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PROTEIN TRANSLOCATION ACROSS THE MEMBRANE OF THE ENDOPLASMIC RETICULUM

by

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DISSERTATION

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PROTEIN TRANSLOCATION ACROSS THE MEMBRANE OF THE ENDOPLASMIC RETICULUM

Pablo D. Garcia

Abstract

The understanding of the mechanism of protein translocation across the membrane of the endoplasmic reticulum (ER) has been approached in the present work by studying this process in both lower and higher eukaryotic organisms. A cell-free system that utilizes components derived from the yeast Saccharomyces cerevisiae and that faithfully reproduces the process in vitro has been developed. In this system translocation of the precursor for the yeast pheromone α-factor can occur efficiently post-translationally, i.e. after the polypeptide has been released from the ribosome. Since this made it possible to study protein translocation in the absence of translation, questions concerning the energy requirements for the process were addressed. Thus, it was found that translocation requires the hydrolysis of ATP and is not affected by the presence of proton and potassium ionophors.

In contrast to the yeast ER membrane, translocation in the mammalian system was shown to be very inefficient when translation was inhibited by cycloheximide. Under this condition only full-length polypeptides that remained associated with the ribosome as nascent chains were translocated. Signal recognition particle (SRP) and SRP receptor, known to be involved in targeting ribosomes synthesizing secretory proteins to the ER membrane, were required in this reaction. Thus, this result indicates that coupling of the translation and translocation machineries is an absolute requirement for protein translocation across the mammalian ER membrane. This reaction

made it possible to analyze translocation of long nascent chains in the absence of their elongation and, thus, study the energy requirements for protein translocation across mammalian ER membranes. I found that the hydrolysis of both ATP and GTP are required for translocation and have determined that these requirements occur after signal recognition by SRP on the ribosomes.

Peter Walter

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

APPENDIX 2:

CHAPTER 1

INTRODUCTION:

PROTEIN TRANSLOCATION ACROSS THE MEMBRANE OF THE ENDOPLASMIC RETICULUM

BACKGROUND

One of the fundamental steps during the evolution of living cells was the acquisition of the plasma membrane, a barrier by which cells could be isolated from their surroundings. This allowed cells to control their intracellular environment and, thus, establish the necessary conditions for the many different metabolic reactions that are essential for life itself. Such an insulating barrier must have some very specific characteristics. Somehow the elements from the environment that are required for cellular metabolism must be captured and selectively internalized. Also, through this barrier cells have to sense the conditions of their surroundings and determine the adjustments required to preserve their optimal intracellular environment. Thus, the plasma membrane evolved as a dynamic boundary between cells and their surrounding, through which signals and compounds are selectively transfered in both directions. Ultimately, cells began to use this membrane to compartmentalize some of their metabolic processes. For example, performing certain potentially harmful reactions outside of the cells is of clear advantage. Eukaryotic cells carried the principle of using membrane barriers to create and control particular environments one step further. They evolved subcellular organelles surrounded by barriers similar to the plasma membranes in which different metabolic functions are compartmentalized.

The evolution of the plasma membrane and its use to compartmentalize different functions resulted in new problems for which solutions must have evolved concomitantly with the membranes themselves. First, since proteins are synthesized in the cytoplasmic compartment, they must be transported outside of the cell or into the intracellular organelle in which their function is performed. Second, since large hydrophilic proteins cannot

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diffuse freely across the hydrophobic core of biological membranes, a mechanism for the specific translocation of these macromolecules across this barrier must exist. Thus, specific proteins must be sorted from the ones that remain in the cytoplasm, targeted to their correct destination and selectively translocated across the membrane in question. In eukaryotic cells, the complexity of this problem is increased due to the multiple destinations. Therefore, protein sorting and targeting mechanisms for the different organelles must exist. Understanding of the mechanism by which these fundamental problems are solved has been the purpose of intensive research in recent years. Consequently, the mechanism of protein secretion in prokaryotes (Benson et al, 1985; Lee & Beckwith, 1986; Randall et al, 1987) and eukaryotes (Walter & Lingappa, 1986; Burgess & Kelly, 1987; Pfeffer & Rothman, 1987), protein import into mitochondria (Tzagollof & Myers, 1986; Roise & Schatz, 1988), chloroplast (Schmidt & Mishkind, 1986), peroxisomes (Lazarow & Fujiki, 1985) and into the nucleus (Dingwall & Laskey, 1986; Newport & Forbes, 1987) has been described in some detail. The theme of this thesis is to understand the mechanism of targeting and translocation across membranes of proteins destined to be secreted from eukaryotic cells. Analogies and comparisons with the mechanism of translocation for other organelles and secretion in bacteria will be discussed when pertinent.

THE SECRETORY PATHWAY IN EUKARYOTIC CELLS

Secreted proteins in eukaryotic cells must traverse a pathway composed of multiple organelles formed by membranous cisternae or vesicles that are functionally interconnected to each other. Through this system, secretory and plasma membrane proteins are transported vectorially from their site of synthesis to the extracellular space. During their traffic through the

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secretory pathway, proteins are subjected to different modifications in each of the comparments of the pathway. The proteins that are permanent residents of the intracisternal space or the membrane of any comparment in the secretory pathway follow the same route as secretory proteins. However, somehow they are selectively retained in the organelle in question. Thus, in analogy with the signals to distinguish secretory proteins from cytoplasmic proteins, a mechanism must exist to sort the proteins in transit from the permanent residents of each organelle. In this section, I will briefly describe the most relevant processes during the traffic through the secretory pathway to put the theme of this thesis in a broad context.

The Endoplasmic Reticulum:

Much of our present knowledge of the secretory pathway is derived from the classic studies of higher eukaryotic cells by Palade and coworkers (Palade, 1975). The first step in the pathway is the synthesis of secretory and most integral membrane proteins on ribosomes attached to the cytoplasmic face of the membranes of the endoplasmic reticulum (ER). This organelle consists of many interconnected cisternae generally located in a perinuclear disposition. Two forms of ER can be distinguished morphologically, the rough and smooth ER. The rough endoplasmic reticulum (RER), whose name is derived from its rough appearance in electron microscopy due to attached ribosomes, is the site of synthesis of secretory and most integral membrane proteins (Palade, 1975). The mechanisms by which ribosomes synthesizing secretory proteins are recognized and targeted to the RER membrane and the mechanism of protein translocation are directly related to the present work and will be described in detail in further sections of this chapter. The smooth endoplasmic reticulum (SER) has no

ribosomes attached but is the site of synthesis of the lipid components of membranes. Thus, in addition to being part of the secretory pathway, the ER is the site of synthesis and assembly of the cellular membranes.

