The HindIII fragment of this plasmid (lacking a signal sequence but containing a sequence encoding an N-linked glycosylation site engineered into the BssHII site) was excised and ligated in place of the corresponding HindIII fragment of pSPGP1 (which lacked the glycosylation addition site). The resulting plasmid, pSPgGP1, differed from pSPGP1 only in the presence of the 24-bp oligonucleotide encoding a glycosylation addition site, and the encoded proteins are

identical except for the insertion of eight amino acids, Ala-His-Asn-Gly-Ser-Gly-Ser-Gly, between amino acids 20 (Gly) and 21 (Ala) in the globin domain of the pSPgGP1 gene product. Since glycosylation is restricted to the lumen of the ER (Rothman, et al., 1978; Lingappa, et al., 1978b), addition of N-linked sugars represents a definitive assay for translocation that is independent of other criteria such as signal peptide cleavage or protection from proteolysis. Thus, addition of carbohydrate to the globin domain encoded by pSPgGP1 upon transcription-linked translation in the presence of microsomal membranes would constitute direct and conclusive evidence for translocation of the globin domain of the globin-prolactin fusion protein.

Fig. 2-6 demonstrates that when pSPgGP1 is transcribed and translated as described previously for pSPGP1, a ~32-kD globin and prolactin immunoreactive protein was synthesized (lanes D and I, arrows pointing downward). The co-translational (but not posttranslational, lane C) addition of microsomal membranes resulted in appearance not only of the 26-kD P1 band (lane J) and the 14-kD gGS1 band (lane E, small arrowhead pointing upward), analogous to the GS1 cleavage product of pSPGP1, but also in the appearance of a 16-kD globin but not prolactin immunoreactive band (lane E, large arrow pointing upward). This new 16-kD band, termed gGS1', was believed to be the glycosylated derivative of gGS1. Consistent with this interpretation, gGS1' was well protected from proteases (lane F), as was P1 (lane K), while gGS1 was relatively poorly protected (lane F, small arrow pointing upward). Protection of gGS1 approximated that of GS1 (20%), presumably representing those chains of gGS1 that were translocated but not glycosylated, an intermediate often observed in glycoprotein biosynthesis both in vivo and in vitro. Also, as was seen with preGSP, the precursor, pregGSP, was not protected.

Figure 2-5. Construction scheme of plasmid, pSPgGP1. Coding regions of all plasmids had been inserted between HindIII and PstI of the vector, pSP64. Only intervening sequences between restriction sites, SphI and PstI, sites of the SP64 vector are shown. Relevant restriction sites are indicated above the lines, with coding regions represented by the bars below. The signal sequence coding region was deleted from pSPSG1 which encodes the β-lactamase signal sequence (S-L) fused precisely to the amino terminus of chimpanzee α-globin into which a synthetic N-linked glycosylation site had been inserted (gG). The 430-bp HindIII fragment of the resulting plasmid, pSPSG3, was then inserted into the HindIII site of pSPGP1 to create pSPgGP1. Details are described in Chapter 7. Stippled areas indicate prolactin-coding regions, black the lactamase signal sequence, and white indicates the globin coding regions. Prolactin signal sequence is indicated (S) as is the mature region (P).

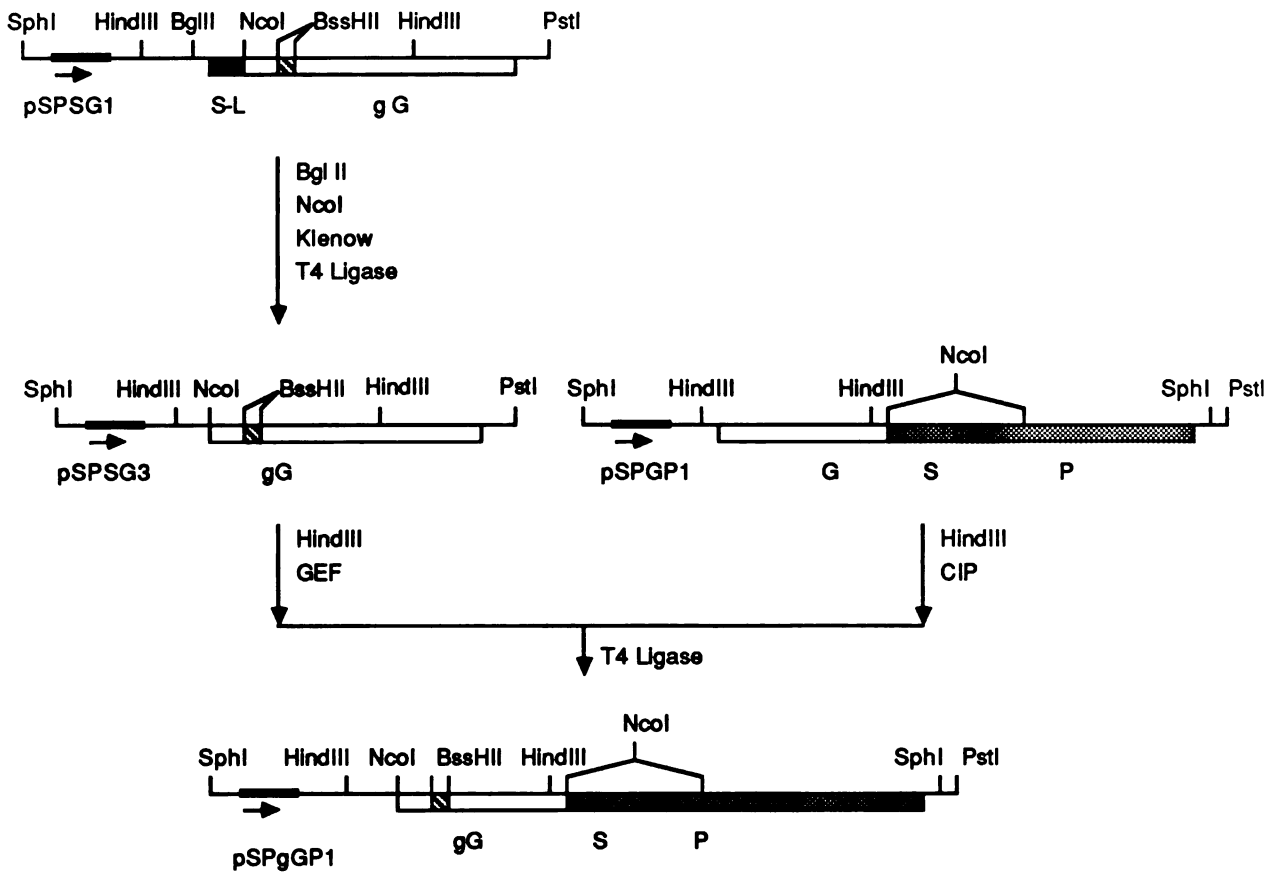
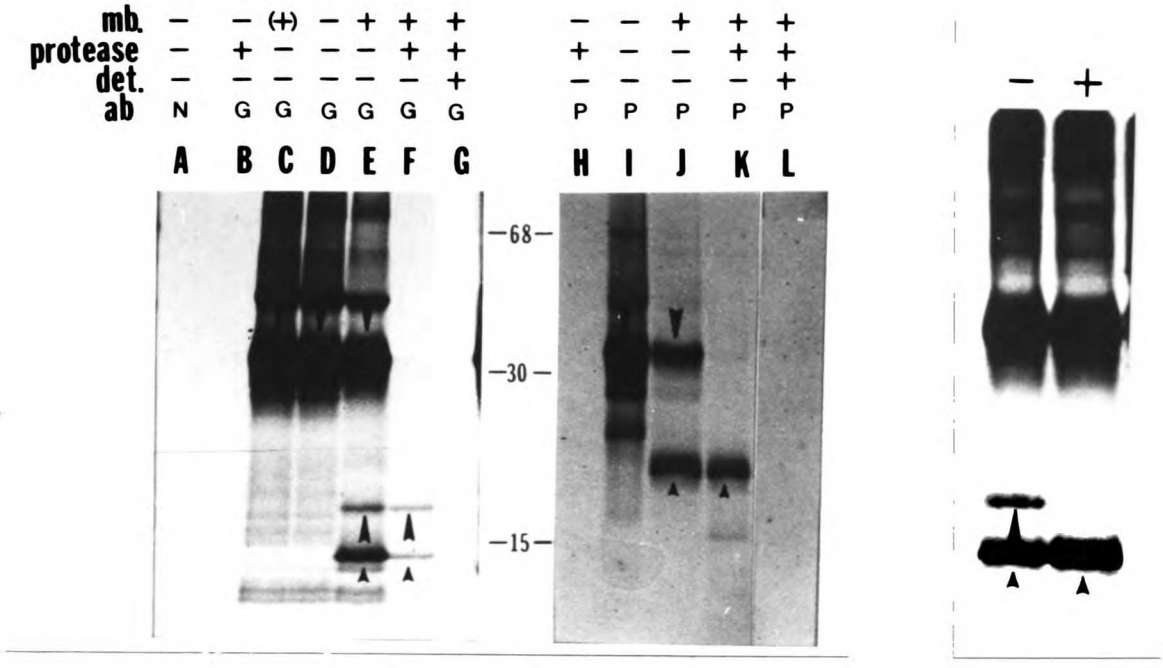

SphI
HindIII
BglII
NcoI
BssHII
HindIII
PstI
pSPSG1
S-L
g G
Bgl II
NcoI
Klenow
T4 Ligase
SphI
HindIII
NcoI
BssHII
HindIII
PstI
pSPSG3
gG
SphI
HindIII
NcoI
HindIII
SphI
PstI
pSPGP1
G
S
P
HindIII
GEF
HindIII
CIP
T4 Ligase
NcoI
SphI
HindIII
NcoI
BssHII
HindIII
SphI
PstI
pSPgGP1
gG
S
P

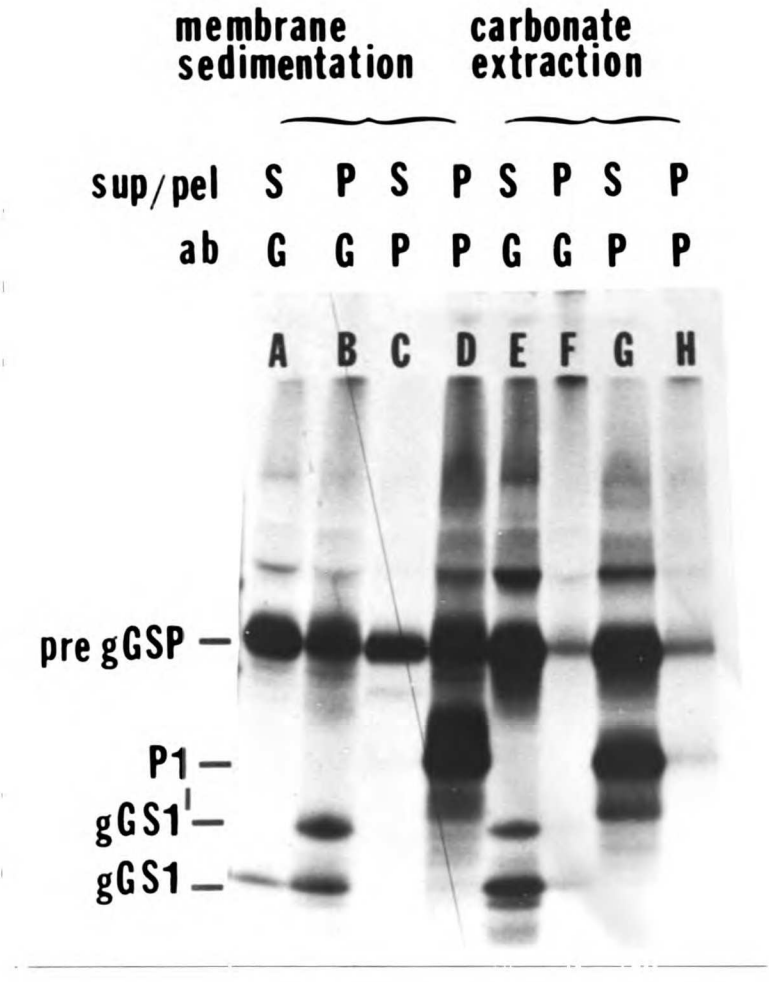
Figure 2-6. Lumenal localization of translation products of pSPgGP1. (A) In vitro translation products encoded by pSPgGP1 and localization by proteolysis. Plasmid pSPgGP1 was transcribed and translated as described in Material and Methods with membranes either absent (- mb., lanes A, B, D, H, and I) or present at a concentration of 4 A_{280} U/ml (+ mb., lanes E-G and J-L) during the translation reaction, or added posttranslationally to the same concentration with additional incubation [60 min at 24°C; (+) mb., lane C]. Some aliquots were then treated with proteinase K (+ protease) in the presence (+ det., lanes G and L) or absence of detergent (- det., lanes B, F, H, and K) as described and immunoprecipitated with globin antiserum (G, lanes B-G), prolactin antiserum (P, lanes H-L), or nonimmune rabbit serum (N, lane A). Samples were prepared and subjected to SDS PAGE and autoradiography. Downward pointing arrowheads indicate the "precursor", gGSP. Small upward pointing arrowheads in lanes E and F indicate gGS1, large arrowheads point to gGS1'. Small upward pointing arrowheads in lanes J and K indicate P1 bands. (B) Endo H digestion of in vitro translation products encoded by pSPgGP1. Plasmid, pSPgGP1 was transcribed and translated in the presence of dog pancreas rough microsomes as in (A). Following immunoprecipitation with anti-globin serum the sample was split in half and either digested with endoglycosidase H (Endo H +, lane B) or mock digested (Endo H -, lane A) as described in Chapter 7. Large upward pointing arrowhead indicates the glycosylated form of the cleaved precursor, gGS1'. The small upward pointing arrowheads indicate the unglycosylated form, gGS1.


mb. – – (+) – + + +
protease – + – – – + +
det. – – – – – – +
ab N G G G G G G
A B C D E F G
mb. – – + + +
protease + – – + +
det. – – – – +
ab P P P P P
H I J K L
68
30
15
– +

Our interpretation of these data was confirmed by treatment with Endo H which shifted the position of gGS1' on SDS PAGE to that of gGS1 (Fig. 2-6 B), thereby demonstrating the presence of carbohydrate on gGS1'. Similar treatment of the protein encoded by pSPSG3, which contains the glycosylation site but lacks a signal sequence to allow its utilization, demonstrates neither shift up on SDS PAGE with co-translational membranes, protection from proteolysis, nor shift down with Endo H digestion (data not shown).

Having demonstrated unequivocally that both P1 and gGS1' were faithfully translocated across microsomal membranes using the internal signal sequence of prolactin encoded in pSPgGP1, we proceeded to study the disposition of these molecules in the lumen. In particular, we wanted to know if gGS1' was integrated into the bilayer on the cisternal side or whether it was soluble in the lumen, i.e., did gGS1 display properties of a secretory or of an integral membrane protein? Similarly we investigated whether pre gGSP and those gGS1 chains that are not protected from proteases (Fig. 2-6, lane F) are peripherally bound to membranes, integrated into membranes, or free in the cytosol. Fig. 2-7 shows the results of sedimentation of membranes in either isotonic sucrose buffer or after extraction with sodium carbonate pH 11.5, a procedure designed to strip off nonintegral proteins and content proteins from microsomal membranes (Fujiki, et al., 1982). It can be seen that all forms (pre gGSP, gGS1, gGS1', and P1) are extracted by carbonate (lanes E-H) and that both P1 and gGS1' sedimented quantitatively with membranes in sucrose buffer (lanes A-D). Control extractions with a translation product known to integrate into membranes co-translationally demonstrated the fidelity of carbonate extraction in our hands (data not shown). Both gGS1 and pre gGSP were found in both the supernate and the pellet after membrane sedimentation in sucrose. The majority of

Figure 2-7. Carbonate extraction and vesicle sedimentation of translation products encoded by pSPgGP1. Plasmid pSPgGP1 was transcribed in vitro and products translated for 1 h at 24 °C in the presence of 4 A_{280} U/ml of dog pancreas membranes. The translation reaction was then split into two equal aliquots, one of which was extracted with carbonate and the other sedimented in isotonic sucrose buffer. Lanes A-D display supernatant (S) and pellet (P) after membrane sedimentation, and lanes E-H are the supernatant (S) and pellet (P) after carbonate extraction. Samples were immunoprecipitated with anti-globin (G, lanes A, B, E, and F) or anti-prolactin (P, lanes C, D, G, and H) serum, electrophoresed on a polyacrylamide gel, and viewed by autoradiography.


membrane sedimentation
carbonate extraction
sup/pel S P S P S P S P
ab G G P P G G P P
A B C D E F G H
pre gGSP
P1
gGS1'
gGS1

gGS1 sedimented with microsomes, while pre gGSP was split approximately evenly between the membrane pellet and the supernate.

These data indicate that gGS1' is not integrated into the vesicle membrane but exists either free in the microsomal lumen or peripherally associated with membrane proteins of the vesicle lumen. Also, those chains of gGS1 that are not protected from proteolysis are apparently in large measure bound to the membranes.

Discussion

Our original purpose in constructing pSPGP1 was to determine if amino terminal signal sequences were functionally related to internal signal sequences by converting a normally amino terminal signal into an internal one.

The existence of internal signal sequences has been proposed (Lingappa, et al., 1979; Blobel, 1980) for integral membrane proteins as a means of accounting for multiple transmembrane loops, since alternating signal and stop transfer sequences in register could serve to stitch the nascent polypeptide into the bilayer in a programed fashion. Recent evidence demonstrates the existence of internal signal sequences directly in the case of vertebrate rhodopsin (Friedlander, M., and G. Blobel, 1985) and hepatitis B virus surface antigen (Eble, B., et al., 1986) by the ability of internal coding regions to translocate what would otherwise remain in the cytoplasm. The existence of internal sequences in secretory proteins is more controversial. Ovalbumin lacks a cleaved signal sequence (Palmiter, et al., 1978), yet its nascent chain competes with nascent preprolactin for membrane receptors for translocation (Lingappa, et al., 1978b), suggesting the existence of an uncleaved signal sequence somewhere in the ovalbumin chain. An internal tryptic fragment from ovalbumin also displayed rough ER-specific competition for membrane receptors involved in prolactin translocation. Because this internal region, but not the amino

terminus, displayed strong homology to amino terminal signal sequences of other proteins of the chicken oviduct, it was proposed that the uncleaved signal sequence of ovalbumin resided in this internal position rather than at the amino terminus and therefore, that amino terminal and internal signal sequences were structurally and functionally similar (Lingappa, et al., 1979). Subsequent work, both indirect (Meek, et al., 1982; Braell and Lodish, 1982) and direct (Tabe, et al., 1984), has demonstrated the existence of a signal sequence for ovalbumin that is closer to, but not necessarily at, the amino terminus, but has not ruled out the existence of a signal sequence at the more internal location.

In principle, internal and amino terminal signal sequences could represent two classes of ligands. For example, amino terminal signal sequences might be capable of directing the translocation only of polypeptide synthesized subsequently while internal signal sequences could translocate both preceeding and subsequently synthesized domains. Our demonstration that the prolactin signal sequence functions when relocated internal to a globin domain, and generates soluble rather than integral membrane products in the lumen, suggests that internal signal sequences can exist in the case of secretory proteins. The observation that the internally relocated prolactin signal will translocate both amino and carboxy flanking domains (albeit with significantly different efficiencies) indicates that internal signal sequences may be structurally and functionally similar to amino terminal signal sequences.

The finding that the two cleavage products are translocated with different efficiencies while using a common signal sequence is surprising. It suggests that GS1 and P1 domains of nascent preGSP are translocated independently. Perhaps this reflects a difference in translocation between domains that are nascent (such as P1 which is synthesized subsequent to the signal sequence) versus those that have

already folded (such as the globin domain that is synthesized before the signal sequence). The location of the signal sequence relative to the translocated domain (i.e., at the carboxy terminus of globin versus at the amino terminus of prolactin) may also effect translocation efficiency of one versus the other domain, as might retention or removal of the signal sequence. Finally, we cannot rule out the possibility that the signal sequence retained by the globin domain can direct the reverse reaction of transport of GS1 from lumen to cytosol, to some extent, hence giving the appearance of inefficient translocation into the lumen.

Translocation of the globin domain of 109 amino acids that is synthesized before the signal sequence is a remarkable observation with surprising implications for the mechanism of chain translocation. Two models for translocation of nascent polypeptides across the ER membrane have been put forth (Blobel, 1980; Engelman and Steitz, 1981). One of these postulates a proteinaceous transport tunnel whose assembly in the plane of the lipid bilayer is directed by the signal sequence (Blobel, 1980). In this view, both targeting and translocation are the result of ligand-receptor-like interactions between domains of nascent chains (topogenic sequences) and membrane proteins serving as receptors and transporters. The other model (Engelman and Steitz, 1981) predicts that the free energy gain from insertion of the hydrophobic signal sequence directly into the lipid bilayer as a helical hairpin more than offsets the unfavorable energetics of pulling the contiguous hydrophilic chain into the bilayer, and thereby provides a thermodynamic basis for spontaneous insertion and translocation across the membrane, with the signal sequence retained in the bilayer after cleavage. This model requires no proteins in the membrane to facilitate translocation, and can be modified to account for signal recognition particle and its receptor as a requirement for targeting rather than translocation.

Since protein folding is likely begun during the window of time between initiation of protein synthesis and extrusion of the signal sequence from the large subunit of the ribosome (Harrison and Durbin, 1985; Karplus and Weaver, 1976), it appears that a (partially) folded globin domain is presented to the membrane for translocation during the synthesis of nascent preGSP. Any hypothesis for translocation must account not only for this observation but also for the apparently soluble, nonintegrated form in which the gGS1' chains are found. The topogenic sequence model can comfortably account for both of the considerations by postulating either an enzyme capable of denaturing the folded globin domain before transport as a component of the translocation machinery, or that the proteinaceous tunnel is of a dimension large enough to accomodate a partially folded domain. While the spontaneous insertion model might accomodate the idea of translocation of a folded globin domain providing it is denatured before the process of transslocation, the observation that the gGS1' chains are not integrated into the microsomal membrane argues that the signal sequence was never inserted directly into the bilayer. Thus, regardless of whether a partially folded or completely denatured domain is translocated, our results suggest that not only targeting (i.e., signal recognition particle-receptor interaction [Walter and Blobel, 1982; Gilmore, et al., 1982; Meyer, et al., 1982]) but also translocation itself, involves proteinaceous machinery in the membrane.