Secretory proteins inside of the ER cisternae are first modified by the addition of oligosaccharides to asparagine (N-linked oligosaccharides; Kornfeld, R. & Kornfeld, S., 1985). This modification occurs when the site of glycosylation reaches the interior of the ER and does not require termination of synthesis or translocation of the entire polypeptide. Also, in the interior of the ER cisternae, the newly synthesized secretory or membrane proteins are thought to acquire their final conformation by the participation of two soluble proteins which reside in the ER: disulfide isomerase (Kaderbhai & Austen, 1985) and heavy chain binding protein or BiP (Pelham, 1986). Disulfide isomerase catalyzes the formation of the correct inter- and intra-molecular disulfide bounds that contribute to the stabilization of the conformation of these proteins (Kaderbhai & Austen, 1985). On the other hand, the function of BiP has not been clearly established. Direct participation in protein folding (Gething et al, 1986), retention in the ER of incorrectly folded proteins (Bole et al, 1986; Gething et al, 1986) and/or solubilizing protein aggregates in the ER (Munro & Pelham, 1986) have been suggested. Although BiP and disulfide isomerase are soluble and interact functionally with proteins that are in transit in the ER, they are not transported to the other organelles down the secretory pathway. The mechanisms of their sorting from other proteins and selective retention in the ER are not completely understood, but it involves the recognition of recently identified signal on the carboxy terminus of both proteins (Munro & Pelham, 1987). Thus, proteins that acquire their final conformation in the ER and that do not contain

retention signals are transported by bulk phase flow (Pfeffer and Rothman, 1987) to the "Golgi Complex", the next organelle down the secretory pathway.

Transport to the other Compartments of the Secretoty Pathway:

Morphological evidence indicates that the transport from ER to Golgi is probably mediated by small membraneous vesicles that are formed by budding of small patches of the ER membrane (Farquhar, 1985; Pfeffer & Rothman, 1987). During the budding process, secretory proteins are encapsulated in vesicles and then are released into the Golgi cisternae upon their fusion with the membranes of this organelle. Since this vesicular transport also results in transfer of membranes from ER to Golgi, integral membrane proteins are transported as such between the organelles. The Golgi complex is composed of several stacks of membraneous cisterns that are arranged in a perinuclear disposition on one side of the nucleus. The golgi stacks close to the nucleus are referred to as the "Cis" Golgi, while the stacks proximal to the plasma membrane are referred to as the "Trans" Golgi. In their traffic through Golgi, the secretory proteins are carried from the cis to the trans stacks also by a similar vesicular transport system (Farquhar, 1985; Pfeffer & Rothman, 1987).

During their traffic through the Golgi stacks, secretory proteins are subjected to two major processes: they acquire most of their post-translational modifications and they are sorted for the targeting to their final destination. The N-linked oligosaccharides that are acquired during their synthesis in the ER are modified by the removal of several mannose residues, and the addition of N-acetylglucosamine and sialic acis in higher eukaryotes (Kornfeld & Kornfeld, 1985; Farquhar, 1985; Hirschberg & Snider, 1987) or by the addition of more mannose groups in yeast

(Kukuruzinska et al, 1987). A second, less well characterized, type of glycosylation (O-linked glycosylation) also occurs in this organelle, in which sugar moieties are added to serine or threonine residues of the secretory proteins (Farquhar, 1985). Also, sulfations and addition of glycosaminoglycans to proteoglycans occur during the transit of these polypeptides through the Golgi complex (Farquhar, 1985). A more conceptually relevant process that occurs in Golgi is the sorting of proteins for their targeting to at least three different organelles: lysosomes, secretory granules and secretory vesicles.

The mechanism of sorting of lysosomal from secretory proteins is one of the most well understood sorting events within the secretory pathway (Farquhar, 1985). By the action of two enzymatic reactions, mannose residues on the N-linked oligosaccharide of lysosomal proteins are phosphorylated to mannose-6-phosphate (Man-6-P). Thus, the enzyme N-acetylglucosamine-1-phosphotransferase recognizes lysosomal proteins by a not well understood mechanism and then adds N-acetylglucosamine-1-phosphate groups to the mannose residues on their N-linked oligosaccharides. The enzyme phosphodiester glycosidase then removes the N-acetylglucosamine groups, leaving the phosphate on the mannose residues. Two identified Man-6-P receptors, both integral membrane proteins, then binds with high affinity to these proteins. This interaction results in the targeting of lysosomal proteins to specific transport vesicles destined to the lysosomes.

During transit through the Golgi, secretory proteins are sorted into two groups: proteins that are constitutively secreted and proteins that are stored in secretory granules and, upon stimulation, are rapidly secreted (Burgess and Kelly, 1987). It is believed that, in analogy to the sorting

of lysosomal proteins, this sorting process occurs by recognition of an unidentified signal on proteins destined to secretory granules, which results in their targeting to this organelle. Associated with their packaging into secretory granules, some secretory proteins are proteolytically processed from the "proprotein" to their mature form (Burgess and Kelly, 1987). Constitutively secreted and integral membrane proteins, on the other hand, appear to be carried by bulk phase flow to secretory vesicles that are constantly moving from Golgi to the plasma membrane.

THE SIGNAL HYPOTHESIS

The "signal hypothesis" was proposed by Blobel and Dobberstein to explain the mechanism by which the ribosomes synthesizing secretory proteins are targeted and attached to the ER membrane (Blobel & Dobberstein, 1975a). The hypothesis was based on the finding that precursors for secretory proteins contained amino-terminal extensions of about 20 amino acids (Milstein et al, 1972; Blobel and Dobberstein, 1975a) that are rapidly removed upon translocation into the ER (Blobel and Dobberstein, 1975a). According to the hypothesis, the peptide extensions act as "signal sequences" (or "signal peptides") that determine the targeting of ribosomes synthesizing secretory proteins to the ER membrane. The hypothesis proposes that the signal sequences are specifically recognized by receptors on the ER membrane, and that this interaction triggers the formation of a protein tunnel in the ER membrane through which the secretory proteins are translocated. This simple hypothesis provides a conceptual explanation for the problems outlined in the first section of this chapter: the signal-receptor interaction explained the specificity of targeting to the correct membrane, and the formation of a "tunnel",

dependent on this interaction, explained the selective translocation of the secretory proteins.