Another implication of the globin domain being synthesized before the signal sequence, is that the energy expended in synthesis of a length of polypeptide cannot be the sole driving force for its translocation. We cannot at this time rule out a role for continued synthesis of the prolactin domain in translocation of the already completed globin domain, although we doubt this possibility. It seems more likely to us that the driving force for translocation resides in the membrane. If this hypothesis

hypothesis is correct, it should be possible to dissociate translocation of arrested nascent domains from continued protein synthesis, although the inability of completed and released chains to be translocated argues for some additional requirement such as the ribosome or other protein cofactors (such as SRP) in the cytoplasm.

The placement of a globin domain at the amino terminus of a signal sequence provides the first marker for localization of a signal sequence after its cleavage. While we cannot rule out that such a bulky group has altered the fate of the signal, these data suggest that the signal is itself translocated, although it may be pulled back into the cytosol for degradation subsequently. In any case it appears not to be retained in the lipid bilayer, in view of our results with carbonate extraction.

We have also demonstrated a novel and direct approach to assaying for transfer of proteins across the ER membrane, that is the engineering of an N-linked glycosylation site (Asn-X-Ser/Thr) into the protein domain of interest. While failure to glycosylate does not rule out translocation, the addition of carbohydrate can be a powerful independent line of evidence in studying these events.

These results change our understanding of the vectorial discharge of proteins synthesized on membrane-bound ribosomes (Redman and Sabatini, 1966). Since in this case, translocation of the globin domain must take place after completion of its synthesis, it would appear that only a subset of the early events culminating in segregation in the ER cisternae need be vectorial.

Finally, the translocation of a globin domain when placed amino terminal to a signal sequence should not be taken to imply that any protein domain regardless of size or of secondary structure would be so translocated. The limits on translocation of domains both amino terminal as well as carboxy terminal to a signal sequence

remain to be determined. There may well exist domains or conformations incompatible with translocation. Replacement of the amino terminal domain of asialoglycoprotein receptor with tubulin inhibited the function of the internal signal sequence of this protein (Spiess and Lodish, 1986). At least two cases of cytoplasmic proteins unable to be translocated by amino terminal signal sequences have been proposed (Moreno, et al., 1980; Kadonaga, et al., 1984).

Chapter 3

FUNCTION OF AN INTERNALIZED SIGNAL SEQUENCE IN VIVO

Introduction

As described previously, our approach to the problem of understanding the mechanism of translocation involves the use of molecular genetic techniques to create plasmids which encode fusion proteins whose translocation or membrane topology may reveal certain aspects of the translocation mechanism. By engineering defined sequences involved in the translocation process (i.e., signal and stop-transfer sequences) to specific locations within a protein, it has been demonstrated that a normally cytosolic protein can be redirected to the endoplasmic reticulum lumen by an amino-terminal, cleaved signal (Lingappa, et al., 1984), and that when translocation is initiated by an amino terminal signal sequence, a stop transfer sequence halts transfer of the protein across the membrane and results in transmembrane integration (Yost, et al., 1983). Expression of a fusion protein consisting of the NH_2-terminal 109 residues of cytoplasmic globin followed by all of preprolactin demonstrated that an amino terminal signal sequence has the intrinsic capacity to translocate not only the protein domain that follows it (the COOH-domain) but also the domain which may precede it (the NH_2-domain; see Chapter Two). One limitation of these studies is that they were all carried out in cell-free translation/translocation systems. Indeed, similar types of experiments carried out in *E. coli* have not always produced the same results as those obtained in eukaryotic cell-free systems (Moreno et al., 1980; Kadonaga et al., 1984; Kuhn, 1987). One explanation for such discrepancies is that translocation in cell-free systems may not faithfully reconstitute the translocation mechanism of a living cell. Alternatively, the differences may reflect real differences in the translocation processes of eukaryotes and prokaryotes (Watanabe and Blobel, 1987) or in the particular fusion

proteins used. Therefore, because of the fundamental significance of translocation of the previously synthesized globin domain by the internalized signal sequence of prolactin described in Chapter Two, we wished to determine whether the observed (apparent low efficiency) translocation of the 109 amino acid NH_2-terminal domain reflects a permissiveness of the cell-free system not observed in living cells.

Expression of heterologous mRNAs in microinjected oocytes from *Xenopus laevis* results in the faithful translation of the encoded proteins (Gurdon et al., 1971) and their appropriate subcellular compartmentalization (Zehavi-Willner and Lane, 1977), secretion (Colman and Morser, 1979) and often appropriate post-translational modification (e.g. removal of signal sequences, phosphorylation or glycosylation; for reviews see Colman et al., 1984; Soreq, 1984).

Microinjection of *Xenopus laevis* oocytes presented several additional advantages for our purposes: many different plasmids can be rapidly introduced by microinjection of the same SP6 transcripts used to program cell-free translation reactions (Contreras, et al., 1982), resulting in very efficient, specific expression (Gurdon, et al., 1971); a variety of other reagents including antibodies (Morgan, et al., 1986; Valle, et al., 1982), cell fractions (Richter and Smith, 1981), synthetic peptides (Koren, et al., 1983); cell-free translation products (Lane et al., 1979) and drugs (Colman, et al., 1981) may also be easily introduced.

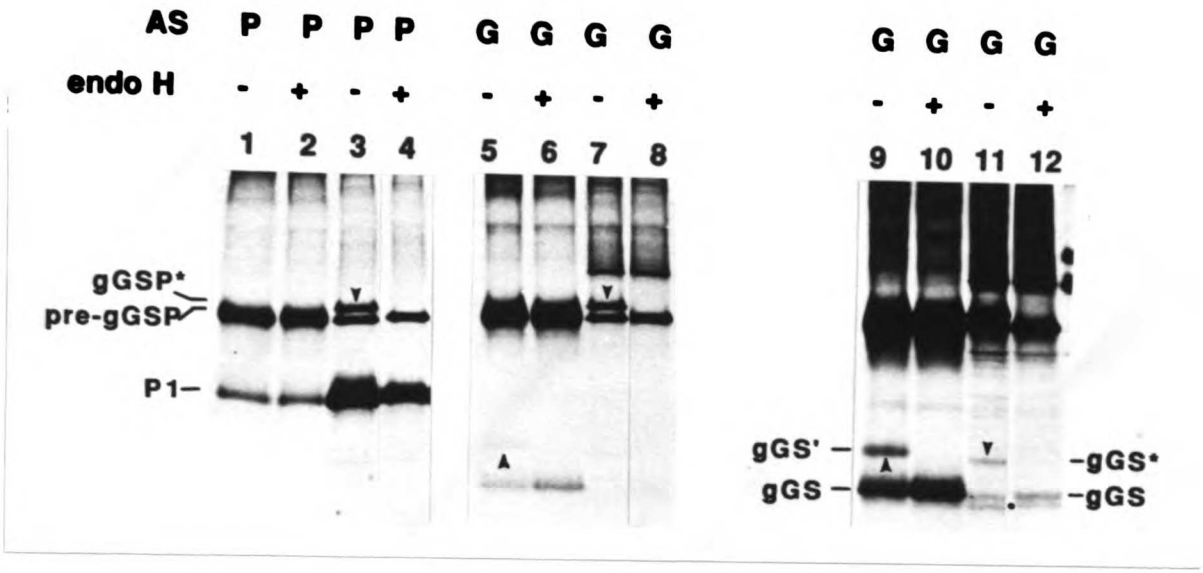
To corroborate and extend the findings described in chapter two we studied the translocation of protein(s) encoded by pSPgGP1 following injection of in vitro transcripts into oocytes of *Xenopus laevis*. This plasmid encodes a fusion protein (described in Chapter 2) consisting of 109 amino acids of chimpanzee α-globin into which a glycosylation site has been inserted, fused to the amino terminus of full-length preprolactin. Glycosylation of the globin domain was assayed to provide definitive evidence of its translocation. The results demonstrate that the

translocation events observed in cell-free systems are, in general, a faithful reflection of events occurring in living cells. However, some additional translocation-related events not reconstituted in vitro are described.

Results

SP6 transcription products of plasmid, pSPgGP1, were either microinjected into *Xenopus* oocytes or expressed in a rabbit reticulocyte lysate translation system supplemented with dog pancreas rough microsomes. Results are displayed in Figure 3-1. A ~32 kDa band which was both prolactin and globin immunoreactive seen in both systems (Fig. 3-1 lanes 1, 3, 5, 7), and referred to as pre-gGSP. Pre-gGSP represents the full-length precursor hybrid protein consisting of the 117 amino acids of globin (including the inserted glycosylation consensus sequence) fused to full-length preprolactin (229 amino acids). A somewhat slower migrating band was seen in Xenopus oocytes, but not in cell-free translation (lanes 3 and 7), that was reactive to both globin and prolactin antisera. This band represented a glycosylated species of pre-gGSP (denoted gGSP*) as demonstrated by sensitivity to endo H digestion (lanes 4 and 8). A ~26 kDa prolactin, but not globin, immunoreactive band which co-migrated exactly with mature prolactin and is termed P1 was observed in both the cell-free system and *Xenopus* oocytes (lanes 1 and 3). In the cell-free system, this product has been demonstrated to be a cleavage product of pre-gGSP, generated by the action of signal peptidase apparently at the normal peptidase cleavage site of preprolactin (see Chapter Two). Specific globin-immunoreactive products can be seen upon longer exposure in lanes 9-12. A ~14 kDa band called gGS and described in Chapter Two was also seen in *Xenopus* oocytes (lanes 9-12). A glycosylated form of gGS1 was seen in both the cell-free system (gGS', lane 9) and in Xenopus oocytes (gGS*, lane 11). Both gGS' and gGS* were shifted to the position of gGS following endo H digestion (lanes 10 and 12). gGS* migrated with a slightly higher

Figure 3-1. Comparison of translation products of pSPgGP1 immunoprecipitated from microinjected *Xenopus laevis* oocytes with cell-free translation products. SP6 transcripts were either microinjected into *Xenopus oocytes* (lanes 3, 4, 7, 8, 11, 12) or translated in reticulocyte lysate in the presence of microsomal membranes (2.5 A_{280} U/ml) (lanes 1, 2, 5, 6, 9, 10). [^{35}S]methionine labelled products were immunoprecipitated with anti-globin (G; lanes 5-12) or anti-prolactin serum (P; lanes 1-4), and subjected to endo H (2, 4, 6, 8, 10, 12) or mock digestion (1, 3, 5, 7, 9, 11). Products were prepared and subjected to SDS-PAGE and analyzed by autoradiography of the fluorographed gel. Lanes 1-8 are from a 38-hour exposure of the autoradiograph. Lanes 9-12 are identical to 5-8 but are from a 1-week exposure of the same gel to visualize the fainter low molecular weight globin-immunoreactive bands. Positions and labels of the identified translation product are indicated. Downward pointing arrowheads in lanes 3 and 7 indicate the glycosylated full-length product, gGSP*. Upward pointing arrowheads in lanes 5 and 9 indicate the glycosylated, globin-immunoreactive cleavage product seen in cell-free systems, termed gGS'. The downward pointing arrowhead in lane 11 refers to the presumably analogous product in oocytes, gGS*. The globin-immunoreactive band which migrates more rapidly than gGS seen in lanes 11 and 12 is indicated by a black dot. This band is consistently seen in oocytes and may represent signal cleavage products of an internal initiation product. It doesn't appear to be the substrate for N-linked glycosylation to produce gGS*, since the intensity of the higher band, denoted gGS is increased following endo H digestion.

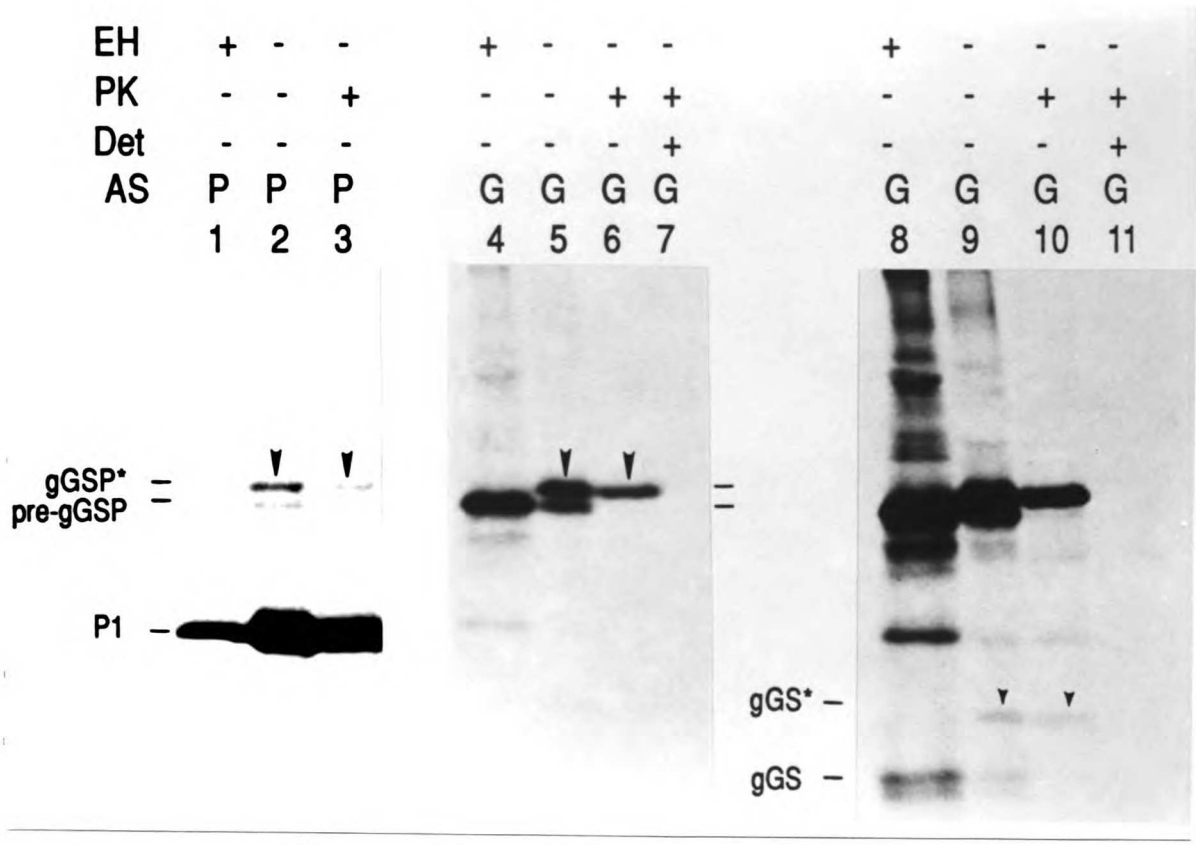

AS
P P P P
G G G G
G G G G
endo H
- + - +
- + - +
- + - +
1 2 3 4
5 6 7 8
9 10 11 12
gGSP*
pre-gGSP
P1–
gGS' –
gGS –
–gGS*
–gGS

electrophoretic mobility than gGS' most likely due to processing of the N-linked oligosaccharide by glucosidases present in the oocyte secretory pathway but lacking in dog pancreas rough microsomes. No major bands were seen in oocytes injected with pSPgGP1 transcripts and immunoprecipitated with non-immune serum nor in uninjected (but biosynthetically labelled) oocytes immunoprecipitated with anti-globin or anti-prolactin sera (data not shown). Thus we are assured that the bands identified are products of the injected transcript of pSPgGP1.

These data suggest that the translocation of pre-gGSP in Xenopus oocytes is similar to that seen in cell-free systems since similar cleaved and glycosylated forms were observed, i.e. that the internalized preprolactin signal sequence is able to facilitate the translocation of both flanking protein domains. However, the glycosylated, full-length fusion protein, gGSP*, not seen in cell-free systems was of interest. We wondered whether it represented a transmembrane form with the uncleaved signal sequence spanning the ER membrane or a secretory form.

To determine the subcellular localization of the products observed in *Xenopus* oocytes, microinjected oocytes were homogenized in an isoosmotic buffer and aliquots incubated with proteinase K either in the presence or absence of the nonionic detergent, Nikkol, or with no additions. Following this treatment, any protein residing in the ER lumen will be resistant to proteolysis unless the integrity of the membrane is abolished by the addition of nonionic detergent (Blobel and Dobberstein, 1975b). Membrane spanning proteins will be reduced in molecular weight by digestion of the cytoplasmically disposed domain(s) (Katz et al., 1977; Lingappa et al., 1978a) and polypeptides located in the cytoplasm will be completely digested by added protease in the absence of detergent (Blobel and Dobberstein, 1975b). As can be seen in Figure 3-2, pre-gGSP was completely degraded by exogenously added protease (lanes 3, 6, 10), as was gGS (lanes 9, 10). The presumed cleavage

Figure 3-2. Proteolysis of pSPgGP1 encoded products in microinjected *Xenopus* oocytes. Oocytes were injected with SP6 transcripts of pSPgGP1, labelled with [^{35}S] methionine for 20 hours and vesicles prepared as described in Chapter 7. Equal aliquots were incubated with 0.3 mg/ml proteinase K (lanes 3, 6, 7, 10, 11) in the presence (lanes 7, 11) or absence of 1% Nikkol (lanes 3, 6, 10) or with no additions (lanes 2, 5, 9). Samples were split evenly and immunoprecipitated either with anti-globin (G; lanes 4-7, 9-11) or anti-prolactin (P; lanes 6, 7) serum. Equivalent untreated samples were immunoprecipitated with anti-prolactin (P; lane 1) or anti-globin serum (G; lanes 4, 8) and subjected to endo H digestion (lanes 1, 4, 8). Samples were prepared and subjected to SDS-PAGE and products were analyzed by autoradiography of the fluorographed gel. Lanes 1-3 are from a 17-hour exposure; lanes 4-7 are from a 5-day exposure and lanes 8-11 are the same as lanes 4-7, but from a 3-week exposure which was necessary to visualize the faint lower molecular weight globin-immunoreactive products. Downward pointing arrowheads in lanes 2, 3, and 6 indicate the glycosylated, protected product, gGSP*. The small downward pointing arrowheads in lanes 9 and 10 indicate the glycosylated, protected product, gGS*.

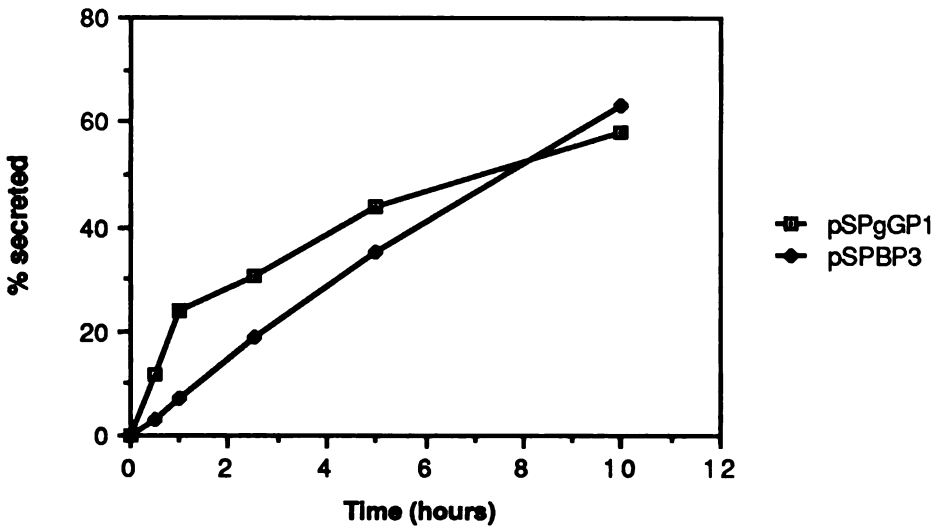

EH + - - + - - - + - - -
PK - - + - - + + - - + +
Det - - - - - - + - - - +
AS P P P G G G G G G G G
1 2 3 4 5 6 7 8 9 10 11
gGSP*
pre-gGSP
P1
gGS*
gGS

products, P1 and gGS*, were well-protected from protease (lanes 2 & 3 and 9 & 10, respectively). These results are similar to those described in chapter two for cell-free translocation of gGSP. The glycosylated full-length, uncleaved, hybrid protein, gGSP*, was also well-protected from protease (lanes 2 & 3 and 5& 6), suggesting that this band represents full-length chains which are translocated completely and glycosylated, but not cleaved by signal peptidase. Our interpretation of these data is that the internalized signal sequence of preprolactin can direct translocation of both the amino and carboxyl terminal flanking domains in living cells as well as in cell-free systems. However, in *Xenopus* oocytes some of the translocated fusion proteins are not cleaved by signal peptidase and these are localized to the vesicle lumen.

Since many products of gGSP1 were apparently localized to the secretory pathway we wondered which, if any, were secreted. To address this question a pulse-chase experiment was performed. Microinjected oocytes were labelled with [^{35}S] methionine, followed by incubation with cycloheximide and unlabelled methionine. Proteins were selectively immunoprecipitated from both cell homogenates and media at given time points. Following chase times up to 40 hours only P1 chains were seen in the medium (data not shown). Figure 3-3 demonstrates that the kinetics of secretion of P1 are indistinguishable from those of authentic, mature prolactin ($t_{1/2}$ ~ 6 hours). This is not surprising since, once cleaved, the P1 chains derived from pre-gGSP should be identical to mature prolactin in the lumen of the ER.

While the protease protection of gGSP* strongly suggested that it was localized entirely within the vesicle lumen, the possibility remained that the hydrophobic signal sequence was inserted in the membrane such that it was inaccessible to exongenously added proteases with both flanking domains in the vesicle lumen. To address the question of whether gGSP* was integrated into the bilayer on the

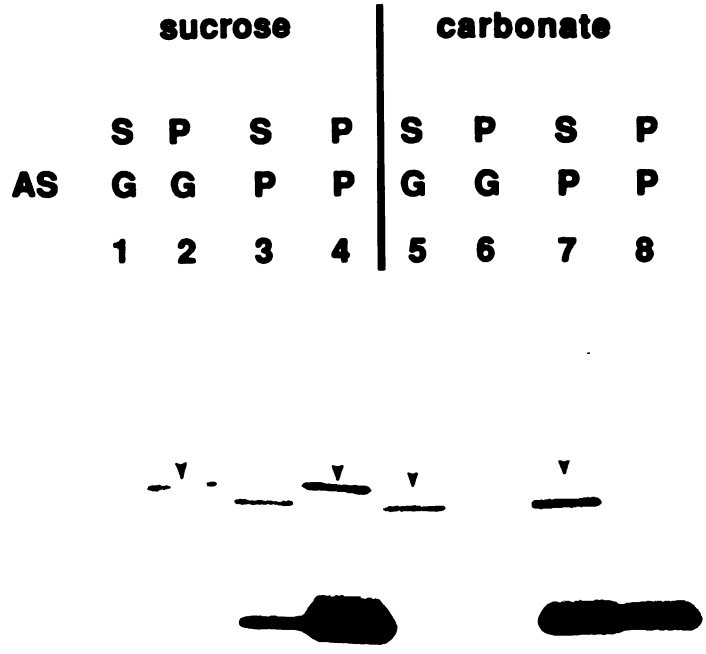
Figure 3-3. Kinetics of secretion of mature prolactin molecules derived from gGSP1 and preprolactin. *Xenopus* oocytes were injected with SP6 transcripts of plasmids encoding either gGSP or authentic preprolactin. Following a 2-hour incubation in medium containing [^{35}S] methionine, oocytes were incubated in batches of 5 in a "chase" medium containing 20 mM methionine, 10^{-4} M cycloheximide and 10% fetal calf serum. Oocytes and incubation media were collected at the indicated time points and samples analyzed by immunoprecipitation followed by SDS-PAGE and autoradiography. Relative intensities of bands were determined by scanning laser densitometry and presented as the percentage of the total cleaved (mature) prolactin fragments found in the medium at each time point.

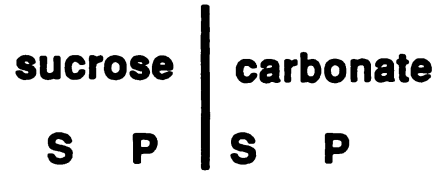

80
60
40
20
0
% secreted
0
2
4
6
8
10
12
Time (hours)
pSPgGP1
pSPBP3

lumenal side or soluble in the lumen (i.e. did gGSP* display properties of a secretory or of an integral membrane protein?), vesicles prepared from oocytes expressing gGSP were extracted with sodium carbonate pH 11.5 This procedure was designed to strip off nonintegral proteins and release content proteins from membrane preparations (Fujiki, et al., 1982). Figure 3-4 A shows the results of sedimentation of oocyte vesicle preparations following expression of pSPgGSP1. Vesicles were sedimented either in isotonic sucrose buffer or following extraction with sodium carbonate. It can be seen that gGSP*, and P1 sedimented quantitatively with membrane vesicles (lanes 1-4), while pre-gGSP was predominantly in the supernatant. All forms (pre-gGSP, gGSP*, and P1) were released to the supernatant followin extraction by carbonate (lanes 5-8). [Note: the P1 appears to be only partially extractable by carbonate treatment. This is a consequence of the overexposure of these bands on this autoradiograph which was necessary to observe the much fainter pre-gGSP and gGSP* bands. From shorter exposures and other experiments, 80-95% of the P1 bands are released following carbonate treatment.] In the same experiment a fusion protein which has been shown to be integrated into microsomal vesicles in vitro (Yost et al., 1983) was expressed in oocytes. This protein sediments quantitatively with membrane vesicles even following extraction with carbonate (Figure 3-4 B), demonstrating the fidelity of this assay in *Xenopus* oocytes.

These data indicate that gGSP*, which contains the uncleaved prolactin signal sequence, is not integrated into the ER membrane. Rather, it appears to be free in the ER lumen or peripherally associated with membrane proteins of the vesicle lumen. This is in agreement with our results obtained in cell-free systems in which signal-containing cleavage products of gGSP1 were not integrated into the microsomal membrane.

Figure 3-4. Membrane sedimentation and carbonate extraction of vesicles prepared from microinjected *Xenopus* oocytes. Oocytes were injected with SP6 transcripts of either pSPgGP1 and labelled for 3 hours with [^{35}S]methionine. Vesicles were prepared as described in Chapter 7. Equal aliquots were diluted 250-fold with either 0.1 M $NaHCO_3$ pH 11.5 (lanes 5-8) or 0.25 M sucrose, 50 mM triethanolamine pH 7.5, 0.1 M KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol (lanes 1-4), incubated for 30 minutes on ice and membranes sedimented as described in Chapter 7. Supernatant (1, 3, 5, 7) and pellet (2, 4, 6, 8) fractions were split and immunoprecipitated with anti-prolactin (3, 4, 7, 8) or anti-globin serum (1, 2, 5, 6). (B) Oocytes injected with SP6 transcription products of pSPG2D (which encodes an integral transmembrane protein) were treated as above and immunoprecipitated. Samples were prepared and subjected to SDS-PAGE and analyzed by autoradiography of the fluorographed gel.

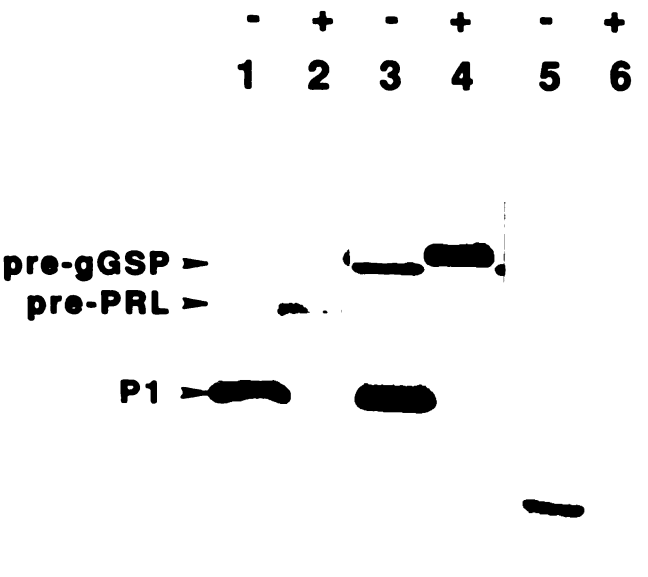

sucrose
carbonate
S P S P S P S P
AS G G P P G G P P
1 2 3 4 5 6 7 8


sucrose
carbonate
S P S P


G2D

From the above analyses, oocyte products, gGS, gGS*, and P1 appear analagous to cell-free cleavage products, gGS, gGS', and P1, respectively (described in Chapter 2 and figure 3-1), according to their apparent molecular weights, immunoreactivity, endo H sensitivity, subcellular localization and membrane association. However, the observed ratios of these products differ between the two systems. In contrast to cell-free systems where gGS plus gGS' chains were generated in a 1:1 ratio with P1, much less gGS1 and gGS* than P1 is observed in oocytes (see figure 3-1, compare intensities of gGS relative to P1 bands in lanes 1, 2, 9, 10 versus 3, 4, 11, 12). The ratio of gGS plus gGS* to P1 chains generated in *Xenopus* oocytes is 1:3, i.e. three times as many prolactin-immunoreactive cleaved chains appeared to be generated compared to globin-reactive cleavage products. One possible explanation for the non-stoichiometric amounts of globin-containing and prolactin chains seen in oocytes is that translation may be initiated preferentially at an internal AUG of the injected pSPSgGP1 transcript. To test this possibility, oocytes were injected with mRNA and with hydroxyleucine, a leucine analogue which, when incorporated into nascent chains, can prevent translocation and signal sequence cleavage by inhibiting the interaction of the leucine-rich preprolactin signal sequence with SRP (Hortin and Boime, 1980; Walter, et al., 1981). By preventing cleavage of the prolactin signal sequence, P1 chains should disappear and the true precursor from which the P1 chains were generated should accumulate. Figure 3-5 shows the results of this experiment. Injection of hydroxyleucine into oocytes which had been preinjected with SP6 transcripts encoding authentic preprolactin resulted in accumulation of preprolactin as expected. Lanes 1 and 2 show mature prolactin generated in oocytes in the absence of hydroxy-leucine and the precursor form which accumulates in the presence of hydroxy-leucine, respectively. Prolactin-immunoprecipitated products of gGSP-injected oocytes are seen in lane 3, showing P1, pre-gGSP and a very minor fraction of gGSP*. When the same transcript is translated by oocytes in the presence

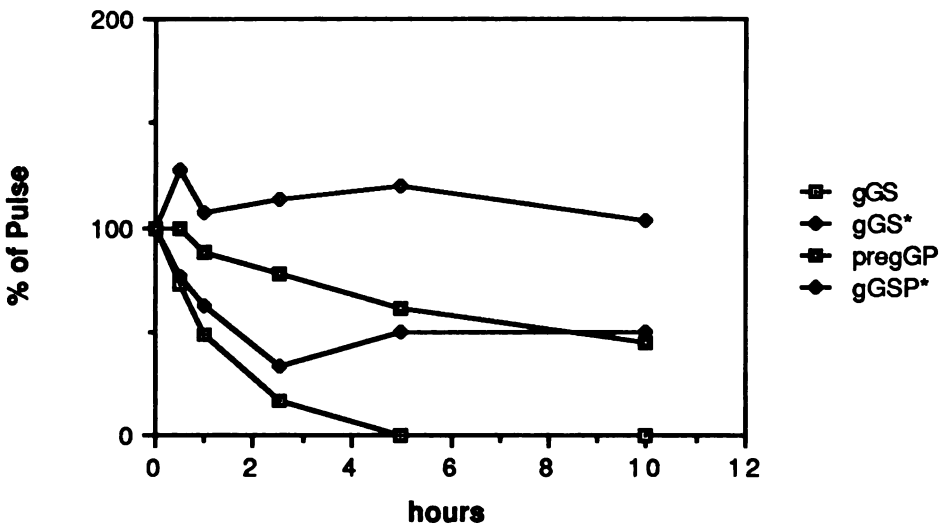
Figure 3-5. Expression of prolactin, SPgGP1 and globin in microinjected *Xenopus* oocytes in the presence and absence of hydroxyleucine. Oocytes were microinjected with SP6 transcripts of pSPgGP1 (lanes 3, 4), pSPBP3 (which encodes authentic preprolactin, lanes 1, 2) or pSPG1E (which encodes authentic α-globin; lanes 5, 6) and incubated at 19°C overnight. Equal numbers of oocytes were subsequently injected with [^{35}S] methionine in the presence (+; lanes 2, 4, 6) or absence (-; lanes 1, 3, 5) of 0.25 M hydroxyleucine. Oocytes were homogenized in triton buffer and immunoprecipitated with anti-prolactin (lanes 1-4) or anti-globin (lanes 5, 6) serum. Samples were prepared and bands separated by SDS PAGE and visualized by autoradiography. Precursors which accumulate in the presence of hydroxyleucine, preprolactin (lane 2) and pregGSP1 (lane 4), are indicated as are processed polypeptides which migrate to the position of mature prolactin (P1, lanes 1, 3). Proteins synthesized in the presence of hydroxyleucine (lanes 2, 4, 6) migrate slightly more slowly than the analagous proteins made in its absence (lanes 1, 3, 5); this is due to the incorporation of hydroxyleucine which has a higher molecular weight than leucine.


- + - + - +
1 2 3 4 5 6
pre-gGSP
pre-PRL
P1

of hydroxy-leucine (lane 4) P1 is greatly diminished and the major band which accumulates is one which migrates slightly more slowly than pre-gGSP and which is also globin-immunoreactive (data not shown). The slight shift in electrophoretic mobility relative to pre-gGSP is most likely due to the incorporation of the hydroxy-leucine analog which has a higher molecular weight than leucine. A similar shift is also seen for authentic globin synthesized by oocytes in the presence of hydroxy-leucine (see lanes 5 and 6). Globin is a cytoplasmic protein which has no cleaved signal sequence, which we didn't expect to generate a precursor in the presence of hydroxy-leucine. We conclude from these results that the major precursor of P1 is pre-gGSP. Thus, the nonstoichiometry of gGS forms and P1 seen in oocytes is not the result of internal initiation of translation of the injected transcript.

An alternative explanation for the disproportion detected between P1 and gGS plus gGS* is that an equivalent number of prolactin and globin-containing domains were initially generated by processing of the precursor, pre-gGSP, but that the gGS and/or gGS* chains were selectively degraded with time. Figure 3-6 shows the results of a pulse-chase experiment in which the products of pSPgGP1 were quantified over time. While pre-gGSP (localized to the cytoplasm), gGSP*, and gGS1* (localized to the ER lumen) appear fairly stable over this time interval (10 hours), the gGS1 chains were relatively rapidly degraded. The ratio of gGS plus gGS* to P1 immediately following labelling was 0.85:1 and declined rapidly with time. This indicates that gGS and gGS* and P1 are cleavage products of the same precursor, pre-gGSP, and initially generated stoichiometrically. However, the signal-containing, globin-immunoreactive cleavage product are preferentially and rapidly degraded. We suggest that this may reflect the normal metabolism of signal sequences following cleavage in living cells which is not reconstituted in cell-free translocation systems.

Figure 3-6. Pulse-chase of gGP1 translation products in *Xenopus* oocytes. Oocytes were injected with SP6 transcripts of pSPgGP1, labeled for 2 hours in [^{35}S] methionine and subsequently chased in incubation medium containing 20 mM unlabelled methionine, 10^{-4} M cycloheximide and 10% fetal calf serum. Products were analyzed at chase times 0, 0.5, 1.0, 2.5, 5.0 and 10.0 hours after pulse. Oocytes were homogenized in triton buffer, split into two equal aliquots which were immunoprecipitated with either globin or prolactin antisera. After SDS PAGE, bands were viewed by autoradiography and quantitated by scanning densitometry. Intensity of each band was determined at time 0 and intensities at each subsequent time point are expressed as a percentage of this. Open squares represent gGS, black diamonds gGS*, black squares pre-gGSP and open diamonds gGSP*.


200
100
0
% of Pulse
0
2
4
6
8
10
12
hours
gGS
gGS*
pregGP
gGSP*

Discussion

These studies were initiated in order to confirm and extend to intact cells the surprising findings for translocation described in chapter two. The results presented here demonstrate that internalized signal sequences can function to translocate both flanking domains in intact cells. As seen in vitro, the cleavage products, P1 and gGS1* (composed of the globin domain with the cleaved signal sequence of preprolactin at its carboxyl terminus) were well-protected from protease and gGS1* was glycosylated. In addition, a product not observed in cell-free translation/translocation systems was seen, the full-length glycosylated hybrid protein which was not cleaved by signal peptidase. This product represented a completely translocated species, as it was completely protected from exogenous proteases, sedimented quantitatively with the membrane fraction and was extracted by sodium carbonate, pH 11.5.

Why this form is seen in *Xenopus* oocytes and not in cell free translation system is not known. Perhaps glycosylation of the globin domain prevents subsequent action of signal peptidase.

The complete translocation of the hybrid protein without cleavage of the signal sequence represented by gGSP* demonstrates that even when the signal sequence is not cleaved, it does not reside in the bilayer but appears to be translocated itself. Thus, signal sequence cleavage, even of a normally cleaved signal sequence, is not a prerequisite for translocation in eukaryotes. Mutations in bacterial signal sequences which prevent cleavage also do not prevent secretion from *E. coli* (Lin et al., 1978; Koshland et al., 1982; Kadonaga et al., 1985).

The topology of gGSP* relative to the ER membrane is also interesting in regard to recent work demonstrating "signal-like" activity for other hydrophobic

stretches of amino acids. Several bitopic transmembrane proteins exist which contain a single internal, uncleaved signal sequence which serves to facilitate translocation of the carboxyl terminus as well as to anchor the protein in the membrane (Bos, et al., 1984; Spiess and Lodish, 1986; Lipp and Dobberstein, 1986; Zerial et al., 1986). Our results suggest that these sequences differ from classical signal sequences in ways other than that they are internal and are not cleaved by signal peptidase. The "classical" signal sequence of prolactin when internalized and not processed by peptidase is completely translocated rather than imbedded in the membrane.

In addition, classical stop transfer sequences have recently been shown to facilitate SRP-mediate translocation in some contexts (Mize et al., 1986; Zerial et al., 1987) as has an artificial stretch of hydrophobic and uncharged amino acids (Zerial et al., 1987). This has led to speculation that functional distinctions between signal and stop transfer sequences may be more a consequence of position in a protein rather than of absolute structure or function of these elements (Coleman et al., 1985; Zerial et al., 1987). Our results would suggest otherwise. While classical stop transfer sequences and even "random" hydrophobic sequences can in some contexts share some functions with classical signal sequences (i.e. SRP arrest and SRP-dependent translocation), they do not share all functions such as recognition by signal peptidase and luminal localization. The complete translocation of the signal sequence of gGSP* should not be considered an artifactual consequence of an artificial hybrid protein since the native secretory protein, ovalbumin, possesses an uncleaved signal sequence which is also completely translocated.

As discussed in Chapter Two for the signal-bearing cleavage products of gGSP, the observation that none of the signal-containing polypeptides derived from gGSP in *Xenopus* oocytes, whether cleaved or uncleaved, are integrated in the vesicle

membrane suggests that signal sequences are not retained within the lipid bilayer. Moreover, from these results it seems possible that signal sequences may never interact directly with membrane lipids since it is difficult to imagine cells possessing a constitutive mechanism to remove integrated signal sequences from the lipid bilayer. Rather, signal sequences may cross the bilayer via interactions with transmembrane proteins or through an aqueous channel (Gilmore and Blobel, 1985).

Cell free translation systems typically yield incomplete conversion of nascent precursors to processed, translocated products. In living cells, however, cytoplasmic precursor forms of secretory proteins are rarely seen. The precursors observed in cell-free systems are believed to result from a relative inefficiency of signal-receptor interactions in the fractionated system relative to living cells. Indeed, by increasing the concentration of receptors (by increasing membrane concentration [Lingappa et al., 1977] or SRP [Siegel and Walter, 1985]) translocation efficiency in vitro is increased. Failure to detect precursor forms in vivo may also reflect an efficient mechanism for clearance of miscompartmentalized products which is not reconstituted in the cell-free systems (Lane et al., 1979). Following injection of SPgGP1 transcripts in oocytes, we observed a major, relatively stable cytoplasmic precursor (pre-gGSP). Decreased efficiency of translocation of gGSP relative to prolactin was seen in vitro also (data not shown). This is probably a consequence of steric interference of the signal sequence by the amino terminal globin domain. The presence of this precursor protein in oocytes is significant however, in that it may allow a sensitive in vivo assay for molecular components of translocation. By measuring changes in relative amounts of cytoplasmic precursor and translocated products of SPgGP1 generated following introduction or manipulation of components involved in translocation, their roles in vivo may be investigated.

It is interesting to note that upon expression of ovalbumin in *Xenopus* oocytes a significant accumulation of unglycosylated cytoplasmic ovalbumin was also observed (Lane et al., 1979). Ovalbumin is a secretory protein with an uncleaved signal sequence (Palmiter et al., 1978) which has been proposed to be internal (Lingappa et al., 1979).

The finding that signal-bearing cleavage fragments were grossly under-represented in *Xenopus* oocytes was at variance with the results from cell-free systems. We determined that signal-bearing globin fragments were preferentially and rapidly degraded. Since full-length precursor, pre-gGSP and authentic globin (data not shown) are relatively stable in the cytosol and P1 and gGSP* and gGS* also appear stable in the ER lumen and secretory pathway, we suspect that the extremely rapid degradation of the processed signal-containing globin products may reflect a fate particular to signal-containing cleavage fragments. A cytoplasmic membrane protein of *E. coli* has been described which is a signal peptide peptidase and which preferentially degrades cleaved signal peptides (Ichihara et al., 1984). A similar activity may exist in the ER membrane of eukaryotes. However, we cannot exclude the possibility that the instability of gGS may be caused by its incomplete translocation across the endoplasmic reticulum membrane or that presence of the signal sequence is a non-specific factor in its instability by preventing folding into a more stable conformation. Rapid degradation of precursors to secretory proteins introduced into the cytoplasm of *Xenopus* oocytes has been reported (Lane et al.,1979) Processed secretory proteins were substantially more stable when introduced into the oocyte cytoplasm as was unglycosylated ovalbumin.

Signal sequences are generated stoichiometrically with secretory and membrane proteins, and in some tissues specialized for secretion (pituitary or pancreas, for example) the quantity of stored secretory product constitutes a large

percentage of total cell protein, thus it seems likely that an efficient mechanism for clearance of cleaved signal peptides must exist. Attempts to study the fate of cleaved signal sequences have been hampered by the difficulty in detecting the small, hydrophobic, cleaved peptide. For this reason, the true fate of cleaved signal sequences has not been determined in eukaryotes. By joining a passenger domain to the amino terminus of a signal sequence, the size of the resultant cleaved, signal-bearing domain has been greatly increased and has been rendered immunoreactive. Thus, the globin domain enabled us to identify the signal-bearing fragment since it can be immunoprecipitated and easily detected by standard SDS PAGE. While we cannot rule out the possibility that such engineering has altered the fate of the cleaved signal itself, the distinctive kinetics of degradation of gGS relative to other products localized either to the secretory pathway or cytosol suggests that its rapid degradation occurred by a mechanism normally used for signal degradation.

To date, most progress in defining the mechanisms of targeting to, and translocation across, the ER membrane has accrued from studies in cell-free systems. While the results of this study confirm that the translocation behavior of altered substrates in cell-free systems is basically faithful to the corresponding events in vivo, our results indicate at least one translocation event which is not reconstituted in currently available cell-free systems; namely, the fate of cleaved signal sequences.