Experimental evidence supporting the signal hypothesis has been accumulated since it was proposed. Most of the evidence has been obtained using an in vitro system capable of protein translocation into RER vesicles contained in a purified rough microsomal subcellular fraction (Blobel and Dobbertein, 1975b). Biochemical fractionation of this in vitro system has permitted the identification and characterization of many of the proposed components of the translocation machinery (described bellow). Thus, the validity of the signal hypothesis has been largely demonstrated. Furthermore, besides being a model to explain protein translocation across the ER membrane, the signal hypothesis also provided a conceptual framework for understanding many of the sorting and topological problems arising from the multi-organellar nature of eukaryotic cells (Blobel, 1980). Therefore, parallels and similarities with targeting and import of proteins into other subcellular organelles have been established. Although the ER system remains the best understood, signal sequences for the import into mitochondria (Tzagollof & Myers, 1986, Roise & Schatz, 1988), chloroplast (Schmidt & Mishkind, 1986) and the nucleus (Dingwall & Laskey, 1986) have been well characterized.

TRANSLOCATION MACHINERY IN HIGHER EUKARYOTES

Protein translocation across purified rough microsomal membranes (RM) can be observed when this fraction is added to a cell-free extract that is translating secretory proteins (Blobel & Dobbertein, 1975b). Depletion of the translocation activity from RM by either high salt extraction (Warren and Dobberstein, 1978; Walter and Blobel, 1980) or mild proteolysis (Walter et al, 1979; Meyer and Dobberstein, 1980a) indicated that proteins from

the translocation machinery that are exposed on the cytoplasmic side of the ER vesicles can be removed by these treatments. Two components of the machinery have been identified because of their absolute requirement in these translocation assays. In wheat germ translation extracts, translocation into salt-extracted rough microsomes was recovered by adding back the high salt soluble fraction (Warren and Dobberstein, 1978; Walter and Blobel, 1980). This assay led to the purification from the high salt extract of a cytoplasmic component of the translocation machinery; the "signal recognition particle" or "SRP" (Walter and Blobel, 1980). In rabbit reticulocyte lysates, reconstitution of translocation activity of trypsin-treated microsomes by the protease-generated soluble extract (Walter et al, 1979; Meyer and Dobberstein, 1980a) led to the purification of the cytoplasmic fragment of a membrane protein required for protein translocation (Meyer & Dobberstein, 1980b). Called "docking protein" by Meyer and Dobberstein because it was believed to be the ribosome attachment component on the ER membranes, this protein is the receptor for the signal recognition particle (Meyer et al, 1982; Gilmore et al, 1982a&b).

The Signal Recognition Particle (SRP):

The function of SRP is to serve as an adaptor between translation and translocation by targeting ribosomes synthesizing secretory proteins to the ER membrane. To perform this function, SRP binds both to ribosomes and to the ER membrane via the SRP receptor. The binding to ribosomes occurs with two different affinities (Walter et al, 1981): low affinity binding (with an apparent K_D of 5×10^{-5} M) to ribosomes not engaged in translation or translating cytoplasmic proteins, and high affinity binding (with an apparent K_D of 8×10^{-9} M) to ribosomes that are synthesizing secretory proteins. The increase in the binding affinity is the direct result of the

recognition by SRP of the signal sequence on the nascent secretory protein (Walter et al, 1981). In the wheat germ translation system, this recognition occurs as soon as the nascent chain reaches the length necessary for the emergence of the signal sequence from the ribosome (Walter and Blobel, 1981b). The high affinity binding of SRP to the ribosome results in a transient inhibition or a kinetic delay of protein synthesis elongation, a phenomenon known as "elongation arrest", of the secretory polypeptide (Walter and Blobel, 1981b). Then, the bound SRP mediates the binding of the complex to microsomal membranes (Walter and Blobel, 1981a) via its interaction with the SRP receptor (Gilmore et al, 1982a&b; Meyer & Dobberstein, 1982).

SRP is a ribonucleoprotein composed of six polypeptides (Walter and Blobel, 1980) and a 300 nucleotide RNA molecule (Walter and Blobel, 1982), known as 7SL or SRP RNA. The first 100 and the last 50 nucleotides of SRP RNA contain sequences that are homologous to the Alu family of highly repetitive genomic sequences (Ullu et al, 1982). The middle 140 nucleotides correspond to a unique sequence that is called "S" region (Ullu et al, 1982). Its secondary structure shows that SRP RNA is mostly base paired, resulting in both Alu regions being on one end of the molecule and the S region being on the other end (Gundelfinger et al, 1984; Zwieb, 1985; Poritz et al, 1988). The six SRP polypeptides can be separated into four protein units (Siegel and Walter, 1985): two heterodimers (68/72-kDa and 9/14-kDa) and two monomers (19-kDa and 54-kDa). Of the four, only the 54-kDa protein is not a nucleic acid binding protein and, therefore, is bound through a protein-protein interaction with the 19-kDa subunit (Walter and Blobel, 1983). The functions of the SRP proteins have been determined by disassembly and reconstitution of the particle (Walter and Blobel,

1983). The 9/14-kDa subunit binds to the Alu sequences of the RNA, forming a domain which is involved in the elongation arrest activity of SRP (Siegel and Walter, 1985, 1986). The 54-kDa protein can be directly crosslinked to the signal peptide of nascent chains, indicating that it is involved in the signal recognition function (Kurzchalia et al, 1986; Krieg et al, 1986). Specific alkylation of this protein results in the inability of SRP to bind ribosomes with high affinity (Siegel and Walter, 1988a), indicating that the particle fails to recognize signal sequences. Thus, the 54-kDa protein constitutes the signal recognition domain of SRP. In similar experiments, particles reconstituted with alkylated 68/72-kDa protein fail to interact with SRP receptor (Siegel and Walter, 1988a). This observation indicated that this protein constitutes or contributes to the SRP domain involved in targeting to the ER membranes. Footprinting analysis of the SRP proteins on its RNA have localized the 68/72-kDa protein arround the middle of the folded RNA, and the 19 kD protein to the end opposite to the Alu domain (Siegel and Walter, 1988b). Taking into consideration all these results, a picture of SRP emerges in which the elongation arrest and signal recognition domains are localized in opposite ends of the particle with the membrane targeting domain in the middle (Siegel and Walter 1988b). This interpretation is consistent with electron microscope pictures which show SRP as an elongated rod-shaped particle in which three domains can be distinguished (Andrews et al, 1985, 1987).

The SRP Receptor:

As indicated above, targeting of the SRP-ribosome complex to the ER membrane is mediated by the SRP-SRP receptor interaction (Gilmore et al, 1982a,b; Meyer et al, 1982). This interaction also results in the release of the elongation arrest of translation (Walter and Blobel, 1981b; Gilmore

et al, 1982a&b), due to the displacement of SRP from the ribosome (Gilmore and Blobel, 1983). This displacement, however, is not accompanied by binding of SRP receptor to the ribosome (Gilmore and Blobel, 1983). Thus, SRP and SRP receptor are involved catalytically in targeting ribosomes that are synthesizing secretory proteins, but they are not directly involved in the attachment of the ribosome to the ER membrane. Presumably, after ribosome attachment occurs, SRP and SRP receptor recycle and can be used for a new round of ribosome targeting (Gilmore and Blobel, 1983). Careful characterization of SRP receptor purified under native conditions indicates that it is composed of two polypeptide subunits (Tajima et al, 1986): α-subunit and β-subunit with molecular weights of 70 kDa and 30 kDa, respectively. Most of the mass of the α-subunit is exposed on the cytoplasmic side of the ER membrane. This is the domain of the protein that can be removed by proteases in the assay that led to the early identification of SRP receptor (Meyer and Dobberstein, 1980a,b). This proteolytic fragment of the α-subunit does not bind to SRP by itself (Lauffer et al, 1985), but can reassociate with the domain that remains in the ER membrane after cleavage, thus reconstituting full activity (Meyer et al, 1980a&b; Gilmore et al, 1982a). The mild proteolysis used to obtain the cytoplasmic fragment of the α-subunit does not result in cleavage of the β-subunit (Andrews et al, 1988). These results indicate that both proteolytic fragments of the α-subunit, perhaps with the β-subunit, are part of the domain of the SRP receptor that is responsible for SRP binding. We have cloned and sequenced a cDNA encoding for the α-subunit of the SRP receptor and found that the protein contains three regions that are rich in positively charged amino acids (Lauffer et al, 1985). These regions shown some homology to nucleic acid binding proteins, suggesting that they

could be in direct contact with SRP RNA during the SRP-SRP receptor interaction (Lauffer et al, 1985).

Ribosome Attachment and Signal Sequence Receptor:

In contrast to the targeting mechanism, the actual translocation process remains poorly characterized. After ribosome attachment, translocation is carried out by an unknown mechanism while the secretory protein is elongated. The nature of the ribosome attachment site on the ER membrane is not fully characterized. Two proteins, ribophorins I and II, have been found to associate in equal molar amounts with membrane-bound ribosomes (Amar-Costesec et al, 1984; Marcantonio et al, 1984). These proteins contain most of their mass exposed in the lumen of the ER, spanning the membrane once and having small cytoplasmically-exposed domains (Crimaudo et al, 1987; Harnik-Ort et al, 1987). Attempts to establish their participation in the translocation process have been inconclusive. Moreover, a ribosome binding activity has recently been reconstituted into liposomes from a fraction of microsomal proteins that lack ribophorins (Yoshida et al, 1987). Thus, the particiption of ribophorins, if any, in protein translocation remains to be determined. Independent of the nature of the ribosome attachment site, the nascent secretory proteins appear to interact with protein components of the ER membrane. When ribosomes are detached from the membrane by puromycin and high salt treatments, the nascent secretory proteins remain tightly bound to the ER membrane (Gilmore and Blobel, 1985). This interaction can be disrupted by addition of 4 M urea, indicating that the binding is due to protein-protein interactions (Gilmore and Blobel, 1985). An ER membrane protein that directly binds to the signal sequence of nascent chains has been recently identified using chemical crosslinking (Wiedmann et al, 1987). This protein, called "Signal

Sequence Receptor" or "SSR", is an integral membrane glycoprotein that appears to function as a second receptor to which the signal sequence is handed after SRP displacement during targeting to the ER membrane (Wiedmann et al, 1987, Walter, 1987). Although no biochemical details are presently known about its function, SSR is the first ER membrane protein that functionally interacts with signal peptides. It thus appears that ribosome attachment and signal sequence recognition by SSR somehow lead to the initiation of translocation of the secretory protein, which occurs by a completely unknown mechanism.

The Signal Peptidase:

To explain the passage of hydrophilic polypeptides through the hydrophobic core of the lipid bilayer, the signal hypothesis proposes that translocation occurs through a protein tunnel (Blobel and Dobberstein, 1975a). Such a tunnel, its protein components, or its functional interactions with the other components of the translocation machinery remain to be described. However, it is known that during the passage of nascent secretory proteins across the membrane, the signal sequence is removed by "signal peptidase" (Blobel and Dobberstein, 1975a). This enzyme is an integral membrane protein whose active site resides within the microsomal vesicles (Jackson and Blobel, 1977), since precursor proteins in the absence of translocation are cleaved only by detergent extracts of RM (Jackson and Blobel, 1977). This activity led to the purification of the enzyme from the detergent extract as a protein complex with of six or seven polypeptides (Evans et al, 1986). This finding was rather surprising since removal of the signal sequence is a relatively simple enzymatic reaction and since the bacterial counterpart purifies as a single polypeptide (Zwizinski and Wickner, 1980; Wolfe et al, 1982, 1983; Wolfe and Wickner,

1984). Thus, this finding raises the posibility that signal peptidase activity is only one of several functions of this complex. Perhaps some of the subunits form part of the protein tunnnel proposed by the signal hypothesis. A detailed characterization of the possible functions of the complex and its subunits should contribute to understand how secretory proteins are translocated.

Are ER Lumenal Proteins Involved in Translocation?

The participation of ER lumenal proteins in the translocation process cannot be ruled out. In particular, it seems reasonable that the ER proteins that participate in folding of secretory proteins could somehow facilitate transfer through the membrane. As indicated before, BiP binds tightly to unfolded or incorrectly folded proteins. It is, therefore, possible that BiP could bind to unfolded nascent secretory proteins as they emerge into the lumen of ER. If BiP function is to actively fold nascent polypeptides, the energy released in the process could be utilized for the protein transfer across the membrane. In collaboration with K. Kassenbrock and R. Kelly, we have tested this posibility by determining if nascent chains are tightly bound to BiP during translocation (Kassenbrock et al, 1988). Our results showed no high affinity association of nascent chains with BiP, although a clear association with terminated polypeptides in the ER lumen was observed (Kassenbrock et al, 1988), suggesting that BiP does not facilitate translocation. However, this conclusion is limited by the fact that in our experiments only high affinity association with incorrectly folded proteins could be measured (Kassenbrock et al, 1988). To conclusively rule out the possible involvement of BiP or other luminal proteins, the translocation assays should be performed with microsomal vesicles that are completely depleted of their lumenal content.