Chapter 4

UNCOUPLING TRANSLOCATION ACROSS THE ENDOPLASMIC RETICULUM MEMBRANE FROM NASCENT CHAIN ELONGATION

Introduction

As discussed previously, a characteristic feature of translocation of proteins across the ER membrane is the tight coupling of translocation with the ongoing synthesis of the polypeptide (Blobel and Dobberstein, 1975 b; Lingappa et al., 1977; Rothman and Lodish, 1977). This "cotranslational" aspect of translocation has posed a great obstacle to elucidation of mechanism, since transport events can be analyzed only during the narrow window of time and under the fastidious conditions required for synthesis of the molecule whose transport is being studied. Thus, for example, it has been unclear whether the functioning ribosome extrudes or "pushes" the growing chain across the lipid bilayer or whether (proteinaceous) machinery in the membrane moves the chain across; does translocation require the expenditure of energy or does it occur independent of an outside energy source.

The data presented in Chapters 2 and 3, demonstrating translocation of a previously synthesized protein domain by an internalized signal sequence, suggested to us that translocation of a protein could be experimentally dissociated from chain elongation. We therefore investigated the coupling of translocation to translation in the following way: messenger RNA (mRNA) was transcribed in vitro from SP6 plasmid DNA which had been cleaved at a restriction site 5' to the termination codon of a coding region of interest. Such truncated transcripts lack a termination codon. When they are used to program cell-free protein synthesis, the initial engaged ribosome should read to the truncated 3' end of the mRNA and be unable to release the nascent chain for lack of a termination codon. Since translocation is initiated well before completion of a protein's synthesis (Blobel and Dobberstein, 1975 a; Ainger and Meyer, 1986), i.e., at a point well before the ribosome has reached the

termination codon, it is likely that a nascent chain translated from such truncated mRNA would be translocation competent. The lack of further elongation was ensured by the addition of pharmacologic inhibitors of both initiation and elongation. Microsomal membranes were then presented to such complexes of nascent polypeptide chain and ribosome and translocation was assayed.

Results

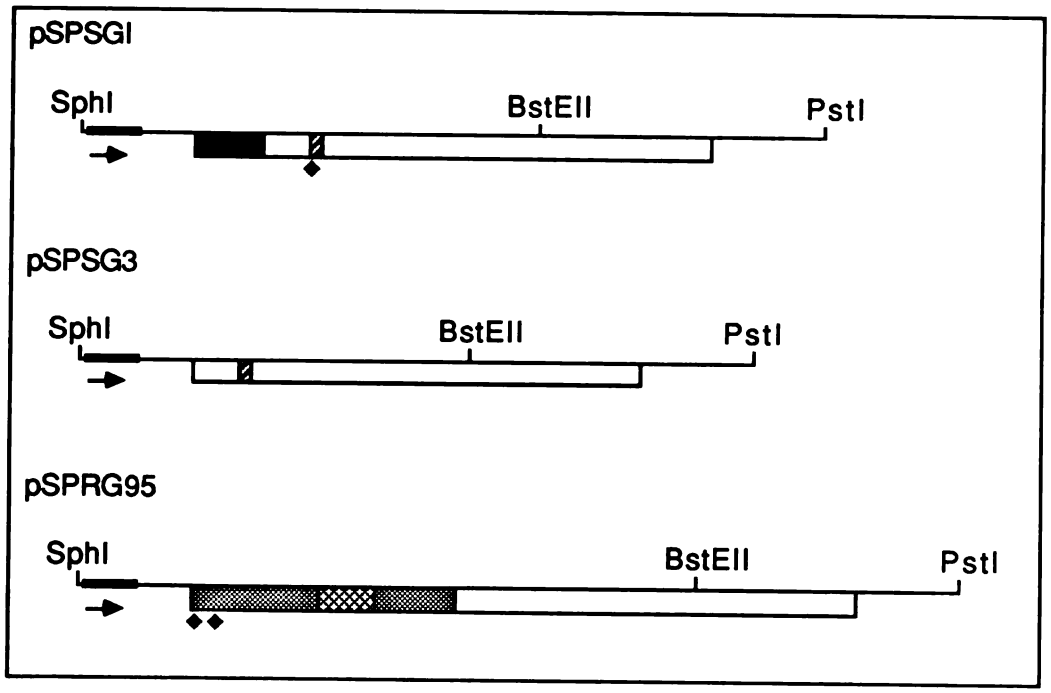
Several criteria have been established for translocation of proteins across microsomal membranes in cell-free systems: (1) Protection from exogenously added proteases except when detergents are present to solubilize the protecting lipid bilayer (Blobel and Dobberstein, 1975 b); (2) Glycosylation of newly synthesized chains (since oligosaccharidyltransferases are exclusively lumenally disposed enzymes) (Katz, et al., 1978; Lingappa, et al., 1978); and (3) Cleavage of signal sequences since signal peptidase of intact membranes appears to be accessible only to translocated chains (Jackson and Blobel, 1977).

For these studies we chose pSPSG1, an SP6 expression plasmid that encodes a fusion protein consisting of the β-lactamase signal sequence fused to the amino terminus of chimpanzee α-globin, and with a glycosylation site engineered into the globin sequence. Expression of this plasmid enabled us to assay translocation by all three independent criteria described above. Restriction maps and coding regions of this plasmid (pSPSG1) and of a related plasmid (pSPSG3), in which the region encoding the signal sequence was deleted, are shown in Figure 4-1A.

Plasmid pSPSG1 was expressed and was shown to program the synthesis of glycosylated and nonglycosylated globin-immunoreactive chains in the expected fashion: in the absence of membranes pSPSG1 encoded pre SG1, a nonglycosylated precursor (Fig. 4-2, lane E). When membranes were present cotranslationally (Fig. 4-

Figure 4-1. Posttranslational translocation: substrates and protocol. (A) Restriction maps of relevant regions of pSPSG1, pSPSG3 and pSPRG95, with defined coding regions represented underneath. The SP6 promoter is indicated by the heavy black lines at the 5' ends of the restriction maps and the direction of transcription by the arrows. Globin amino acid sequences are represented by open bars and the β-lactamase signal sequence by the black bar. The inserted concensus sequence for N-linked glycosylation is represented by the diagonal striped bars. Rhodopsin amino acid sequences are represented by the stippled bars and its transmembrane segment by the cross-hatched bar. Sites which are utilized for addition of core oligosaccharide are indicated (*). (B) Protocol for expression of plasmids, with and without termination codons. Messenger RNA lacking a termination codon was prepared by in vitro transcription (Kreig and Melton, 1983) of plasmids linearized with BstEII, which cleaves the globin coding regions 35 codons upstream from the termination codon. Full-length transcripts including the termination codon were prepared in separate reactions. Full-length and truncated mRNA's were incubated separately in transcription-linked translation reactions with rabbit reticulocyte lysate. Reactions were carried out at 24°C for 15 minutes; 10^{-4} M aurin tricarboxylic acid (ATA) was added to inhibit initiation, and after another 15 minutes emetine was added to 10^{-4} M to block further elongation. Expected translation products of full-length (left) and truncated (right) coding regions are schematically represented. Translation of full-length mRNA yields released full-length polypeptide, free ribosomes, and mRNA while translation of truncated transcripts produces intact polysomes with "arrested" nascent polypeptide chains emerging from the ribosomes. Polysomes were first incubated with or without 1mM puromycin before addition of dog pancreas microsomal membranes (5 A_{280} U/ml) and further incubation at 24°C for 20 minutes. Translation products from full-length plasmids were not treated with puromycin before incubation with membranes.

A.


pSPSGI
SphI
BstEII
PstI
pSPSG3
SphI
BstEII
PstI
pSPRG95
SphI
BstEII
PstI

B.

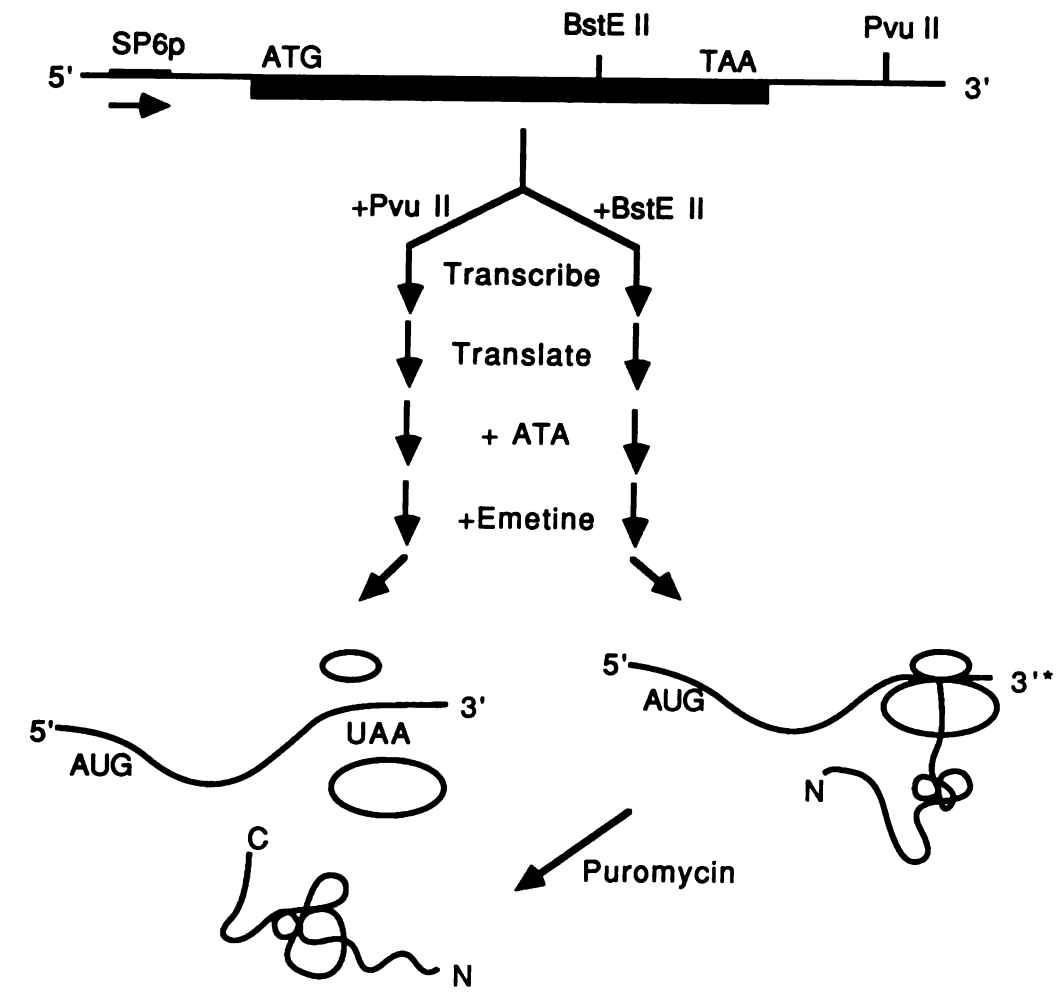
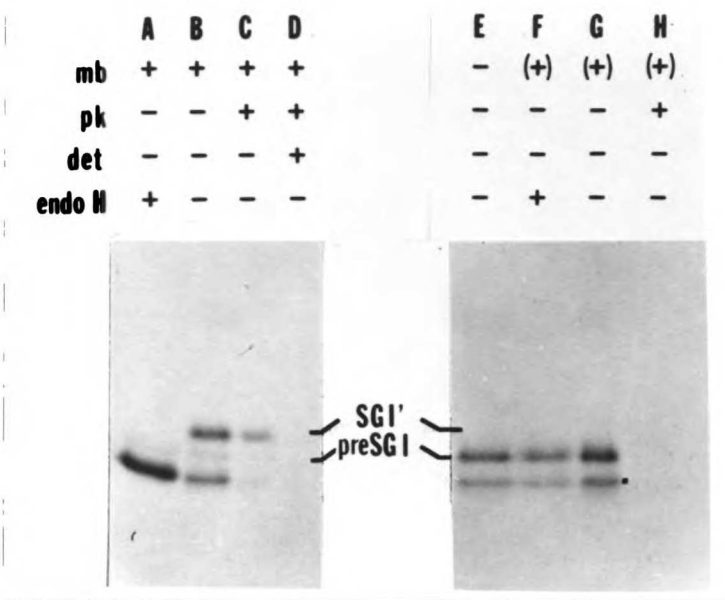

SP6p
ATG
BstE II
TAA
Pvu II
5'
3'
+Pvu II
+BstE II
Transcribe
Translate
+ ATA
+Emetine
5'
AUG
UAA
3'
5'
AUG
3'*
N
C
N
Puromycin

Figure 4-2. Cotranslational translocation of SG1 across microsomal membranes. Plasmid pSPSG1 was transcribed in vitro as described (Kreig and Melton, 1983). Transcription linked translation reactions were carried out either in the presence (+MB; lanes A to D) or absence (- MB; lane E) of microsomal membranes (2.5 A_{280} U/ml) or with membranes added posttranslationally ([+] MB; lanes F to H; see legend to Fig. 1). Some samples were treated with endoglycosidase H (+ endo H; A and F), others were treated with proteinase K at 0.1 mg/ml (+ PK) in the presence (+ det; lane D) or absence (- det; lanes C and H) of 1 percent Nikkol (a nonionic detergent). Proteolysis and endo H digestion are described in Chapter 7. The positions of the glycosylated species SG1' and the precursor form, preSG1, are indicated between lanes D and E. The lower molecular weight band in lane G, indicated by a black dot, is an unidentified translation product of pSPSG1 which comigrates with the processed and unglycosylated form of SG1 (see lane A). This band occurs in the presence and absence of membranes, is protease-sensitive and appears to be an internal initiation product. All samples were immunoprecipitated with rabbit antiserum to human hemoglobin prepared as described and subjected to SDS-PAGE. Gels were fluorographed, and labeled proteins were viewed by autoradiography.


A B C D E F G H
mb + + + + − (+) (+) (+)
pk − − + + − − − +
det − − − + − − − −
endo H + − − − − + − −
SGI'
preSGI

2, lanes A to D), nascent preSG1 was converted to a higher molecular weight form called SG1'. Endo H digestion converted SG1' to a form of lower molecular weight than preSG1, demonstrating that SG1' was processed (signal sequence cleaved) as well as glycosylated (Fig. 4-2, lane A). Protease digestion established that SG1', but not residual preSG1, was translocated across microsomal membranes (Fig. 4-2, lane C). Addition of detergent abolished all protease resistance, an indication that protection was conferred by the lipid bialyer and was not a property intrinsic to the processed chain (Fig. 4-2, lane D). However, when membranes were added posttranslationally (Fig. 4-2, lanes F to H), preSG1 was the only species observed. From these results we conclude that only nascent preSG1 is transported across the ER membrane and hence that translocation of SG1 chains appears coupled to their translation.

Following the logic described in the introduction to this chapter and depicted in Figure 4-1B, we found a restriction site that would allow us to truncate the globin coding region prior to its termination codon. This site, for restriction endonuclease BstEII, was found 138 codons from the initiation codon (AUG), and 34 codons from that of termination. Plasmid pSPSG1 was cleaved with BstEII and the resulting linear DNA was transcribed. This truncated transcript was called SG1/B. Its translation products were immunoreactive with antiserum to globin and migrated on SDS-PAGE at approximately 12 kD, 3 kD smaller than full-length pre-SG1, as expected for faithful expression of the molecules truncated at BstEII. In order to demonstrate that these polypeptide chains were maintained while emerging from ribosomes and were not released as ribosomes fell off truncated mRNA's, completed translation reactions were sedimented in sucrose density gradients. Analysis of gradient fractions by SDS-PAGE revealed that almost all full-length chains were sedimented under conditions which resulted in pelleted ribosomes (data not shown). When SG1/B was translated with membranes present during

translation (that is, cotranslationally), efficient translocation was demonstrated by all criteria, that is, protection from protease (Fig. 4-3, lane C), addition of carbohydrate (Fig. 4-3, lanes A and B) and signal sequence cleavage (compare Figure 4-3, lanes A, B), as observed for SG1 (Fig. 4-2). The product was fully protease resistant, presumably because the ribosome protected the portion of the chain which it was anchoring on the cytoplasmic face of the ER membrane. In contrast to SG1, when membranes were added following the translation of SG1/B and in the presence of inhibitors of initiation and elongation, translocation was again observed by the same criteria (Fig. 4-3, lanes E to H), although with somewhat reduced efficiency. In the case of posttranslational membrane addition, either emetine (Fig. 4-3) or cycloheximide (data not shown) were used to prevent further protein synthesis. Thus, translation of mRNA lacking 35 codons at the 3' terminus (including the termination codon) permits transport across the ER membrane without ongoing chain elongation; that is, we have achieved an uncoupling of translocation from translation.

In order to probe the basis for this uncoupling we treated the translation reaction with puromycin, an aminoacyl transfer RNA (tRNA) analog that causes termination and release of nascent chains (Lodish, et al., 1971; Blobel and Sabatini, 1971). As can be seen from Table I, treatment with puromycin resulted in release of nascent chains to the post-ribosomal supernatant following high speed centrifugation. We found that puromycin treatment (Fig. 4-3, lanes I to K) abolished (posttranslational) translocation of chains encoded by SGI/B. These data suggest a role for the ribosome in transport across the ER membrane that is distinct from its role in protein synthesis.

Nucleoside triphosphates and other small molecules were removed from completed translation reactions by gel filtration. Incubation of these preparations

Figure 4-3. Co- and post-translational translocation of SG1/B across microsomal membranes. Plasmid pSPSG1, linearized with BstEII, was transcribed to produce mRNA lacking a termination codon (see Figure 1). The transcript was translated in vitro, as described, etiher in the presence (+ MB; lanes A to D) or absence (-MB; lanes E to K; see legend to Fig. 1) of 2.5 A_{280} U/ml of microsomal membranes. Some samples translated without membranes were first incubated in 1 mM puromycin (+ Puro; lanes I to K) for 20 minutes before the addition of emetine to 10^{-4} M with subsequent incubation with membranes at 5 A_{280} U/ml ([+] MB). Other samples were treated with emetine alone (-Puro; lanes E to H), before incubation with membranes. Some samples were treated with endo H (+ endo H; lanes A, E, and I), some treated with proteinase K (0.1 mg/ml) (+ PK) in the presence (+ det; lanes D and H) or absence (- det; lanes C, G, and K) of 1 percent Nikkol. All samples were immunoprecipitated, subjected to electrophoresis on SDS-PAGE and viewed by autoradiography.

	A	B	C	D	E	F	G	H	I	J	K
puro	−	−	−	−	−	−	−	−	+	+	+
mb	+	+	+	+	(+)	(+)	(+)	(+)	(+)	(+)	(+)
pk	−	−	+	+	−	−	+	+	−	−	+
det	−	−	−	+	−	−	−	+	−	−	−
endo H	+	−	−	−	+	−	−	−	+	−	−

with microsomal membranes and nucleoside triphosphates (Fig. 4-4, lane C), but not with membranes alone (Fig. 4-4, lane B), resulted in translocation. The requirement for the energy supplement [consisting of adenosine triphosphate (ATP), guanosine triphosphate (GTP), and creatine phosphate with creatine kinase] indicated that translocation did not proceed spontaneously but rather displayed an energy requirement independent of protein synthesis.

Plasmid pSPSG3 encodes a protein called SG3 which is identical to SGI except that it lacks the β-lactamase signal sequence. The mobility of SG3 was identical in the presence or absence of membranes added either co- or post-translationally (Fig. 4-5, lanes B-D). None of the SG3 chains were protected from proteases (Fig. 4-5, lanes A, E, and F). Likewise, no translocation either co- or posttranslationally was observed for the similarly truncated form of pSPSG3, termed SG3/B (which lacks the signal sequence as well as the termination codon). This is consistent with the established role of the signal sequence in directing chain translocation.

We have extended our findings for a cleaved amino terminal signal sequence of a secretory protein to an uncleaved internal signal sequence of an integral membrane protein. Plasmid pSPRG95 encoded a fusion protein consisting of the amino terminal glycosylated domain of bovine rhodopsin and the first rhodopsin transmembrane helix, which includes both a signal and a stop transfer sequence (Friedlander and Blobel, 1985), followed by codons 19 to 143 of globin (see Figure 4-1 A). This protein is integrated into membranes and glycosylation of its amino terminus reflects its translocation as has been shown for secretory glycoproteins Lingappa, et al., 1978). Figure 4-6A shows that RG95, the protein encoded by pSPRG95, is glycosylated and, hence, integrated in an obligate cotranslational fashion when the termination codon is present. However, truncation of the coding region at the BstEII site of globin results in uncoupling of integration from elongation:

Figure 4-4. Reconstitution of translocation from energy depleted translation reactions. SG/BstEII was translated in reticulocyte lysate, translation inhibited by incubation with 10^{-4} M ATA followed by addition of 10^{-4} M emetine. Translation products were fractionated by high speed centrifugation. The pelleted fraction was resuspended in a buffer with the same ionic composition of the reticulocyte translation reaction and then subjected to two rounds of centrifuge desalting on Sephadex G-25 (see Chapter 7). Aliquots were incubated for 20 min at 24°C in the presence (lanes B and C) or absence (lane A) of microsomal membranes, with (lane C) or without (lanes A and B) supplementation with 1 mM ATP, 1 mM GTP and 10 mM creatine phosphate.

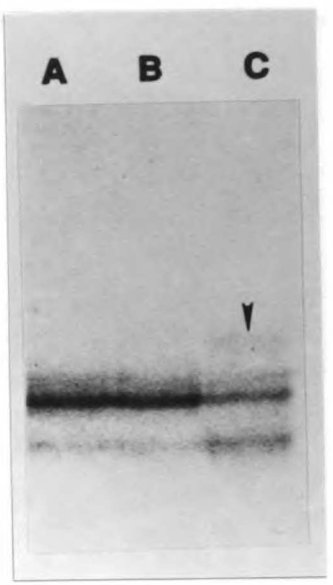

A
B
C

Figure 4-5. Co- and posttranslational incubation of SG3 in the presence of microsomal membranes. Plasmid pSPSG3 (Fig.4-1) was transcribed and translated. Translation reactions were carried out either in the presence (+ MB; lanes D to F) or absence (- MB; lane C) of microsomal membranes (2.5 A_{280} U/ml), or with membranes added posttranslationally ([+] MB; lanes A and B; 5 A_{280} U/ml). Some reactions were then subjected to proteolysis with proteinase K at 0.1 mg/ml (+ PK; lanes A, E, and F) in the presence (+ det; lane F) or absence (- det; lanes A and E) and 1 percent Nikkol. All samples were immunoprecipitated, subjected to SDS-PAGE and bands were viewed by autoradiography.