SIGNAL SEQUENCES AND OTHER TOPOGENIC SEQUENCES

The Signal Sequence:

According to the signal hypothesis, the signal sequence contains all the information required for targeting of secretory proteins to the ER membrane. Thus, the question of what constitutes a signal sequence is of vital importance for the understanding of the mechanism of signal sequence recognition. Two striking features were immediately evident from early characterizations of signal sequences: compilations of many signal sequences revealed no primary sequence conservation (Watson, 1984), and the nature of signal sequences appears to be conserved from bacteria to higher eukaryotes (Fraser and Bruce, 1978; Talmadge et al, 1980a&b; Muller et al, 1982). Despite the lack of conservation, all signal sequences contain certain common features (Watson, 1984; vonHeijne, 1985; Briggs and Gierach, 1986): i) One to three basic amino acids are usually found at the amino terminus of the signal peptide, ii) 8 to 15 hydrophobic amino acids follow these basic amino acids, and iii) a polar region of 5 to 6 amino acids follows, with amino acids of small side chain preferentially found at positions 1 and 3 prior to the cleavage site. Conservation of these features has lead to the suggestion that they contribute to the structural motif involved on signal recognition (vonHeijne, 1984 and 1985; Briggs and Gierach, 1986).

Numerous experiments have provided unequivocal evidence that the signal peptide contains the information required for targeting and translocation of secretory proteins. The most conclusive evidence came from the experiments in which globin, a cytoplasmic protein, was translocated across mammalian microsomes when the signal peptide of β-lactamase was fused to its amino terminus (Lingappa et al, 1984).

Furthermore, experiments in which signal sequences are specifically altered have provided indications that some of the conserved features constitute the information that is recognized by signal sequence receptors. For example, the incorporation of β-hydroxy leucine into the hydrophobic core of the preprolactin signal peptide results in reduced hydrophobicity and blocks signal recognition by SRP (Walter et al, 1981). Also, deletions that reduce the size of the hydrophobic core or mutations that introduce charged residues into the core result in impairment of protein secretion and cytoplasmic accumulation of precursors in bacteria (for review see Silhavy et al, 1983, Benson et al, 1985 and Briggs and Gierasch, 1986). These experiments clearly demostrate that the length and hydrophobicity of the central core of the signal peptide is one of the motifs involved in signal recognition, both in bacterial and mammalian cells.

The involvement in signal recognition of the positively charged domain of signal peptides has been tested genetically. Replacement of basic amino acids by acidic residues in this domain of the Escherichia coli outer membrane lipoprotein signal peptide results in a delay in protein secretion. This observation indicates that, although the positive charges are not absolutely required for translocation, they are somehow involved in determining the efficiency by which the protein is translocated in bacterial systems (Inouye et al, 1982; Vlasuk et al, 1983). This conclusion correlates well with the observation that all the bacterial signal peptides contain at least one basic amino acid at their amino terminus (vonHeijne, 1984; Watson, 1984). A few eukaryotic signal sequences, however, contain negative net charges at their amino terminus (vonHeijne, 1984; Watson, 1984). Therefore, we tested directly the involvement of this domain in signal recognition in eukaryotes. For this

purpose, we used the signal peptide mutants of the outer membrane lipoprotein (see Appendix 1; Garcia et al, 1987). We found that all the mutants were recognized by SRP and translocated across mammalian microsomal membranes at efficiencies comparable to that of the wild type protein (Appendix 1; Garcia et al, 1987). Thus, although the majority of the eukaryotic signal peptides contain a positively charged domain, it appears that they are not absolutely required or involved in signal recognition by the translocation machinery of higher eukaryotes.

Analysis of the signal peptidase cleavage site from all the known signal peptides, in both bacterial and mammalian secretory proteins, has revealed an amino acid distribution pattern that has been suggested to represent the feature that determines cleavage specificity (vonHeijne, 1984). This distribution pattern shows that amino acids with a small side chain are found in the first and third positions before the cleavage site (-1 and -3 positions respectively). Alanine is by far the most common amino acid at these positions, but glycine, serine, cysteine and threonine are found occasionally (Watson, 1984; vonHeijne, 1984). The amino acid at position -2 is more variable but frequently contains a large aromatic or hydrophobic side chain (Watson, 1984; vonHeijne, 1984). Substitution of valine for alanine in position -1 of the yeast invertase signal peptide results in lack of cleavage at the correct site and a small amount of cleavage at a cryptic site (Schauer et al, 1985). Thus, it appears that the amino acids in these positions somehow determine the specificity of the cleavage site. The pattern is more random in the other positions before the cleavage site, although polar amino acids are usually found (Watson, 1984; vonHeijne, 1984). Any amino acid can be found after the cleavage site (Watson, 1984; vonHeijne, 1984). Therefore, no restrictions in the

amino terminus of the mature secretory protein are added by the cleavage specificity determinants. The outer membrane lipoprotein signal peptides from bacteria represent an exception of this rule. However, they are processed by an specialized signal peptidase as part of a complex processing pattern (see appendix 1 for details and references). On the other hand, the distance of the cleavage site from the hydrophobic core of the signal peptide is remarkably constant (about 5 or 6 amino acids; see Watson, 1984 and vonHeijne, 1984). This observation led to the suggestion that this distance may also play a role in determining cleavage specificity (vonHeijne, 1984; Watson, 1984). The use of these distribution patterns as "rules" has been successfully used to predict the cleavage site for many signal peptides, including the cases reported by us in appendixes 1 and 2 (see also Garcia et al, 1987 and 1988). Therefore, it is believed that the amino acids at positions -1 and -3 and their distance from the hydrophobic core of the signal peptide are the determinants for signal peptidase recognition and cleavage specificity.

Although the signal sequences from most secretory proteins are cleaved during translocation with remarkable efficiency and specificity, their removal is not an absolute requirement for protein translocation. This fact is demonstrated by the existence of a secretory protein (Palmiter et al, 1978; Lingappa et al, 1979; Braell and Lodish, 1982) and many membrane proteins (for review see Wickner and Lodish, 1985) which retain their signal sequences on their mature forms. Therefore, since neither the positively charged domain nor removal of the signal sequence are absolute requirements for translocation, the questions of what constitutes the signal and what is the nature of the signal-receptor interaction remain to be resolved. It has recently been shown that a remarkable number of random

amino acid sequences can function as signal peptides in vivo, when inserted in place of the yeast invertase signal sequence (Kaiser el al, 1987). Although most of these peptides work at low efficiencies, which correlate primarily to their overall hydrophobicity, it appears that the specificity with which they are recognized must be surprisingly low (Kaiser el al, 1987). This finding and the observations that only mutations in the hydrophobic core of signals sequences result in absolute defective translocation phenotypes seems to indicate that the other conserved features may indeed be dispensable and may at most add fidelity to the process.

Transmembrane and Stop-Transfer Sequences:

The basic characteristic of integral membrane proteins is that the polypeptide chain contains domains on both sides of the lipid bilayer, with a "transmembrane sequence" traversing the membrane. These sequences are 20 to 25 amino acids long and contain only hydrophobic amino acids disposed in an α-helical conformation (for review see Wickner and Lodish, 1985), in which all the polar groups of peptide bonds are paired. Therefore, the transmembrane sequences are completely hydrophobic and in a stable interaction with the hydrophobic core of the lipid bilayer. During their biogenesis, the extracytoplasmic domains of integral membrane proteins use the same machinery as secretory proteins for translocation across the membrane (Anderson et al, 1982). After the ribosome is targeted to the ER membrane, translocation of the extracytoplasmic domains proceeds until the transmembrane sequence reaches the membrane (Yost et al, 1983). Then, the transfer across the membrane somehow stops and the protein is integrated into the lipid bilayer (Yost et al, 1983). Therefore, it is assumed that transmembrane sequences encode "stop-tranfer" information that somehow

determine the uncoupling of the protein from the translocation machinery (Yost et al, 1983). Besides their stop-transfer and membrane anchor functions, some of the transmembrane sequences also function as the signal sequence for the integral membrane proteins (for review see Wickner and Lodish, 1985). Since transmembrane sequences serve multiple functions, it is assumed that they contain domains that interact with different components of the translocation machinery.

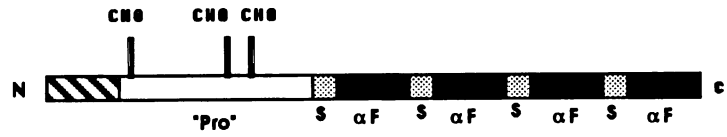
Although it is believed that all these functions are encoded in the transmembrane sequences, it has not been possible to experimentally dissect them and independently study each of the interactions with components of the translocation machinery. However, during the characterization of a signal peptide encoded by the "pre-core" region of the Hepatitis B Virus, we made a striking observation that might represent a case of stop-transfer function not accompanied by membrane integration (see appendix 2; Garcia et al, 1988). We found that after removal of the signal peptide, translocation of the protein is not completed, in analogy with the stop-transfer function of transmembrane sequences. However, the protein does not contain any hydrophobic sequence similar to typical transmembrane sequences. Therefore, after translocation is stopped, the protein cannot be integrated into the membrane and is released back into the cytoplasm (Appendix 2; Garcia et al, 1988). Although we have not yet determined if this behavior can be assigned to a discrete sequence in the protein, a reasonable explanation of our observation is that a domain of the protein interacts with the translocation machinery in a way that mimics the interaction of stop-transfer sequences of integral membrane proteins. It is possible that further characterization of this observation might provide some insights of the stop-transfer function of transmembrane sequences.

THE YEAST PREPRO-α-FACTOR AS A MODEL SECRETORY PROTEIN

For reasons outlined in the following section of this chapter, we have chosen the precursor for the yeast pheromone α-factor, prepro-α-factor, as substrate for protein translocation. For a clear understanding of some experimental assays used in the present work, I will describe in this section the most relevant steps of the biogenesis of α-factor. The mature pheromone is a 13 amino acid long peptide that is secreted by haploid cells of α mating type of the yeast Saccharomyces cerevisiae. The structure of prepro-α-factor (pαF) is schematically represented in figure 1. It contains a 20 amino acid signal peptide (shaded in fig. 1) at the amino terminus as part of an 80 amino acid "pro" region that contains three N-glycosylation sites (indicated by "CHO" in fig. 1). Carboxy-terminal to the pro-region are four tandem repeats of the α-factor peptide (in black in fig. 1) separated by 6-8 amino acid spacer peptides (marked "S" in fig. 1; Kurjan and Herskowitz, 1982).

The signal peptide of pαF is removed by signal peptidase upon translocation into the lumen of the ER (Waters et al, 1988). Also in the ER, it becomes modified by the addition of three core oligosaccharides to the N-glycosylation sites in the "pro" region (Julius et al., 1984a). Then pαF traverses the yeast secretory pathway. Late in the Golgi apparatus or in secretory granules, pαF is processed by proteolytic cleavage performed by the KEX2 gene product, generating α-factor molecules that still contain the spacer peptide at their amino-terminal end (Julius et al., 1984b). Further processing by the membrane-bound dipeptidyl-aminopeptidase A (Julius et al., 1983) and the KEX1 gene product (Dmochowska et al, 1987) results in the production of mature α-factor that is secreted from the cell.

Figure 1: Structure of prepro-α-factor.


CHO
CHO CHO
N
C
"Pro"
S αF S αF S αF S αF

THE OBJECTIVE OF THIS STUDY:

Most of the current knowledge of the process of protein translocation across the ER membrane has been deduced from biochemical dissection of the *in vitro* assay developed by Blobel and Dobberstein (1975a,b). Therefore, little is known about the *in vivo* significance of the functions of the translocation machinery described above. Moreover, by concentrating on the phenomena that can be studied *in vitro*, some important processes for protein translocation may have been overlooked. An ideal situation for studying this or any other complex biological phenomena is to have an experimental system in which the role of each of the known components involved can be studied both *in vivo* and *in vitro*. The ability to genetically manipulate the components of the translocation machinery will be of clear advantage to both understanding their *in vivo* functions and identifying other yet unknown components. As indicated earlier in this chapter, the best understood system is the translocation of proteins across the mammalian ER membrane. Obviously, genetic manipulations of such organisms are extremelly difficult. Therefore, we have decided to study other eukaryotes in which genetic and biochemical experiments can be carried out. The yeast *Saccharomyces cerevisiae* is by far the most accessible eukaryote from the genetic point of view. However, atthe onset of these studies nothing was known about the protein translocation machinery in the yeast ER. Therefore, in a first step toward developing an experimental system accessible to both genetic and biochemical methods, we have developed an *in vitro* assay for protein translocation across the yeast ER membrane. This thesis describe such an assay and extensively compares the mechanism of translocation in the yeast and mammalian systems.