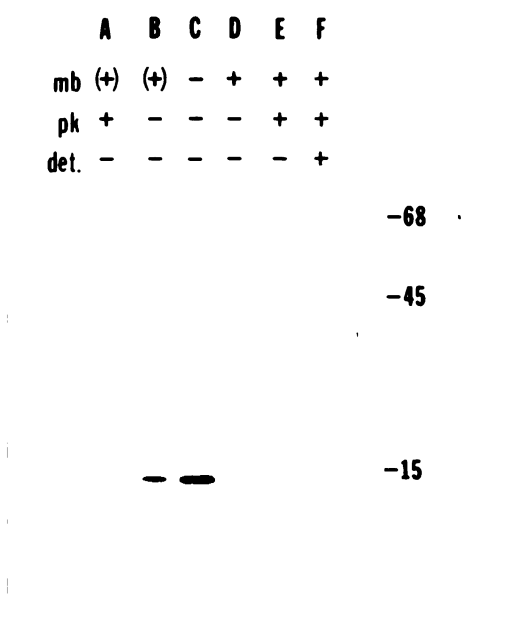
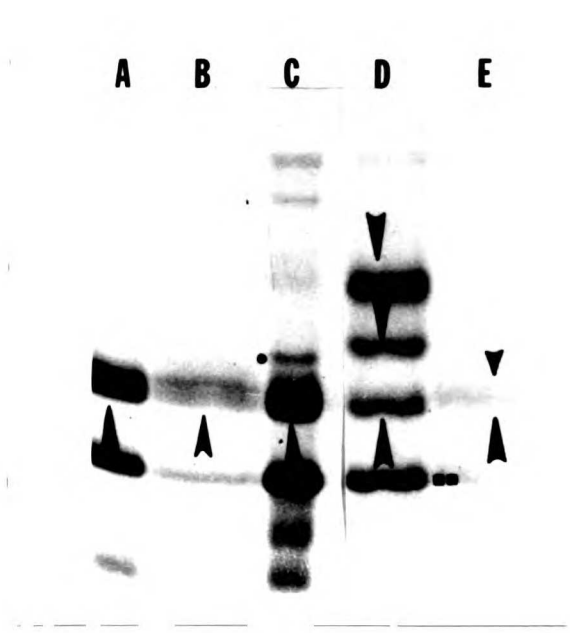
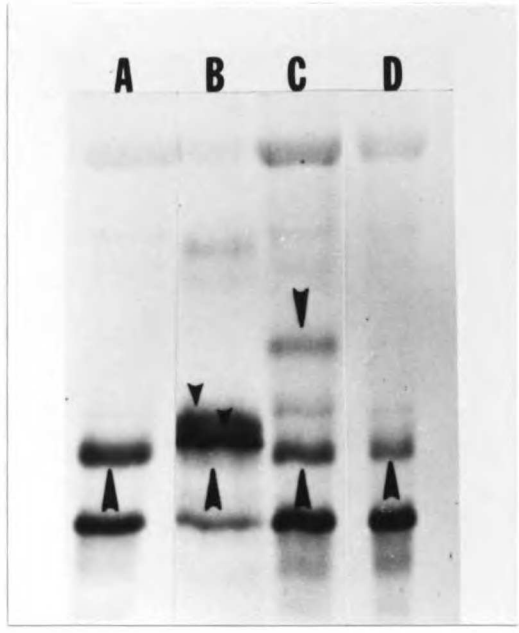

A B C D E F
mb (+) (+) – + + +
pk + – – – + +
det. – – – – – +
–68
–45
–15

Figure 4-6 (A). Co- and post-translational translocation of RG95. Transcription-linked translation was carried out with plasmid pSPRG95 as described. Translation reactions were carried out either without microsomal membranes (lanes A to C) or with microsomal membranes present at a final concentration of 2.5 A_{280} U/ml during translation (lanes D and E). Some portions were supplemented with microsomal membranes at a final concentration of 5 A_{280} U/ml after completion of translation and addition of emetine to 10^{-4} M (lanes A and B). All samples were immunoprecipitated with antiserum to globin and some were treated with endo H (lane B, E), before SDS-PAGE. Upward pointing arrow indicates the precursor form of RG95. Downward pointing arrows in lane D, indicate position of glycosylated bands resulting from transmembrane integration of RG95. Downward pointing arrowhead of lane E indicates position of the endo H-digested form of glycosylated RG95. The dot to the left of lane C indicates the position of a minor artifact band presumed to result from initiation at an internal AUG. (B) Posttranslational translocation of RG95/B. Plasmid pSPRG95 was cleaved with BstEII and subjected to transcription-linked translation as before. The translation reaction in lane A contained no microsomal membranes. Lane C had microsomal membranes added to a final concentration of 5 A_{280} U/ml, after 20 minutes of translation and after addition of emetine to 10^{-4} M. Lane B is the same as for lane C except that products were treated with endo H. Lane D was the same as indicated for lane C except that sample was treated with 1 mM puromycin for 20 minutes at 24°C before incubation with microsomal membranes. All incubations with membranes were for 20 minutes at 24°C.



A.



B.

(glycosylation can be achieved even when incubation with membranes is carried out following the translation of RG95/B and in the presence of elongation inhibitors (Fig. 4-6B). As in the case of SG1/B, post-translational translocation was ribosome dependent because treatment with puromycin before incubation with membranes prevented glycosylation.

Discussion

We have shown here that expression of certain in vitro transcribed mRNA's that lack termination codons permits the uncoupling of a protein's translocation across microsomal membranes from its synthesis. Furthermore, we demonstrated that in the cases presented here translocation, whether coupled or uncoupled to translation, is ribosome dependent. We demonstrated these findings for both a secretory protein (SG1) and for an integral membrane protein (RG95); and for both an amino terminal-cleaved signal (that of β-lactamase) and an internal, uncleaved signal (that of rhodopsin). We conclude that, in general, translocation across the ER membrane is not dependent on concurrent synthesis of the protein being transported. Our data have several implications, both theoretical and practical, for the problem of protein transport across the ER membrane.

The demonstration that puromycin treatment or deletion of the signal sequence abolishes translocation, even when it has been uncoupled from protein synthesis, suggests that both the ribosome and the signal sequence participate in maintaining a "translocation competent state" of nascent chains, at least in higher eukaryotes. This is consistent with the observation that SRP can interact with signal sequences of nascent secretory proteins only in the context of the synthesizing ribosome (Krieg et al., 1986; Wiedmann et al., 1987a). The ribosome may be required not only to maintain a translocation competent state, but also as a ligand for translocation, i.e. to form a functional ribosome membrane junction required for

translocation (Connolly and Gilmore, 1986). Thus, it appears that the coupling of translation to translocation may reflect a role of the synthesizing ribosome in translocation apart from its role in protein synthesis.

A number of proteins studied in higher eukaryotic systems have displayed a strict cotranslational feature to their translocation. However, in bacteria (Randall, 1983; Randall and Hardy, 1986; Koshland and Botstein, 1982), translocation of newly synthesized proteins has been shown to lack the obligate coupling to protein synthesis observed in higher eukaryotic systems. Moreover, in a recently developed homologous yeast cell-free system, completed chains of prepro-alpha-factor can be demonstrated to cross microsomal membranes (Hansen et al., 1986; Rothblatt and Meyer, 1986a; Waters and Blobel, 1986), even in the absence of any ribosomes (Hansen, et al., 1986). This is a property of yeast membranes and not of prepro-α–factor, since post-translational translocation of prepro-α–factor across canine pancreas rough microsomes is ribosome dependent (Garcia and Walter, in press).

Translocation which has been uncoupled from translation requires a source of energy in the form of nucleoside triphosphates. Thus, as has been observed in both bacteria (Chen and Tai, 1985) and yeast (Hansen, et al., 1986; Waters and Blobel, 1986; Rothblatt and Meyer, 1986b), translocation in higher eukaryotes displays an energy requirement independent of that of protein synthesis (see also Mueckler and Lodish, 1986b). The energy-dependence of the uncoupled translocation reaction argues that translocation is not a spontaneous process.

While these experiments indicate that the energy derived from protein synthesis is not required for translocation when an alternate energy source is provided, it cannot be concluded that the energy of protein synthesis is not involved in translocation normally. The work of Chen and Tai (1987) has addressed the question of the involvement of ATP in cotranslational translocation in a cell-free

system derived from *E. coli*. The finding that a non-hydrolyzable ATP analog which had no detectable effect on protein synthesis was able to block translocation suggests that ATP hydrolysis is required even for cotranslational translocation.

It remains to be determined whether expenditure of energy is for translocation per se, for assembly and maintenance of the translocation competent state (e.g. denaturing the partially folded polypeptide chain) or possibly for assembly and maintenance of a transmembrane channel through which the translocating protein may pass.

Our results also have practical implications for addressing the problem of protein translocation across the ER membrane. Progress in defining the mechanism of translocation events as well as in isolating the putative membrane components has been hampered by the heretofore coupled nature of the assay: ongoing synthesis of the chain whose translocation was to be studied. The novel assay presented here permits discrimination between requirements for these two events by separating the processes of transport from those of synthesis. It should now be possible to distinguish between steps in the transport process, a necessary prerequisite for fractionation and reconstitution of translocation components.

Chapter 5

RIBOSOME DEPENDENT AND RIBOSOME INDEPENDENT TRANSLOCATION DURING DIFFERENT STAGES OF NASCENT CHAIN ELONGATION

Introduction

Early work on translocation of proteins across the ER membrane focused on the membrane bound ribosomes of the ER which synthesize secretory and transmembrane proteins (Palade, 1975) and on the obligate co-translational feature of this process (Blobel and Dobberstein, 1975 b). These characteristics have distinguished transfer of proteins across the ER membrane from mitochondrial protein translocation (Schatz and Butow, 1983) and export of bacterial proteins (Randall, 1983), both of which are post-translational processes. The previous chapter described an experimental uncoupling of nascent chain translocation from elongation for nascent polypeptides whose translocation is normally tightly coupled to translation. Translocation was observed only for polypeptides which remained associated with the ribosome and was termed "ribosome dependent" translocation (see also Connolly and Gilmore, 1986; Mueckler and Lodish, 1986b; Caulfield et al., 1986; Chao et al., 1987). Thus, the ribosome appears to play a critical role in translocation distinct from its role in protein synthesis, formation of a functional ribosome-membrane junction (Connolly and Gilmore, 1986) may activate translocation machinery in the membrane.

However, translocation of completed and released chains (ribosome independent translocation) has also been described recently in eukaryotic cell-free systems. Ribosome independent translocation has been described for the yeast mating pheromone, prepro-α-factor, across yeast microsomes (Hansen et al., 1986) but not mammalian microsomes (Garcia and Walter, in press). A ribosome independent process for insertion into, and translocation across, mammalian microsomes has been reported for honeybee prepromelittin (Zimmermann and Mollay, 1986), M-13 procoat protein (Watts et al., 1983; Wiech et al., 1987), and frog prepropeptide GLa

(Schlenstedt and Zimmermann, 1987). Translocation of these three proteins can occur independent of SRP and its receptor. Thus, ribosome independent translocation may represent transport via an independent mechanism from that of ribosome dependent translocation. Alternatively, ribosome dependent and ribosome independent translocation may occur via the same mechanism, utilizing a subset of common receptors in the membrane, while cytoplasmic components (SRP) may or may not be used depending on the requirements of the precursor proteins.

As a first step towards reconciling ribosome dependent and ribosome independent translocation, we have carried out serial truncations of the coding regions of several plasmids encoding secretory proteins. One truncation product of a fusion of the β-lactamase signal sequence with α-globin was generated which could translocate independent of the ribosome and the translocation of this previously synthesized protein as well as ribosome-associated polypeptides was studied in living cells.

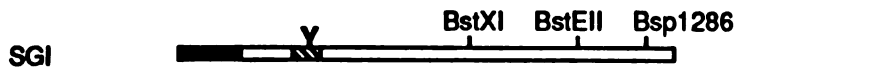
Results

Serial truncations were carried out on several plasmids encoding fusion proteins such as SGI (a fusion of the β-lactamase signal sequence with α-globin) and bPG (a fusion of the amino terminal 83 amino acids of preprolactin with full-length α-globin) as well as a "native" protein, preprolactin. Cell-free transcription and translation of such truncated plasmids produces a truncated polypeptide chain which remains associated with the synthesizing ribosome as a peptidyl tRNA. Pharmacologic inhibitors of initiation and elongation can be added to study translocation in the absence of protein synthesis (post-translational)and the polypeptide chain can be released from the ribosome by treatment with the amino-acyl tRNA analog, puromycin. All translation products were incubated with microsomal membranes either during translation or after addition of initiation and

elongation inhibitors with or without preceding treatment with puromycin. Translocation was assayed by glycosylation or signal peptide cleavage as determined by changes in electrophoretic mobilities on SDS-PAGE. The results are summarized in Figure 5-I. As can be seen, translocation of all full-length proteins was strictly co-translational but the translocation of all truncation products tested (from 82 to 219 codons in length) could be uncoupled from translation, and, with one exception, all translocation was abolished by treatment with puromycin. Thus, in general, failure of polypeptides to translocate once released from the ribosome does not appear to be due to exposure of the 35-45 amino acids buried in the ribosome since allowing the ribosome to translate an additional 100 codons does not abolish ribosome dependent translocation; similarly, the ribosome does not appear to hold the nascent polypeptide chain in a translocation competent state merely by sequestering the carboxy terminal 35-45 amino acids since further truncation of a ribosome dependent substrate does not always allow translocation to occur independent of the ribosome. Treatment with puromycin to release the "nascent" chain from the ribosome abolished translocation with one exception. When pSPSGI (which encodes the β-lactamase signal sequence fused to α-globin into which a glycosylation site has been inserted) was truncated at codon 100 by digestion with restriction endonuclease, BstXI, the encoded polypeptide, SG/BstXI, was seen to be glycosylated (and thus translocated) with similar efficiency either before or after treatment of the polysomes with puromycin. Thus, its translocation appeared to be ribosome independent.

Translocation of SG/BstXI is shown in figure 5-2 A. Translocation occurred when microsomal membranes were present during translation (lanes 1 and 2) as evidenced by the appearance of the glycosylated, higher molecular weight band indicated by the downward pointing arrowhead in lane 1, and referred to as SG/BstXI'. When membranes were added following inhibition of elongation the

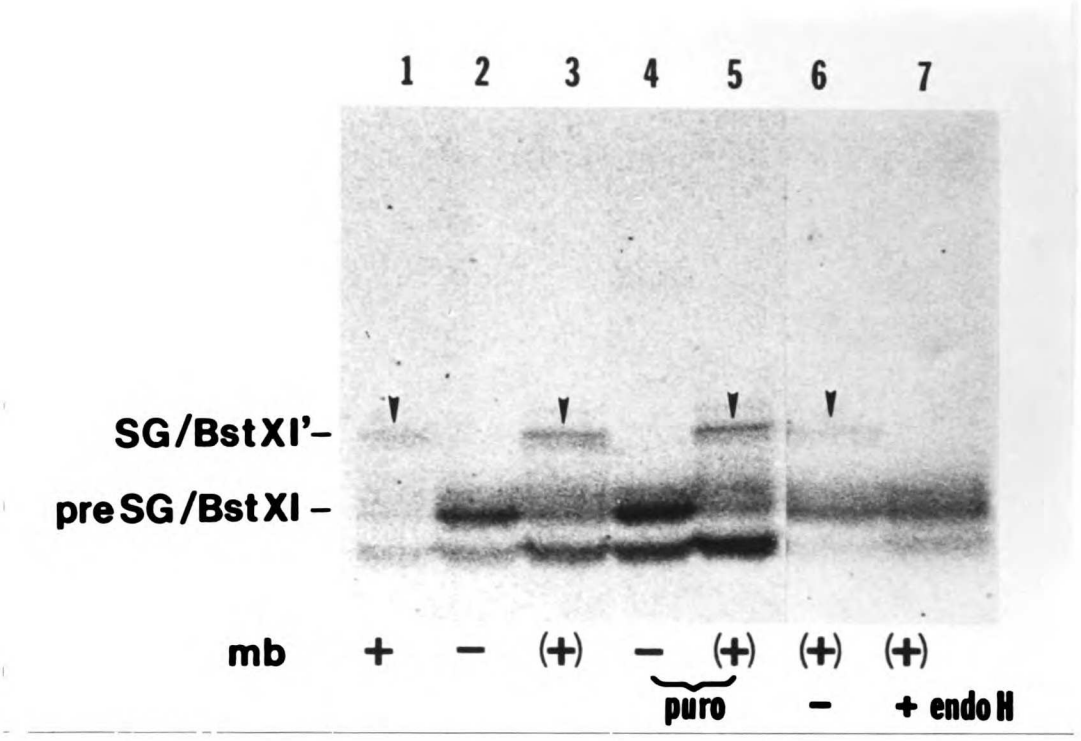
Figure 5-1. Translocation of various cell-free translation products across microsomal membranes. Translation products of plasmids, pSPSGI, pSPbPG, and pSPbPI are schematically represented. Open bars represent globin sequences, the black bar indicates the β-lactamase signal sequence and stippled bars indicate prolactin sequences. Signal sequence cleavage sites are indicated by triangles and the site for N-linked oligosaccharide addition demarked by a branched symbol. The expected sizes of translation products generated following truncation of the plasmids with the indicated restriction endonucleases is indicated both above the diagram of the full-length protein and as different sized lines below. The size of the resulting coding region of a given truncated or full-length plasmid is indicated immediately to the right in codons. Each of these plasmids was transcribed in vitro and translated in a rabbit reticulocyte lysate system which was supplemented with microsomal membranes (5 A_{280} U/ml) either during translation (Co-) or following treatment of translated products with emetine (Post-) or puromycin (Post- Puro). Following incubation, products were immunoprecipitated, prepared, applied to SDS PAGE and bands viewed by autoradiography. Translocation was assessed by membrane dependent processing (either removal of the signal sequence or addition of N-linked oligosaccharide) as determined by changes in electrophoretic mobilities. * indicates that signal cleavage of bPI/PvuII required the release of the nascent chain from the ribosome by puromycin after incubation with membranes (Connolly and Gilmore, 1986).



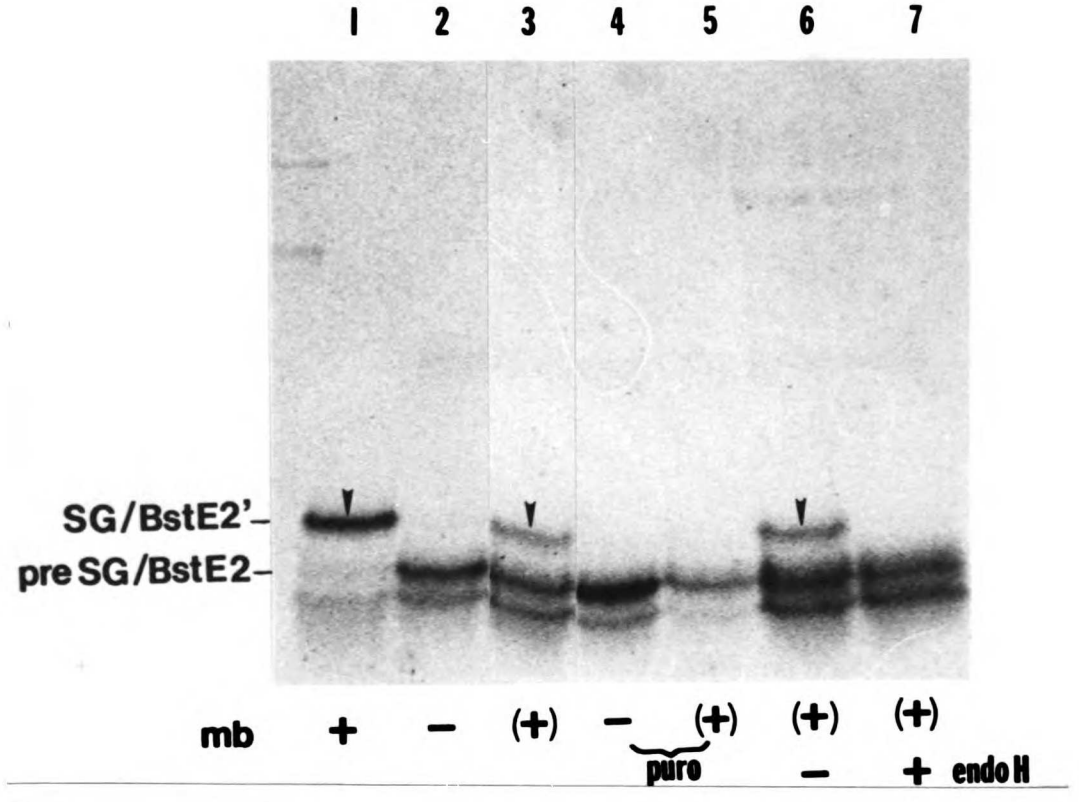
	Template (Codons)	Size	Translocation Co-	Post-	Post-Puro
SGI	173		+	-	N/A
SG/Bsp	162		+	+	-
SG/BstEII	138		+	+	-
SG/BstXI	99		+	+	+
bPG	224		+	-	N/A
bPG/Bsp	213		+	+	-
bPG/BstEII	189		+	+	-
bPG/BstXI	150		+	+	-
bPG/BssHII	100		+	+	-
bPI	229		+	+	N/A
bPI/FspI	205		+	+	-
bPI/BanII	136		+	+	-
bPI/PvuII	82		+*	+*	-

Figure 5-2. Ribosome dependent and ribosome independent translocation. (A) Co- and post-translational translocation of pSPSG/BstXI encoded translation products in vitro. Plasmid pSPSGI was digested with restriction endonuclease, BstXI (which linearized the plasmid at a position 99 codons from the initiation codon) and transcribed and translated as described in 4-1. Translations in rabbit reticulocyte lysate were carried out in the presence (+ mb; lane 1) and absence of canine pancreas rough microsomes (lanes 2-7). After incubation at 24°C for 20 minutes translation reactions were treated with 10^{-4} M aurin tricarboxylic acid (ATA) for 10 minutes to inhibit initiation. Further elongation was subsequently inhibited with 10^{-4} M emetine. Some samples were then treated with puromycin (1 mM) to release peptidyl tRNAs from the synthesizing ribosomes. Separate samples were then incubated simultaneously for 20 minutes in the presence ([+] mb; lanes 3, 5, 6, 7) or absence of rough microsomes (- mb; lanes 2 and 4). Samples were subsequently diluted in a 1% Triton X-100 buffer and immunoprecipitated with globin antiserum. Following immunoprecipitation two identical samples were prepared and digested with endo H (+ endo H; lane 7) or mock digested (- endo H; lane 6). Samples were prepared and subjected to SDS-PAGE and bands viewed by autoradiography. Translocated and glycosylated bands, SG/BstEII', are indicated by arrowheads. (B) Co- and post-translational translocation of pSPSG/BstEII encoded translation products. Plasmid, pSPSGI was digested with restriction endonuclease BstEII which linearizes the plasmid 138 codons from the initiation codon (of the β-lactamase signal sequence). Transcription, translation, and post-translational incubations were performed as described in (A). Microsomal membranes were present either during (+ mb; lane 1) or after translation ([+] mb; lanes 3, 5, 6, 7). Some samples were treated with puromycin (+ puro) prior to further incubation in the presence (lane 5) or absence of membranes (lane 4). Identical samples which had been incubated post-translationally with membranes were treated with endo H (+ endo H; lane 7) or mock

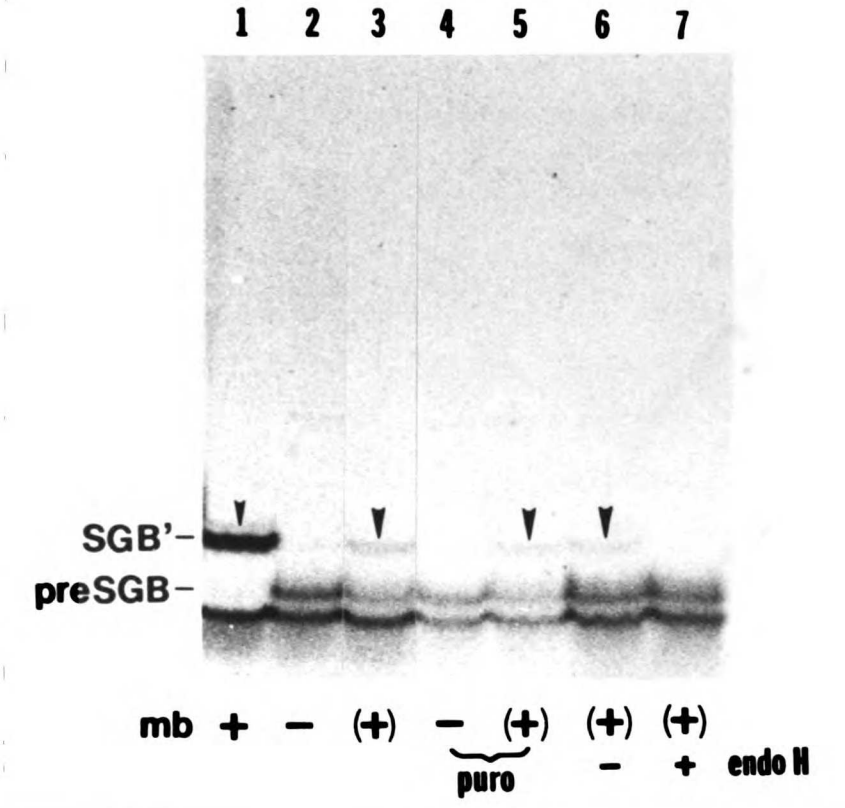
mock digested (- endo H; lane 6). SG/BstE2', the glycosylated translocated protein is indicated by arrowheads. (C) Translocation of nascent and completed translation products of pSPSGB. Plasmid, pSPSGB, was constructed as described in Chapter 7 by inserting a termination codon at the BstXI site of pSPSGI. Transcription, translation, and post-translational treatments were as described above. Microsomal membranes were present either during (+ mb; lane 1) or after translation ([+] mb; lanes 3, 5, 6, 7). Some samples were treated with puromycin (puro) prior to further incubation in the presence (lane 5) or absence of membranes (lane 4). Identical samples which had been incubated post-translationally with membranes were treated with endo H (+ endo H; lane 7) or mock digested (- endo H; lane 6). Translocated, glycosylated bands, SGB', are indicated by downward pointing arrowheads.


1 2 3 4 5 6 7
SG/BstXI'–
preSG/BstXI –
mb + – (+) – (+) (+) (+)
puro
– + endo H

A.


1 2 3 4 5 6 7
SG/BstE2'–
preSG/BstE2–
mb + – (+) – (+) (+) (+)
puro
– + endo H

B.



C.

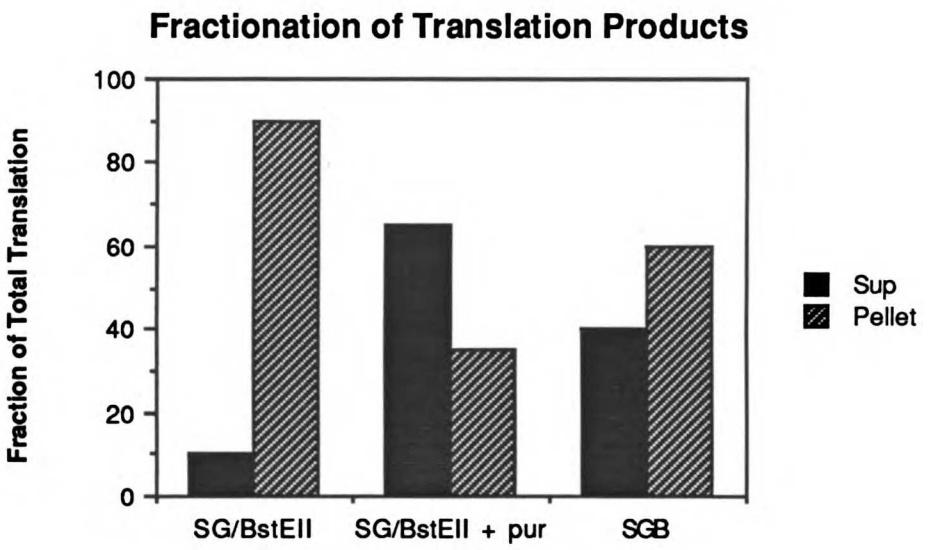
same glycosylated, endo H sensitive band was observed (lanes 3, 6, 7). Treatment of the translation mix with puromycin (to release the nascent polypeptide from the ribosome) prior to incubation with microsomes had no effect on conversion of preSG/BstXI (lane 4) to SG/BstXI' (lane 5). Translocation of the ribosome-dependent substrate, SG/BstEII (see fig. 5-1) is shown in figure 5-2 B. Translocation across membranes added post-translationally (in the presence of inhibitors of initiation and elongation) occurred (lanes 3, 6, 7), producing the glycosylated form, SG/BstEII', unless the chains were released from the ribosome by treatment with puromycin (lanes 4, 5).

We inserted a termination codon at the BstXI site of pSPSGI to create a plasmid called pSPSGB (for construction protocol see Chapter 7), whose completed and released translation product could be studied. Co- and post-translational translocation of the encoded protein is depicted in Figure 5-2 C. Translocation was evidenced by appearance of a glycosylated, endo H sensitive band termed SGB' (indicated by large downward-pointing arrowheads), which was protected from protease (data not shown).

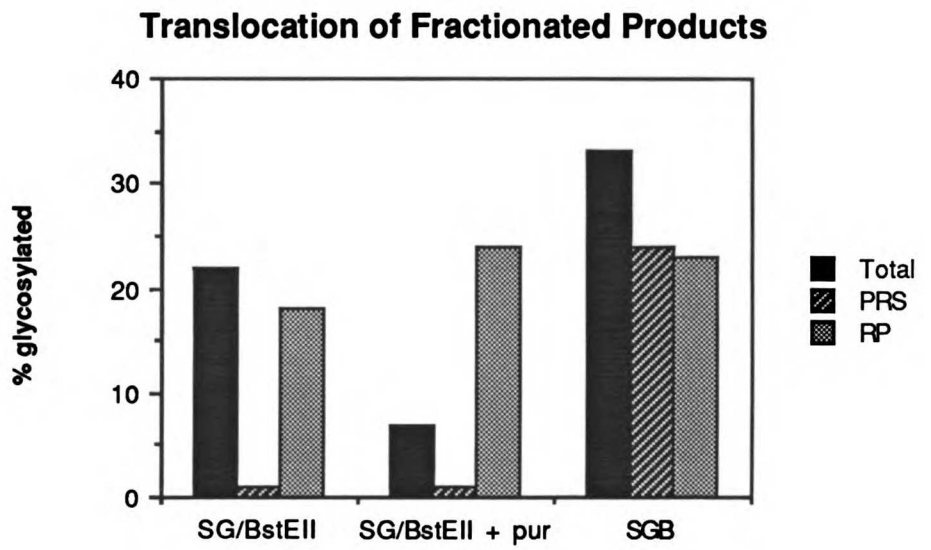
To demonstrate that the ribosome was not involved in the translocation of SGB we prepared a ribosome-depleted high speed supernatant containing in vitro synthesized SGB. In vitro synthesized SG/BstEII before and after puromycin treatment was prepared similarly. Figure 5-3A shows the partitioning of these precursors in the pellet and post-ribosomal supernatant fractions. While 90% of the truncated products, preSG/BstEII, pelleted with the ribosomes, preSGB was split approximately evenly between the ribosomal pellet and post-ribosomal supernatant, as was preSG/BstEII following treatment with puromycin. Post-translational translocation of unfractionated translation products and of the pellet and post-ribosomal supernatant fractions were assayed by appearance of the glycosylated

Figure 5-3. Post-translational translocation of fractionated cell-free translation products. (A) Fractionation of translation products by high speed centrifugation. SG/BstEII and SGB were translated in vitro as described and an aliquot of SG/BstEII translation mix incubated for 15 minutes with puromycin to release the nascent polypeptide chains from the ribosomes. The three samples were subjected to high speed centrifugation to sediment ribosomes and associated macromolecules (see Chapter 7). Post-ribosomal supernatants and pellet fractions were separated and each immunoprecipitated with globin antiserum and subsequently prepared and subjected to SDS-PAGE. Band intensities were quantified by scanning laser densitometry and are displayed as the percentage of the total in each fraction. Post-ribosomal supernatant fractions (PRS) are represented by hatched bars and the pellet fractions (RP) are represented by black bars. (B) Translocation of fractionated translation products. Translation products of SG/BstEII which had been incubated in the presence or absence of puromycin and completed translations of SGB were subjected to high speed centrifugation. Pelleted material was dissolved in reticulocyte translation buffer supplemented with 10^{-4} M each ATA and emetine. Unfractionated translation reactions (Total; solid black bars), dissolved pellet fractions (RP; stippled bars) and post-ribosomal supernatants (PRS; hatched bars) were incubated in the presence and absence of microsomal membranes and ATA and emetine as described. Samples were immunoprecipitated, prepared, subjected to SDS-PAGE and radiolabelled bands were viewed by autoradiography. Bands were quantified by scanning laser densitometry. For each fraction the percentage of precursor chains which had been converted to the glycosylated chains by incubation with microsomal membranes was calculated.

A.


Fractionation of Translation Products
Fraction of Total Translation
100
80
60
40
20
0
SG/BstEII
SG/BstEII + pur
SGB
Sup
Pellet

B.

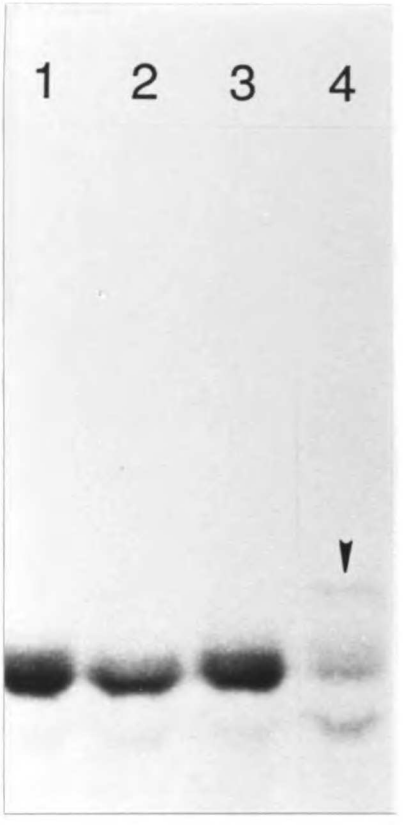

Translocation of Fractionated Products
% glycosylated
40
30
20
10
0
SG/BstEII
SG/BstEII + pur
SGB
Total
PRS
RP

species following incubation with microsomal membranes in the presence of inhibitors of elongation. Results are summarized in Figure 5-3B. As can be seen, SG/BstEII products in the post-ribosomal supernatant fraction were not translocated, while products in the pelleted fraction were translocated as efficiently as those in the unfractionated translation mix. Failure to observe translocation from the post-ribosomal supernatant fraction was not a consequence of the low yield in this fraction since treatment with puromycin released 60% of the translation products to the post-ribosomal supernatant and, again, no translocation from this fraction was seen. In contrast, SGB translation products in the post-ribosomal supernatant were translocated equally as efficiently as those in the pellet or unfractionated mix. Thus it appears that this truncation product of SGI, 99 amino acids in length, 39 amino acids shorter than SG/BstEII, is capable of translocating post-translationally independent of an association with the synthesizing ribosome. Therefore, the ribosome does not appear to be required for the process of translocation per se.

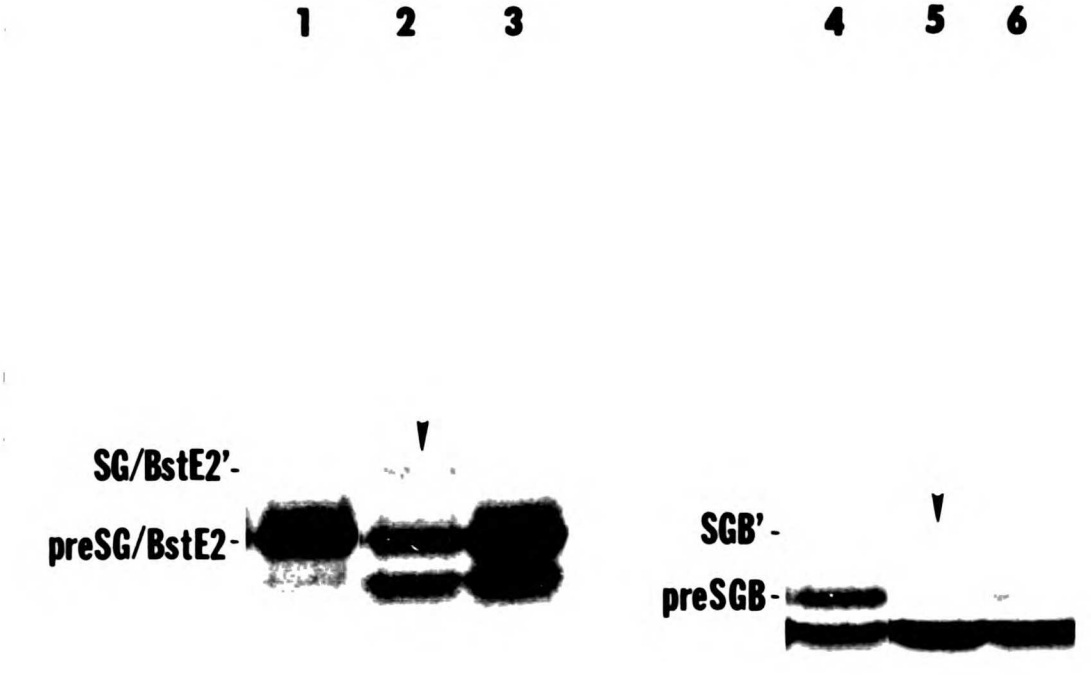
The results shown in figure 5-4 demonstrate that the ribosome independent translocation of SGB is not a spontaneous process, but that it requires ATP and the participation of membrane proteins. Following transcription and translation of pSPSG/BstXI, truncated nascent chains were released from the synthesizing ribosomes by treatment with puromycin followed by high speed centrifugation. When the post-ribosomal supernatant fraction was depleted of ATP prior to the addition of microsomes by treatment with glycerokinase (which is ATP-specific; Hayashi and Lin, 1967; Thorner and Paulus, 1973), neither signal cleavage nor glycosylation occurred (Figure 5-4A, lane 3). Translocation activity could not be restored to glycerokinase treated reactions even when GTP and a regenerating system were added (lane 2). Alkylation of membranes with N-ethyl maleimide (NEM), a treatment which has been shown previously to render membranes translocation-

Figure 5-4. (A) Energy dependence of ribosome independent translocation. Transcription and translation of pSPSG/BstXI was carried out as described. Translation reactions were subjected to high-speed centrifugation and the pellets dissolved in reticulocyte lysate translation buffer adjusted to 5% glycerol, 10-4 M each ATA and emetine. Following a 15 minute incubation with 1 mM puromycin at 24°C, the sample was again subjected to high-speed centrifugation and the post-ribosomal supernatant collected. Aliquots of this fraction were incubated with (lanes 2 and 3) or without (lanes 1 and 4) glycerokinase and were subsequently incubated in the presence (lanes 2-4) or absence (lane1) of microsomal membranes with (lanes 1, 2, and 4) or without (lane 3) supplementation with an energy cocktail consisting of 1 mM ATP, 1 mM GTP, and 0.8 mM creatine phosphate. Samples were prepared and analyzed as usual. (B) Incubation of ribosome dependent and ribosome independent substrates with NEM-treated membranes following inhibition of protein synthesis. SGB and SG/BstEII were expressed in vitro as described, translation inhibited by incubation with 10^{-4} M ATA followed by addition of 10^{-4} M emetine. Membranes were treated with NEM and mock treated as described in Chapter 7. Total translation mixes were incubated in the presence of mock treated membranes (lanes 2 and 5), NEM-treated membranes (lanes 3 and 6), both at 5 A_{280} U/ml, or in the absence of any membranes (lanes 1 and 4). Samples were prepared and analyzed as usual.

A.



B.

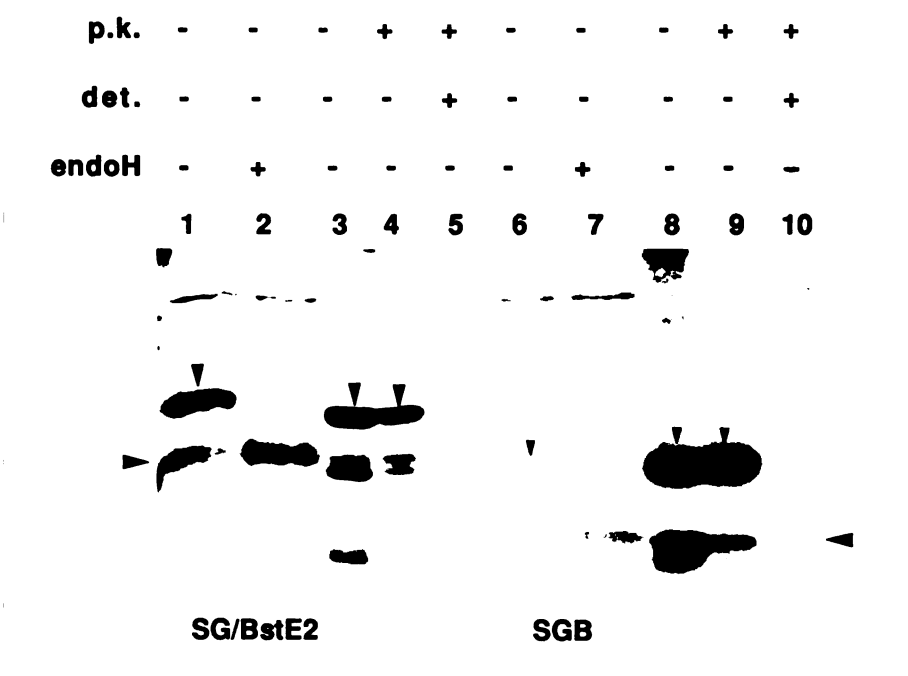


incompetent (Gilmore et al., 1982a; Hansen et al., 1986; Hortsch et al., 1986), inhibited post-translational translocation of both ribosome dependent (SG/BstEII; Fig. 5-4 B, lanes 1-3) and ribosome independent (SGB; Fig. 5-4 B, lanes 4-6) substrates. We cannot rule out the possibility that core-oligosaccharide transferase or signal peptidase is inhibited by alkylation of the cytoplasmic face of the microsomes but protease protection of precursor proteins incubated with NEM-treated microsomes was not seen (data not shown).

As a first step toward understanding the coupling of translation with translocation across the ER membrane in living cells, we studied the translocation of ribosome dependent and ribosome independent translocation substrates in *Xenopus* oocytes. Injection of heterologous messenger RNAs or cell-free transcription products of cloned genes into *Xenopus* oocytes results in efficient expression (Tabe et al., 1984), appropriate subcellular localization (Zehavi-Willner and Lane, 1977) and often post-translational modification (Colman et al., 1984; Soreq, 1984) of the encoded proteins. In addition, microinjection of nuclear proteins into *Xenopus* oocytes has been used successfully to study the process of nuclear localization (Feldherr et al., 1984). Therefore, to achieve both a temporal and spatial separation of translation from translocation in vivo we first translated polypeptides in vitro and then injected them into the cytoplasm of *Xenopus* oocytes.