CHAPTER 2

IN VITRO PROTEIN TRANSLOCATION ACROSS THE ENDOPLASMIC RETICULUM MEMBRANE OF THE YEAST *SACCHAROMYCES CEREVISIAE*

ABSTRACT

We have developed a procedure to isolate microsomal vesicles (yRM) from the yeast Saccharomyces cerevisiae that are active in translocation of precursors for secretory proteins. The in vitro synthesized precursor of the α-factor pheromone (prepro-α-factor, pαF) was efficiently translocated into the lumen of these vesicles in either an homologous or wheat germ cell-free translation system. The fidelity of the translocation process was shown by monitored the addition of N-linked oligosacharides to pαF, its cosedimentation with yRM vesicles and its resistance to externally added proteases. The translocated pαF was soluble after alkaline sodium carbonate treatment, indicating that pαF was properly sequestered within yRM vesicles and not integrated into the lipid bilayer.

INTRODUCTION

An early event in the biosynthetic pathway of secretory, lysosomal, and a variety of integral membrane proteins in eukaryotic cells is the selective translocation of these proteins or some of their domains (as is the case for integral membrane proteins) across the lipid bilayer of the endoplasmic reticulum (ER) membrane (for review see Walter et al., 1984 and Walter and Lingappa, 1986). Much of the knowledge about the molecular mechanism of this phenomenom is derived from our ability to reconstitute this process in vitro with components derived from a variety of higher eukaryotic cells. From these studies a model evolved in which protein translation and its translocation across the membrane are strictly coupled (Blobel and Dobberstein, 1975a&b). Two components, signal recognition particle (SRP) and SRP receptor, of the cellular machinery promoting this process have been purified (Walter and Blobel, 1980,: Gilmore et al., 1982; Meyer et al, 1982). These components function primarily to target the

ribosomes that are synthesizing secretory proteins to the ER membrane. In vitro their molecular function can be described as a sequence of the following steps: i) signal recognition by SRP involves the decoding of the targeting information that is contained in the signal peptide as part of the nascent chain. As a result SRP binds with high affinity to the translating polysome (Walter et al., 1981) and transiently arrests elongation (Walter and Blobel, 1981b; Siegel and Walter, 1985 & 1986). ii) Targeting to the ER is mediated through a direct interaction of the SRP bound to the ribosome with an integral membrane protein (the SRP receptor or docking protein (Gilmore et al., 1982a&b: Meyer et al., 1982) of the ER membrane (Hortsch and Meyer, 1985; Tajima et al, 1986). Interaction of SRP with its receptor causes the elongation arrest to be released and a loss of the high affinity of SRP for the ribosome/nascent chain complex (Walter and Blobel, 1981b). iii) Translocation of the nascent polypeptide across the membrane occurs by a yet uncharacterized, but co-translational mechanism(s). iv) SRP and SRP receptor are recycled after the ribosome binds to the membrane to form a functional ribosome/membrane junction (Gilmore and Blobel, 1983).

Because of the nature of the biochemical approach to the problem, the precise role of the various steps outlined above in protein secretion in vivo remain to be established. For this reason we are beginning to explore yeast as an experimental system that should allow us to link the results of biochemical exploration with the physiological requirements of living cells. Our approach is to first identify components of the yeast protein translocation machinery using in vitro assays analogous to those that were used to characterize the mammalian components. Because to the small size of the genome, it is comparatively easy to clone the genes for isolated

proteins. Modern genetic techniques make it then possible to delete or selectively alter the cloned genes and to study the consequences of such perturbations in vivo.

We chose as a model secretory protein the yeast mating factor produced by α cells (α-factor). A genetic approach to the study of the secretory process in yeast has produced conditional mutants which affect protein processing and secretion (for review see Schekman and Novick, 1982 and Schekman, 1985). Consequently, the biosynthetic pathway of pαF is one of the best understood for a peptide hormone. α-Factor is synthesized in yeast cells of the α mating type as a larger precursor molecule (prepro-α-factor, pαF) of 18.5 kd that contains four copies of the 13 amino acids long α-factor at its carboxyterminal end (see Fig. 1 of chapter 1; Kurjan and Herskowitz, 1982,: Julius et al., 1983). The amino terminus of pαF has a stretch of 20 nonpolar amino acids that function as a signal sequence. In the lumen of the ER, pαF becomes glycosylated at three asparagine residues (Julius et al., 1984). In this work we have used the change in mobility on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) that is associated with this covalent modification to monitor the translocation of pαF across yeast ER membranes and to establish an assay that allows us to characterize this process in vitro.

METHODS

In vitro transcription.

The plasmid pDJ100 (obtained from Dr. D. Julius) was constructed by ligation of BamHI linkers at the upstream HinfI site closest to the initiating ATG and to the downstream SalI restriction sites of the pαF coding sequence, and insertion of this fragment into the BamHI restriction site of the pSP65 vector (D. Julius, personal communication). The plasmid

was linearized by digestion with XbaI, and transcribed in vitro by SP6 phage RNA polymerase (Promega Biotech) (Krieg and Melton, 1984 & 1987). The transcription was carried out in a 20 µl reaction containing 40 mM Tris-HCl pH 7.5, 6 mM magnesium chloride, 2 mM spermidine, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM UTP, 0.1 mM GTP, 0.5 mM GpppG (PL- Pharmacia), 10 mM DTT, 1,000 U/ml of human placental ribonuclease inhibitor, 0.1 mg/ml of linearized plasmid and 500 U/ml of SP6 RNA polymerase. The reactions were incubated at 40 ^{o}C for 60 min, and were stopped by phenol chloroform extraction. The nucleic acids were ethanol precipitated and dissolved in 40 µl of water. One µl of this solution was sufficient to obtain translation products that were easily visible after overnight exposures of the SDS polyacryamide gels without fluorography.

Yeast in vitro translation assay.