Figure 5-5 demonstrates that, when expressed in *Xenopus* oocytes, both SG/EII and SGB were (co-translationally) localized to the ER lumen. Following injection of SP6 transcription products of pSPSG/BstEII or pSPSGB, appropriately sized translation products were generated, which were endo H sensitive (lanes 1, 2, 6, 7), cleaved by signal peptidase (positions of cleaved, unglycosylated chains are indicated by sideways arrowheads) and protected from exogenously added protease (lanes 3, 4, 8, 9) except in the presence of detergent (lanes 5, 10). Neither protein was secreted

Figure 5-5. Subcellular localization and processing of SG/BstEII and SGB expressed in *Xenopus laevis* oocytes. Plasmids, pSPSGB and pSPSGI/BstEII were transcribed in vitro. Cell-free transcripts were microinjected into Xenopus laevis oocytes with [^{35}S]-methionine. Following a four-hour incubation vesicles were prepared from the oocytes as described in Chapter 7. One aliquot of each injected group (SGB or SG/BstEII) was adjusted to 1% Triton X-100 for immunoprecipitation and subsequent endo H digestion (lanes 2 and 7) or mock digestion (lanes 1 and 6). Equal aliquots of the remainder were incubated in the presence (lanes 4, 5, 9, 10) or absence (lanes 3 and 8) of 0.3 mg/ml proteinase K, some in the presence of 1% Triton (lanes 5 and 10) for 3 hours at 0°C. Protease treatment was stopped as described, and samples immunoprecipitated and prepared as usual. Following SDS-PAGE, the gel was fluorographed and bands views by autoradiography. Large downward pointing arrowheads in lanes 1, 3, and 4 indicate the position of SGI/BstEII' which is glycosylated and protected from protease. Small arrowheads in lanes 6, 8, and 9 indicate SGB' which is also glycosylated and protected from protease. Sideways arrowheads indicate the positions of the unglycosylated chains whose signals have been cleaved.

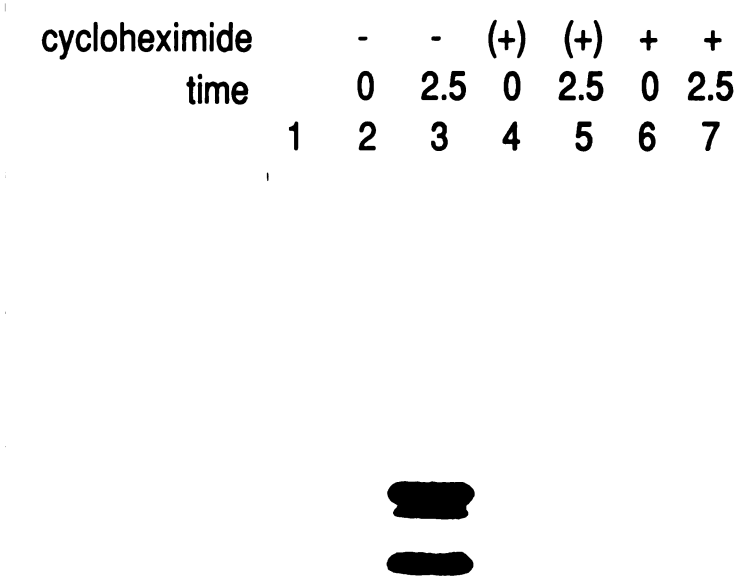

p.k. - - - + + - - - + +
det. - - - - + - - - - +
endoH - + - - - - + - - -
1 2 3 4 5 6 7 8 9 10
SG/BstE2
SGB

into the medium (data not shown). Having determined that both products could be recognized and appropriately processed by translocation machinery upon expression in the oocyte, we proceeded to investigate the translocation of previously synthesized translocation substrates in *Xenopus* oocytes.

To study elongation independent translocation in living cells, polypeptides were first translated in cell-free reactions, treated with inhibitors of initiation and elongation and then microinjected into *Xenopus laevis* oocytes. Since translation reactions contain both translatable transcript and [^{35}S] methionine, it was necessary to first ensure that continued synthesis by the oocyte from the injected template could not occur. Figure 5-6 shows the results from an experiment in which translation mixes of SGI which had been allowed only to initiate by incubation at 24°C for 2 minutes were co-injected (in ~30-50 nl volumes) with 1mM cycloheximide either into oocytes which had been previously injected with cycloheximide several hours earlier or oocytes which had not been treated. As can be seen in lane 1, no translation products were observed in the translation mix which was used for injection, nor were any products specifically immunoprecipitated immediately following microinjection under any condition (lanes 2, 4, 6). Either preinjection or co-injection with cycloheximide was sufficient to block translation of the injected transcript by the oocytes (compare lanes 5 and 7 to lane 3). Immunoprecipitable translation products can be seen in lane 3 after a 2.5 hour incubation, following injection of translation mix in the absence of cycloheximide into untreated oocytes. In the following experiments all translation products were co-injected into *Xenopus* oocytes with 1 mM cycloheximide.

The ribosome dependent translocation substrate, preSG/BstEII, was prepared as described previously by transcription of pSPSGI which had been digested with restriction endonuclease, BstEII, and subsequent translation in rabbit reticulocyte

Figure 5-6. Inhibition of translation of microinjected transcript by *Xenopus laevis* oocytes. In vitro translation of pSPSGI was allowed to incubate just 2 minutes at 24°C and immediately frozen at -80°C. After thawing, the translation mix was split in half and cycloheximide added to 1mM to one (lanes 4-7). The untreated translation mix was injected into untreated oocytes (lanes 2, 3) and the translation mix supplemented with 1mM cycloheximide was injected into either untreated oocytes (lanes 6, 7) or oocytes which had been injected 3 hours earlier with 1 mM cycloheximide (lanes 4, 5). For each group of oocytes half were collected immediately after injection and frozen and half were incubated for 2.5 hours at 19°C. Oocytes were homogenized and immunoprecipitated as usual and a sample of the untreated translation mix was also immunoprecipitated (lane 1). Samples were prepared and subjected to SDS-PAGE and bands viewed by autoradiography of the fluorographed gel.


cycloheximide - - (+) (+) + +
time 0 2.5 0 2.5 0 2.5
1 2 3 4 5 6 7

lysate. Initiation was stopped by addition of aurin tricarboxylic acid (ATA) and further elongation inhibited with 1 mM cycloheximide. Following microinjection into *Xenopus* oocytes, translation products were immunoprecipitated from oocyte homogenates after various periods of incubation and localization of immunoprecipitable products to the ER lumen was assayed by the standard assays of protease protection and endo H sensitivity.

Immediately following microinjection, time 0, only precursor forms were seen in the oocyte, which appear to be identical to cell-free translation products (Figure 5-7, compare lanes 1 and 3). Following a 30 minute incubation, the glycosylated form, SG/BstEII' was observed (compare lanes 2 and 4), which was endo H sensitive (lanes 7 and 8) and protected from exogenously added protease (lanes 5 and 6). These data demonstrate that SG/BstEII was fully translocated across the ER membrane following microinjection into the cytoplasm of the oocytes. Glycosylation of the injected ribosome-associated polypeptide was seen at the earliest time point examined, that is fifteen minutes following injection (data not shown).

Translocation of SG/BstEII in *Xenopus* oocytes was dependent upon both the signal sequence and the ribosome (Figure 5-8). Following injection of reticulocyte translation products of SG3/BstEII (the translation product is identical to that of SG/BstEII except that it lacks the entire β-lactamase signal sequence), the polypeptide was neither glycosylated (Figure 5-8 A, lanes 1-4) nor protected from proteolysis (lanes 5 and 6). Likewise, treatment of the translation reaction of preSG/BstEII with puromycin prior to microinjection also abolished translocation (Figure 5-8 B). Thirty minutes following injection, no glycosylated products were generated (lanes 1, 2) nor were the injected products protected from exogenously added protease (lanes 3, 4). No glycosylation was seen even as many as four hours following injection (data not shown). Thus, localization to the ER and processing of this microinjected "nascent"

Figure 5-7. Subcellular localization of SG/BstEII following microinjection of cell-free translation products into *Xenopus* oocytes. SG/BstEII was prepared by cell-free transcription and translation, as previously described. The translation reaction was adjusted to 1mM cycloheximide and injected into 60 Xenopus laevis oocytes (~40 nl per oocyte). The first ten injected oocytes were collected and frozen immediately. The remaining oocytes were incubated at 19°C for 30 minutes. Vesicles were prepared as described in Chapter 7 and divided into five even aliquots, 2 of which were incubated at 0°C in the presence (lane 6) or absence (lane 5) of 0.3 mg/ml proteinase K. After 3 hours reactions were treated wtih 1 mM phenyl-methyl sulfonyl fluoride (PMSF) and vesicles sedimented through a 0.5 M sucrose cushion containing 1 mM PMSF as described in Chapter 7. Pellets were dissolved and immunoprecipitated. The remaining 3 aliquots were immunoprecipitated and two were subsequently treated with endo H (lane 8) or mock digested (lane 7). A sample of translation product used for microinjection was also immunoprecipitated (lane 1) as was a sample in which membranes had been been present co-translationally. Samples were prepared and applied to SDS-PAGE and bands viewed by autoradiography of fluorographed gels. Position of translocated, glycosylated bands is indicated.

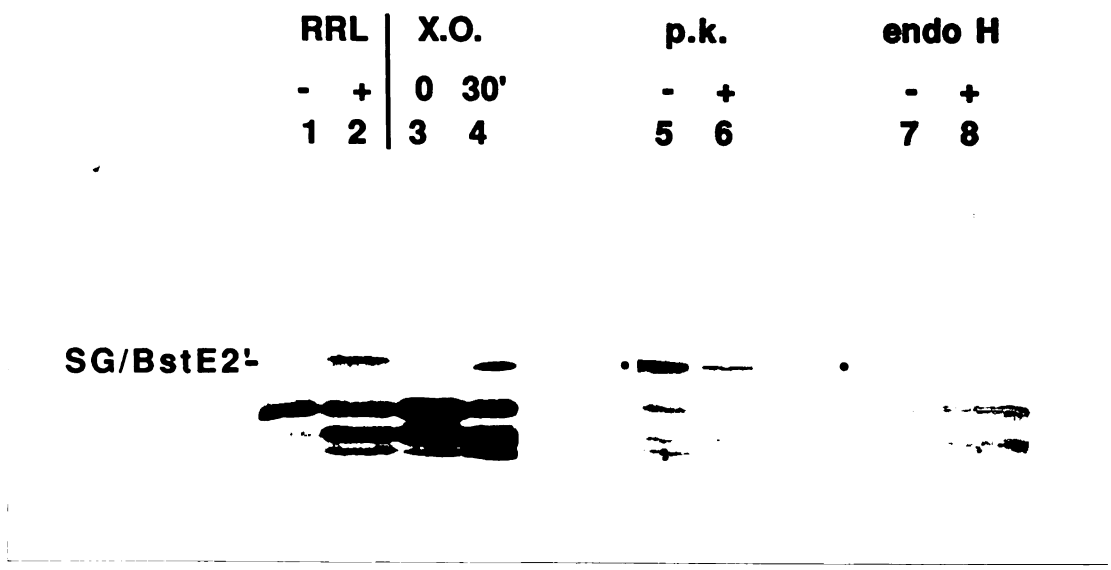

RRL
X.O.
p.k.
endo H
- +
0 30'
- +
- +
1 2
3 4
5 6
7 8
SG/BstE2-

Figure 5-8. Subcellular localization of SG3/BstEII and puromycin-treated SGI/BstEII following microinjection into *Xenopus* oocytes. (A) Subcellular localization of SG3/BstEII following microinjection into *Xenopus* oocytes. Plasmid, pSPSG3 , which encodes globin into which a glycosylation site has been inserted but which lacks the β-lactamase signal sequence coding region present in pSPSGI was linearized with restriction endonuclease BstEII, transcribed and translated as usual. Translation products were adjusted to 1mM cycloheximide and microinjected into 40 Xenopus oocytes. The first 10 were collected immediately following injection and frozen. The others were incubated at 19°C for 30 minutes, following which vesicles were prepared as described and divided into three equal aliquots. Two aliquots were incubated in the presence (lane 6) or absence (lane 5) of 0.3 mg/ml proteinase K as described in figure legend 5-7. The other aliquot (lane 4) and the homogenate of the first 10 oocytes (lane 3) were also immunoprecipitated, as was the translation mix used for injection following incubation in the presence ([+]; lane 2) or absence (-; lane 1) of microsomal membranes. Samples were prepared, applied to SDS-PAGE and bands viewed by autoradiography of the fluorographed gel. (B) Translation of SG/BstEII was carried out as described and the translation mix incubated with 1mM puromycin at 24°C for 15 minutes, supplemented with 1mM cycloheximide and microinjected into 50 Xenopus oocytes. The first 10 oocytes were collected immediately following injection and frozen. The remainder were incubated for 30 minutes at 19°C following which vesicles were prepared as usual and divided into 4 equal aliquots. Three were incubated in the presence (lanes 4 and 5) or absence (lane 3) of 0.3 mg/ml proteinase K, one with 1% Triton X-100 (lane 5) as described. All samples were immunoprecipitated, prepared and applied to SDS-PAGE and bands viewed by autoradiography of the fluorographed gel.

RRL		X.O.		p.k.	
-	(+)	0	30'	-	+
1	2	3	4	5	6

[redacted]

0	30'	-	+
1	2	3	4

[redacted]

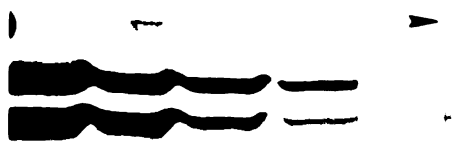
polypeptide appears to occur via the translocation pathway which has been described in cell-free systems (Chapter 4).

To determine whether a protein which could translocate across microsomes in a ribosome independent fashion in cell-free systems could do so in a living cell, we injected cell-free translation products of pSPSGB. The results are depicted in Figure 5-9. As can be seen, immediately after injection immunoprecipitated products appeared to be identical to cell-free translation products (compare lanes 1 and 3). After a 30 minute incubation, no glycosylated forms of this protein were seen (lane 4), nor was the unprocessed form protected from protease (lanes 5, 6). Even following up to four hours of incubation, no glycosylated form was seen (data not shown). These same translation products were, however, translocated across microsomal membranes and glycosylated in vitro (lanes 1 and 2). Thus, this polypeptide which is translocated co-translationally in vivo (see Fig. 5-5, lanes 6-10) and which can translocate post-translationally in a cell-free system does not appear to be translocated post-translationally in intact cells. Failure to observe translocation does not appear to be due to a more rapid degradation of the microinjected polypeptide relative to the ribosome-associated polypeptide since bands from both products appear to be of roughly equal intensity up to 2 hours post-injection (data not shown). Rather, it appears that the completed polypeptide is unable to be targeted and/or translocated in the oocyte.

The apparent lack of ribosome independent translocation in *Xenopus* oocytes suggests that ribosome independent translocation may be a feature only of fractionated cell-free systems and not physiologically significant.

Figure 5-9. Subcellular localization of SGB following microinjection into *Xenopus laevis* oocytes. Plasmid, pSPSGB was transcribed and translated in reticulocyte lysate as usual. Following incubation with 10^{-4} M ATA, the translation mix was adjusted to 1mM cycloheximide and either microinjected into oocytes (lanes 3-6) or incubated in the presence (lane 2) or absence (lane 1) of microsomal membranes. Of the 80 oocytes injected, 20 were collected immediately after microinjection, while the remainder were incubated for 30 minutes at 19°C. Following incubation, vesicles were prepared as described and divided into three equal aliquots. Two were the incubated in the presence (lane 6) or absence (lane 5) of proteinase K as described in figure legend 5-7. All samples were then immunoprecipitated, applied to SDS-PAGE and bands viewed by autoradiography of the fluorographed gel.

RRL		X.O.		p.k.	
-	(+)	0	30'	-	+
1	2	3	4	5	6



Discussion

The data presented in Chapter 4 suggested that translocation across microsomal membranes could proceed independent of ongoing protein synthesis provided the nascent chain remained associated with the ribosome (see also Caulfield et al., 1986; Chao et al., 1987), indicating a role for the ribosome in translocation distinct from that of protein synthesis. However, translocation of several completed and released polypeptides has been reported to occur in the absence of ribosomes (Zimmermann and Mollay, 1986; Hansen, et al., 1986; Schlenstedt and Zimmermann, 1987). Since ribosome independent substrates are in general quite small, we attempted to generate such a substrate by truncation. We found that while most sizes of most chains were dependent on association with the synthesizing ribosome for translocation, one small polypeptide was not. The translocation of completed and released chains (ribosome independent translocation) of SGB (a 99 amino acid fusion protein consisting of the β-lactamase signal sequence fused to the first 68 amino acids of α globin into which a concensus sequence for N-linked glycosylation had been inserted) suggests that the ribosome is not required for the process of translocation per se. Rather, the role of the ribosome may be to maintain nascent chains in a "translocation competent state" and/or to act as a ligand for targeting to the ER membrane, most likely in conjunction with SRP.

From these results it seems reasonable to suggest that nascent chains may pass through sequential stages of elongation in which translocation competence is first independent of, and subsequently maintained by, the ribosome. Finally, at a certain point in chain growth translocation competence may be lost. The "translocation competent state" may be considered in molecular terms as accessibility of the signal sequence to its receptor(s) in the ER membrane. Thus, chains which can translocate independent of the ribosome may do so because their signal sequences are capable of

interacting directly with a signal sequence receptor in the ER membrane (Wiedmann, et al., 1987b). Other proteins which can translocate independent of the ribosome do not require SRP or SRP receptor (Watts, et al., 1983; Zimmermann and Mollay, 1986; Schlenstedt and Zimmermann, 1987; Wiech et al., 1987). Whether SRP is involved in the ribosome independent translocation process described here remains to be determined. However, no affinity of SRP for signal sequences of polypeptides released from the ribosome has yet been demonstrated (Krieg et al., 1986; Wiedmann et al., 1987a). Thus the ribosome dependence of longer polypeptide chains for translocation may reflect their inability to interact directly with the signal sequence receptor in the ER membrane. The ribosome may act as a ligand for SRP, allowing it to bind the signal sequence and deliver it to the ER membrane signal sequence receptor. Ribosome dependent translocation does utilize SRP and SRP receptor (Mueckler and Lodish, 1986a; R. E. Rothman and V. R. Lingappa, unpublished observations). Translocation competence of very long nascent chains may be lost altogether (Chao et al., 1987) because the signal sequence is obscured by the long polypeptide chain preventing SRP from binding, alternatively the chain may be properly targeted to the ER membrane but unable to be translocated because of its large size.

An additional function of the ribosome (which may be interrelated with its proposed role as a ligand for SRP) may be to maintain the nascent chain in a relatively unfolded conformation which might be necessary for translocation. Post-translational translocation of proteins across other membrane systems requires that the substrates be at least partly unfolded (Randall and Hardy, 1986; Eilers and Schatz, 1987).

It appears likely that the ribosome dependent and ribosome independent translocation described in this chapter occur via the same mechanism, utilizing a

common subset of translocation receptors in the microsomal membrane. Since SGB is a fusion protein consisting of the β-lactamase signal sequence and the first half of the cytoplasmic protein globin, it is assumed that translocation of this molecule is entirely a consequence of the signal sequence and not of any specialized property of the translocated domain (Lingappa et al., 1984). Thus it would seem unlikely that a truncation product of a larger protein whose translocation proceeds via the classical pathway, involving SRP (V. R. Lingappa, unpublished observations), would possess information to utilize an independent transport mechanism. However, we cannot rule out the possibility that these manipulations fortuitously "unmask" cryptic properties of this fusion peptide which would permit utilization of a (putative) separate mechanism (Hurt and Schatz, 1987). A mechanism for the membrane insertion of prepromelittin has been suggested for which a role for the mature region of the protein in its mode of membrane insertion has been suggested (Müller and Zimmermann, 1987) and which may require a trypsin and NEM sensitive membrane protein not required for classical (SRP-dependent) co-translational translocation (Zimmermann and Mollay, 1986). This issue can be addressed by experiments using ribosome independent and ribosome dependent substrates to compete for translocation across microsomal membranes. Should the ribosome dependent and ribosome independent substrates described here be shown to utilize the same pathway for translocation, they should be useful tools for the dissection of steps in membrane recognition and translocation and the determination of the molecular requirements for those steps (Connolly and Gilmore, 1986).

Our results following microinjection of in vitro synthesized polypeptides into *Xenopus* oocytes also suggest that translocation requires a functional association of the polypeptide chain with the ribosome even in the absence of chain elongation. In addition, it appears that ribosome independent translocation may not occur in

and may be a feature only of reconstituted cell-free systems. Ribosome-associated nascent chains (SG/BstEII) were rapidly and efficiently targeted to the ER membrane and translocated as determined by signal processing, glycosylation and protease resistance only when associated with the ribosome. A protein which translocates across microsomal membranes in vitro following synthesis and release from the ribosome (SGB) did not appear to be translocated following injection into the cytoplasm of *Xenopus* oocytes. One explanation for this finding, considering the requirements for known targeting components for ribosome independent translocation of other proteins (Zimmermann and Mollay, 1986; Schlenstedt and Zimmermann, 1987), would be that without the aid of SRP, chains cannot be efficiently targeted to the ER membrane in vivo. This inefficiency may be overcome in vitro by increasing the concentration of microsomal membranes. This is consistent with our results which indicate that efficient ribosome independent translocation requires a higher membrane concentration than does ribosome dependent post-translational translocation (data not shown). We cannot exclude the possibility, however, that translation products of SGB are rendered translocation incompetent following introduction into the oocyte cytoplasm perhaps by adopting a different conformation in which the signal sequence is obscured. We also cannot rule out the possibility that ribosome independent translocation in vitro is an artifactual consequence of microsomal membrane preparation for in vitro assays.

Another possible explanation for the observed lack of translocation of SGB translation products in oocytes may be that ribosome independent translocation requires participation of a labile protein which is not required for ribosome dependent translocation. Treatment of the cell with cycloheximide would prevent synthesis of such a factor. This explanation seems unlikely since the event can occur in vitro in the presence of inhibitors of protein synthesis and using fractionated

components which have been treated with nuclease; such a labile protein would be unlikely to persist in these systems. However, this possibility can be addressed directly by treating the translation products to be injected with a nuclease which could be inactivated prior to injection and thus prevent further translation of the injected polypeptide in the oocyte without blocking expression of endogenous oocyte proteins.

The data presented here suggest that the reason translocation across the ER membrane is coupled to protein synthesis is because the synthesizing ribosome plays a crucial role in nascent chain targeting and/or translocation which is distinct from its role in chain elongation.

Chapter 6

SUMMARY

We have used molecular genetics techniques to customize translocation substrates to probe the mechanism of translocation of proteins across the ER membrane. By studying the translocation of these substrates under defined conditions either in cell-free translation systems supplemented with microsomal membranes or in intact cells, insights into several aspects of the translocation process have been gained.

Expression of a fusion protein containing an internalized signal sequence

We engineered a fusion plasmid which encoded the initial 109 amino acids of α globin followed by the entire preprolactin sequence. The translocation of this fusion protein was studied following expression either in a cell-free translation/translocation system or in *Xenopus* oocytes. Surprisingly, translocation of both flanking protein domains was observed (albeit with different efficiencies) and in most cases the signal sequence appeared to be cleaved accurately. The cleavage products genereated were mature prolactin and the 109 amino acid globin domain with the cleaved signal sequence at its carboxy terminus. The presence of this relatively large, immunoreactive domain now attached to the signal sequence provided a marker for the cleaved signal sequence and allowed us to follow its fate. While we cannot rule out the possibility that attachment of this bulky polypeptide altered the fate of the signal sequence, our results suggest that the signal sequence is translocated itself.

Disposition of cleaved signal sequences relative to the membrane

Approximately 20% of the globin-signal cleavage products were localized to the microsomal lumen. These chains were entirely luminal as determined by complete resistance to exogenously added proteases. Treatment of microsomal membranes or vesicles from oocytes with sodium carbonate to release content or

peripheral proteins of the vesicles (Fujiki et al., 1982) resulted in release of all globin-signal products from the membranes. Thus, the cleaved signal sequence does not reside in the bilayer as has been proposed (Engelman and Steitz, 1981; von Heijne and Blomberg, 1979; Briggs et al., 1985; 1986).

Functional definition of a bona fide signal sequence

When expressed in *Xenopus* oocytes both protein domains of the globin-prolactin fusion protein were translocated. However, a significant fraction of chains were translocated without being cleaved by signal peptidase. These chains were localized completely in the ER lumen and were extracted with carbonate treatment. Thus, even uncleaved signal sequences can be translocated across the membrane. The luminal localization of the signal sequence is a functional distinction of a bona fide signal sequence. Signal sequences have previously been functionally defined as amino acid sequences which can direct translocation of a normally cytoplasmic protein (Lingappa et al., 1984; Kaiser et al., 1987) or as sequences which bind to the signal recognition particle (SRP) (Friedlander and Blobel, 1985). However, it has been shown recently that "stop transfer" sequences (Mize et al., 1986; Zerial et al., 1987) and even "random" hydrophobic amino acid sequences (Zerial et al., 1987) can facilitate SRP-dependent nascent chain translocation . However, these sequences are not localized to the ER lumen themselves, distinguishing them from classical signal sequences.

Cleaved signal peptides are rapidly degraded in living cells

While both the prolactin and globin-signal cleavage products of the globin-prolactin fusion protein were quite stable in cell-free systems, the globin-signal fragment was very short-lived in the oocyte. We cannot rule out the possibility that its degradation may be a non-specific consequence of the attached hydrophobic signal

sequence. However, its extremely rapid degradation relative to the other "foreign" fusion protein products (including those whose signals were not cleaved) in the cytoplasm suggests that a clearance mechanism may exist for cleaved signal peptides in intact cells which is not reconstituted in cell-free systems.

Translocation of a previously synthesized protein domain

The translocation of the globin domain, whose synthesis is complete by the time the signal sequence emerges from the ribosome, was a surprising observation. This 109 amino acid polypeptide domain must have begun folding by the time the signal sequence was exposed and the polysome targeted to the membrane. Thus, the mechanism for translocation must be able to accomodate a (partially) folded protein domain, either by translocating it in its folded conformation or by denaturing it prior to translocation. In addition, this result demonstrates that translocation of a given polypeptide domain need not occur concomitant with its synthesis and suggested to us that translocation could be uncoupled from ongoing chain elongation experimentally.

Dissociation of translocation from nascent chain elongation

To uncouple translocation from protein synthesis, expression plasmids encoding secretory or transmembrane proteins were truncated within their coding regions 5' to the termination codon by restriction endonuclease digestion. Expression of these plasmids yielded nascent polypeptide chains which remained associated with the ribosomes as peptidyl tRNAs. The translocation of such substrates was studied both in cell-free systems and in intact cells following pharmacologic inhibition of elongation or puromycin-induced termination.

A role for the ribosome

An association of the nascent chain with the ribosome appears to be a general requirement for translocation, even in the absence of chain elongation (ribosome dependent translocation). This observation suggests an explanation for the coupling between translocation and translation. The nascent chain is not extruded across the bilayer as it is synthesized by the membrane bound ribosome. Instead, coupling between translocation and protein synthesis appears to reflect a role for the ribosome in translocation which is distinct from its role in protein synthesis.

A short fusion protein was generated which was translocated following its release from the ribosome (ribosome independent translocation). This finding suggests that an association of a polypeptide chain with the ribosome is not required for the process of translocation per se. Rather, the ribosome may play a role, probably in conjunction with SRP, in maintaining the polypeptide chain in a translocation competent state, presumably by preventing the signal sequence from being obscured by the nascent chain and/or by preventing folding of the nascent chain.

Translocation is not a spontaneous process

The uncoupling of the processes of translocation and nascent chain elongation allowed us to determine directly whether the translocation process required energy substrates. Depletion of energy substrates abolished both ribosome dependent and ribosome independent translocation, as did alkylation of cytoplasmically exposed microsomal membrane proteins. Thus, translocation across the ER membrane is not a spontaneous process, as has been proposed by some (von Heijne and Blomberg, 1979; Engelman and Steitz, 1981), but instead, requires an energy source and the participation of proteins in the ER membrane.

Prospectus

The use of customized substrates for translocation has provided many insights into the process of translocation. Here we have described two very similar molecules whose requirements for translocation in vitro differ in that one requires an association with the ribosome and one does not. Additional comparison and analysis of these substrates in vitro should permit further dissection of the steps of membrane recognition and translocation and allow determination of the molecular requirements of those steps. In addition, an assay for elongation uncoupled translocation in living cells may help to define the role(s) of the ribosome and/or associated components in vivo.

Chapter 7

MATERIALS AND METHODS

MATERIALS

All restriction endonucleases, nuclease Bal 31, mung bean nuclease, calf intestinal alkaline phosphatase, SP6 RNA polymerase, T4 DNA ligase, and Klenow fragment of *E. coli* DNA polymerase I, *E. coli* glycerol kinase, trypsin, trasylol, puromycin, cycloheximide, emetine, aurintricarboxylic acid (ATA), nucleoside triphosphates, calf-liver tRNA, creatine phosphokinase, and creatine phosphate were from Boehringer Mannheim Diagnostics, Inc., Houston, TX, or from New England BioLabs, Beverly, MA. RNase inhibitor was from Promega Biotec, Madison, WI; staphylococcal protein A-Sepharose was from Pharmacia, Inc., Piscataway, NJ; rabbit anti-human hemoglobin serum was from Cappel Laboratories, Cochranville, PA; rabbit anti-ovine prolactin was from United States Biochemical Corp., Cleveland, OH; proteinase K was obtained from Merck, RFG; endoglycosidase H (Endo H) and [^{35}S]-methionine (translation grade, >800 Ci/mmol) were from New England Nuclear, Boston, MA; Nikkol (octa-ethleneglyco-mono-n-dodecyl ether, a nonionic detergent) was from Nikko Chemicals Co., Ltd., Tokyo, Japan. *Xenopus laevis* were obtained from Nasco, Fort Atkinson, WI. Plasmids, pSPBP3 and pSPBP4 (aka pSPbPI) were constructed by William Hansen, Department of Biochemistry and Biophysics, University of California at San Francisco, using bovine preprolactin cDNA (Sasavage, et al., 1982). All globin encoding plasmids were derived from pMC18 (Yost, et al., 1983).

METHODS

Construction of SP6 expression plasmids

Construction of Globin-Prolactin fusion Plasmid, pSPGP1

As depicted in Fig. 1, plasmid pSPBP3, containing the entire coding region for bovine preprolactin, was linearized with NcoI in the presence of ethidium bromide and the

overhang filled in by treatment with *E. coli* DNA polymerase I Klenow fragment in the presence of all four dNTPs. The plasmid was then cut with PstI and the 850-base pair (bp) fragment containing the preprolactin coding region was purified on, and eluted from, a 1% low melting point agarose gel. Plasmid pSPG1E was cut with BstE II, the 5' overhang filled in as described above, then cut with PstI and the 3-kilobase (kb) vector gel purified. The purified pSPBP3 fragment and pSPG1E vector were treated with T4 DNA ligase. After transformation of *E. coli*, plasmid DNA was prepared from individual ampicillin-resistant colonies and screened by restriction enzyme analysis with NcoI and SphI for appropriate sized fragments (see Fig. 2-1 B). All procedures were essentially as described (Maniatis et al., 1982).

Insertion of N-Linked Glycosylation Site into pSPGP1

To introduce an N-linked glycosylation site into the globin domain of pSPGP1 we used plasmid pSPSG1 (see Fig. 2-5), an exact fusion of the β-lactamase signal sequence and chimpanzee α-globin in which a synthetic oligonucleotide encoding Ala-His-*Asn-Gly-Ser*-Gly-Ser-Gly had been inserted into the BssHII site of the globin coding region. The translation product of this plasmid is translocated across the ER membrane and is core glycosylated in vitro (Figure 4-2). The region encoding the β-lactamase signal sequence was deleted by digestion with NcoI and BglII, treated with Klenow fragment to fill in the 5' overhangs, and recircularized with T4 DNA ligase. The resulting plasmids were used to transform *E. coli*, and plasmid DNA was prepared from individual ampicillin-resistant colonies and screened by restriction enzyme analysis with HindIII. The 430-bp HindIII fragment of the positive clone, pSPSG3, was inserted into pSPGP1 which had been digested to completion with HindIII and treated with calf intestinal alkaline phosphatase to prevent self-ligation. *E. coli* were transformed and DNA prepared from individual ampicillin-resistant

colonies was screened with NcoI to determine the presence and correct orientation of the HindIII insert.

Construction of pSPSGB

Plasmid, pSPSGI was digested to completion with restriction endonuclease, BstXI, and treated with mung bean nuclease to remove single-stranded overhangs. Following digestion with XbaI, the 5' overhang was filled in with Klenow fragment from *E. coli* DNA polymerase I. The plasmid was then recircularized by treatment with T4 DNA ligase. After transformation of *E. coli*, plasmid DNA was prepared from individual ampicillin-resistant colonies and screened by restriction endonuclease analysis with BamHI to determine appropriate deletions.

In Vitro Transcription of SP6 Plasmids

Cesium-purified SP6 plasmids were linearized at sites in the 3' untranslated region (except as noted) by restriction endonuclease digestion, extracted with phenol/chloroform, ethanol precipitated, and dissolved in water. Transcription reactions (Krieg and Melton, 1983) were carried out in 10-100 µl volumes containing 0.2 mg/ml DNA in a reaction mix containing 40 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol, 25 mg/ml calf-liver tRNA, 0.5 mM each ATP, CTP, and UTP, 0.1 mM GTP, 0.5 mM GpppG, 0.9 U/µl RNAsin, 0.9 U/ml SP6 RNA polymerase. Reactions were carried out at 40°C for 1 h. Addition of the cap reagent, GpppG, results in increased expression of in vitro transcripts in cell-free translations and is essential for expression in *Xenopus* oocytes (Contreras et al., 1982).

Rabbit Reticulocyte Lysate Transcription-coupled Translation

In vitro transcription reaction mixtures were used directly in transcription-linked translations in the rabbit reticulocyte lysate cell-free system at a concentration of 20%. Translation reactions were carried out in 10-200 µl vol that contained 40-45% rabbit

reticulocyte lysate (prepared as described; Merrick, 1983), 100 mM KCl, 2 mM $MgCl_2$, 0.9 mM GTP, 1 mM ATP, 10 mM creatine phosphate, 0.2 mM each of 19 amino acids minus methionine, 16 mM Tris HCl (pH 7.5). 0.44 mM spermidine, 2 mM dithiothreitol, 0.4 mg/ml creatine phosphokinase, 0.1 mg/ml calf-liver tRNA, and 1 mCI/ml [^{35}S]methionine. Reaction mixtures were incubated at 24°C for 60 min. unless otherwise noted.

Posttranslational Analyses of Translation Products

Quantitation of protein processing

In vitro transcripts of SP6 plasmids were translated separately in a rabbit reticulocyte cell-free system in the presence or absence of intact dog pancreas rough microsomes (prepared as described, Walter and Blobel, 1983c) or injected into *Xenopus laevis* oocytes. Translation products were immunoprecipitated and separated by SDS PAGE. Bands were localized by autoradiography and quantitated by densitometer scanning, using an LKD 2202 Ultroscan Laser Densitometer from LKB Instruments, Inc., Gaithersburg, MD.

To determine percentage processing of pSPGP1 translation products, intensities of preGSP, GS1, and P1 bands were quantified by densitometry. Percentage processing of preGSP to GS1 forms was determined by [(GS1 x 11/4)/preGSP + (GS1 x 11/4)] x 100 and processing to P1 by [(P1 x 11/7)/preGSP + (P1x11/7)] x 100 to compensate for the differential methionine contents of the three proteins (preGSP contains eleven, P1 seven, and GS1 four). Processing of pre-gGSP was calculated in the same way except that, since there were two gGS forms, gGS and gGS' (in vitro) or gGS* (in oocytes, the values for these two products were summed and used as GS1 above.

Protease protection

Protease protection experiments were performed as follows: After 1 h at 24°C, translation reaction mixtures were chilled on ice, adjusted to 10 mM $CaCl_2$, and divided into equal aliquots of 5 or 10 µl. Some were treated with proteinase K (dissolved in 10 mM $CaCl_2$, 50 mM Tris pH 7.5 and preincubated at 37°C for 15 min) at a final concentration of 0.1-0.4 mg/ml either in the presence or absence of 1% Nikkol or Triton X-100 (nonionic detergents used to disrupt the lipid bilayer). Samples were incubated at 0°C for 1 h (unless indicated otherwise). Protease digestion was stopped by the addition of 2 mM phenylmethylsulfonyl fluoride and samples were immediately transferred to 4-5 vol 1% SDS, 0.1 M Tris-HCl (pH 8.9) which had been preheated to 100°C, then incubated at 100°C for 10-15 min. Samples were diluted 10-20 fold in a solution of 1% Triton, 0.1M Tris pH 8.0, 10 mM EDTA, 100 mM NaCl for immunoprecipitation. Bands from autoradiographs following SDS PAGE were quantified by densitometric scanning. Percentage protection was determined for all bands by [band (+ protease)/band (- protease)] x 100.

Endoglycosidase H Digestion

Endoglycosidase H (endo H) digestion was used to determine core glycosylation of translation products. Endo H removes simple core oligosaccharides from Asn residues, causing a shift to a lower apparent molecular weight upon SDS PAGE. Translation products obtained in the presence of membranes or following expression in *Xenopus* oocytes were immunoprecipitated and eluted from Protein A Sepharose by boiling in 100 µl of 0.1 M sodium citrate pH 5.5, 0.1% SDS for 2 min. Supernates were removed and divided into two aliquots. Endo H was added to one aliquot to a final concentration of 1 µg/ml, and both aliquots were incubated at 37°C for 12 h. After digestion, 10 µg of carrier bovine serum albumin (BSA) was added, and samples

were chilled and adjusted to 15% ice-cold tricholoroacetic acid, precipitates collected by centrifugation, and samples prepared for SDS PAGE as usual.

Membrane Sedimentation and Carbonate Extraction

Cell-free translation products (10 µl) obtained in the presence of 4 A_{280} U/ml dog pancreas rough microsomes, or vesicles prepared from microinjected *Xenopus* oocytes, were diluted 250-fold with either ice-cold 0.1 M sodium carbonate pH 11.5 (Fujiki et al., 1982) or ice-cold 0.25 M sucrose, 5 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM triethanolamine pH 7.4, 0.1 M KCl and incubated at 0°C for 30 min. The samples were centrifuged at 0°C for 1 h at 50,000 rpm in polycarbonate tubes in a Beckman 70.1 Ti rotor (Beckman Instruments, Inc., Palo Alto, CA). The supernatants were removed, the sides of the tube carefully dried with a kimwipe, and the membrane pellets dissolved in 1% SDS, 0.1 M Tris pH 8.9 and diluted 20-fold into immunoprecipitation buffer. The pH of the carbonate samples was adjusted to 7-7.5 with acetic acid, and all samples were immunoprecipitated, prepared and subjected to SDS PAGE and autoradiography as usual.

Immunoprecipitations

Translation mixtures were diluted at least ten-fold in 1% Triton X-100, 0.1M Tris (pH 8.0), 0.1M NaCl, 1 mM EDTA (TxSWB). Antisera were added and incubated at 4°C 8-24 hours after which Protein A Sepharose were added and incubated 2-4 hours with continuous mixing. Samples were then washed three times with chilled TxSWB and two times with 0.1M Tris (pH 8.0), 0.1 M NaCl. Samples were eluted with 1% SDS, 0.1M Tris (pH 8.9), 2mM EDTA, 10% glycerol, 0.01% bromophenol blue, 0.5-1.0 M dithiothreitol, incubated 30 minutes at 37°C and boiled 2-5 minutes prior to application to SDS-PAGE. Immunopreceipitation from oocyte homogenates was essentially the except that yolk platelets and pigment granules were cleared from the

homogenates by spinning 15 minutes at 4°C in a microfuge before addition of antisera to the supernatant and again before addition of Protein A Sepharose.

Alkylation of microsomal membranes

Dog pancreas rough microsomes prepared as described (Walter and Blobel, 1983c) at 50 A_{280} U/ml were incubated at 20°C for 30 minutes in the presence of 5 mM N-ethyl maleimide (NEM) with subsequent addition of dithiothreitol to 10 mM. Control membranes were prepared by adding dithiothreitol prior to incubation with NEM. This treatment has no effect on translocation.

Energy depletion of cell-free translation reactions

Cell-free translation reactions were carried out as described for 20 minutes, initiation was inhibited by10^{-4} M ATA and elongation allowed to continue for an additional10 minutes before addition of emetine to10^{-4} M. The reaction was desalted at 4°C by two successive applications (2 min at 1500 x g) to spin columns consisting of 10 sample volumes of Sephadex G-25 medium equilibrated in 10 mM Tris pH 7.6, 0.1 M KCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, 0.5% bovine serum albumin, 10^{-4} M emetine. Equivalent fractions of the centrifugal eluate were incubated for 20 min in the presence of microsomal membranes (5 A_{280} U/ml) in the presence or absence of energy substrates as described in the legend to Figure 4-4.

High speed centrifugation for preparation of ribosome-depleted supernatants

Translation reactions were performed as described and some treated with 1mM puromycin for 15 min at 24°C to release nascent polypeptides from the ribosome. Following addition of 10^{-4} M each ATA and emetine, reactions were spun at 4°C in a Beckman airfuge at 28 psi for 30 min. Supernatants were very carefully removed and the visible pellets resuspended in an equal volume of a buffer with the same ionic

composition as the reticulocyte lysate translation reaction and supplemented with ATA and emetine to 10^{-4} M.

Microinjection of *Xenopus laevis* oocytes

Oocytes from *Xenopus laevis* were manually dissected, injected as described (Gurdon, et al., 1971, Tabe, et al., 1984) and incubated in modified Barth's saline (MBS: 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 10 mM Hepes (pH 7.6) solution at 18-19°C.

Pulse-chase labeling of translation products of *Xenopus laevis* oocytes

Oocytes were injected with SP6 transcripts, preincubated 5-12 hours, and pulse-labeled for 1-3 hours in MBS containing 2-5 mCi/ml [^{35}S] methionine. Labeling medium was then removed, oocytes were washed with MBS and incubated subsequently in MBS containing 10% fetal calf serum, 20 mM methionine, and 10^{-4} M cycloheximide. Addition of fetal calf serum is intended to inhibit the action of oocyte proteases which may be secreted into the medium.

Preparation of vesicles from microinjected *Xenopus* oocytes

Labeled oocytes were homogenized a ground glass homogenizer at 4°C in an isoosmotic buffer: 0.25 M sucrose, 0.1 M KCl, 50 mM triethanolamine pH 7.5, 5 mM $MgCl_2$, 10 mM $CaCl_2$, 1 mM dithiothreitol (10-30 µl/oocyte). Homogenates were then used in protease protection experiments or vesicles sedimented or extracted with sodium carbonate as described.

Recovery of vesicles from oocyte homogenates

Vesicles were prepared from *Xenopus* oocytes following injection of cell-free translation products. Equal aliquots were incubated at 0°C for 120 min in the presence or absence of 0.3 mg/ml proteinase K. Samples were adjusted to 1 mM phenyl methyl sulfonyl fluoride (PMSF), layered over a 50 µl cushion composed of 0.5 M sucrose, 50 mM triethanolamine pH 7.5, 5 mM $MgCl_2$, 0.1 M KCl, 1 mM dithiothreitol, 1mM PMSF and spun at 4°C in a Beckman airfuge at 28 psi for 10 min. The supernatant and cushion were removed and the pellet dissolved in immunoprecipitation buffer.

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