Yeast translation extracts were prepared by a modification of the method described by Gasior et al.(1979). The ade6 pep4-3 MATa strain of Saccharomyces cerevisiae (which contains reduced levels of vacuolar proteases (Hemmings et al., 1981)) was grown in 4 liters of YEP medium (Mortimer and Hawthorne, 1969) containing 2% glucose to 1 OD_{600}/ml (1 OD_{600} of cells/ml corresponds to 10^7 cells/ml). Cells were collected by centrifugation at 3,000 x g for 5 min, washed with distilled water and resuspended in 200 ml of 50 mM potasium phosphate pH 7.5, 40 mM 2-mercaptoethanol and 1.4 M sorbitol. Zymolyase 5,000 (Kirin Brewery, Japan) was added to 50 µg/ml and the suspension was incubated at room temperature for one hour. These conditions were found to cause optimal spheroplasting for this particular strain. Spheroplasts were harvested by centrifugation at 3,000 x g for 5 min and resuspended in 400 ml of YM-5 (Hartwell, 1967) medium containing 0.4 M magnesium sulfate and incubated at

room temperature for 90 min. The culture was cooled to 0 °C. All the following procedures were performed at 4 °C. Regenerated spheroplasts were harvested by centrifugation at 3,000 x g for 5 min, washed by centrifugation in 1.4 M sorbitol and resuspended in 16 ml of lysis buffer (20 mM Hepes/KOH pH 7.5, 0.1 M potassium or ammonium acetate, 2 mM magnesium acetate, 2 mM DTT and 0.5 mM PMSF). The suspension was homogenized with 10 strokes in a motor driven Potter homogenizer and centrifuged at 27,000 x g for 15 min in a Beckman Ti 50 rotor. The supernatant was collected and centrifuged at 100,000 x g for 30 min after reaching speed in the same rotor. The resulting supernatant was passed over an 80 ml Sephadex G-25 gel filtration column equilibrated with the lysis buffer containing 20% glycerol. Fractions with absorbances of more than 20 A_{260} units/ml were pooled. The pooled peak (12 ml) was adjusted to 0.1 mM calcium chloride and 300 U/ml of micrococcal nuclease was added. After 15 min incubation at 20 oC, EGTA was added to a final concentration of 1.8 mM. The extract was aliquoted, quick-frozen in liquid nitrogen and could be stored at -80 oC for several months without loss of activity.

The translation reactions (20 µl), containing 40-50% by volume of the yeast extract above, were incubated for one hour (except where noted) at 20 °C with the following additions: 1 mM ATP, 80 µM GTP, 17.5 mM creatine phosphate, 30 µM of each of the 19 amino acids excluding methionine, 200 U/ml of human placental RNase inhibitor, 2 mM putrescine, 0.2 mg/ml creatine phosphate kinase, 2.4 mM DTT, 3.1 mM magnesium acetate, 150 mM potassium or ammonium acetate, 20 mM Hepes/KOH pH 7.5, 8 µM S-adenosyl methionine and 75 mM sucrose, 500 µCi/ml 35S-methionine (Amersham, 1000 Ci/mmol), in vitro transcribed messenger RNA and 100 µg/ml yeast tRNA (Sigma).

Preparation of yeast microsomes.

The same Saccharomyces cerevisiae strain used for preparation of the translation extract, was grown in YEP medium containing 2% glucose to 2-3 OD_{600}/ml. The cells from a one liter culture were spheroplasted, regenerated and homogenized as described above, except that the lysis buffer was 4 ml of 20 mM Hepes/KOH pH 7.5, 500 mM sucrose, 1 mM DTT, 3 mM magnesium acetate, 1 mM EGTA, 1 mM EDTA, 100 U/ml Trasylol, 0.5 mM PMSF, and 2 µg/ml each of pepstatin A, chymostatin, antipain and leupeptin. The homogenate was centrifuged in half-filled tubes in a Beckman JS-13 swinging bucket rotor at 8,000 rpm (10,000 x g) for 10 min to obtain the S-10 supernatant. The pellet was resuspended in 4 ml of the lysis buffer, homogenized with 10 strokes in a Potter homogenizer and centrifuged under the same conditions. Both S-10 supernatants were pooled and recentrifuged as above. The sample (3 ml) was loaded on top of 18 ml of a solution containing 35% Percoll in homogenization buffer, but without the protease inhibitors. The samples were centrifuged at 29,000 rpm (76,000 x g) for one hour in the Ti 50.2 rotor. Two turbid bands were visible within the generated Percoll gradient, the upper one (containing the ER, see Figure 2) was collected with a Pasteur pipette. Calcium chloride (1.2 mM) and micrococcal nuclease (1 U/A_{280} unit) were added and the mixture was incubated at 20 ^{o}C for 20 min. The digestion was terminated by addition of 0.5 mM EGTA. After the addition of an equal volume of 20 mM Hepes/KOH pH 7.5, 250 mM sucrose, 50 mM EDTA and 1 mM DTT the sample was incubated on ice for 15 min. Aliquots of 1.4 ml were loaded on top of a two-step gradient containing 0.3 ml of 50% percoll, 20 mM HepesKOH pH 7.5, 250 mM sucrose and 1 mM DTT at the bottom overlayed with 0.4 ml of 20 mM Hepes/KOH pH 7.5, 500 mM sucrose and 1 mM DTT. The gradients were centrifuged in a

swinging bucket rotor (TLS 55) in a Beckman TL 100 ultracentrifuge at 46,000 rpm (140,000 x g) for 50 min. The turbid band on top of the Percoll cushion was collected. The sample was diluted with 20 mM HepesKOH pH 7.5, 250 mM sucrose, 1mM DTT to a final concentration of 25 A_{280}/ml (measured in a 1% SDS solution). One liter of culture yielded approximately 150 µl of this suspension. Small aliquots were frozen in liquid nitrogen and stored at -80 °C. The membranes could be thawed and refrozen at least once without detectable loss of activity.

Endoglycosidase H digestions.

SDS and DTT were added to final concentrations of 2% and 75 mM respectively to 20 µl translation reactions followed by boiling for 5 min. The samples were diluted to 400 µl and digestions were carried out at 37 °C for 12 hours in the presence of 100 mM sodium citrate pH 5.5, 5 mM sodium azide, 0.15 % SDS, 100 U/ml Trasylol and 0.25 µg/ml of endoglycosidase H (New England Nuclear). The reactions were terminated by addition of TCA to 15% on ice and the precipitated proteins were solubilized in SDS-PAGE sample buffer.

Protease protection.

Translation reations (20 µl) were chilled in an ice-water bath to 0 °C and calcium chloride was added to 10 mM. A solution of proteinase K (1 mg/ml) was preincubated for 15 min at 37 °C in Tris-HCl pH 7.5 and 10 mM calcium chloride to degrade contaminating lipases. Two µl of this protease solution were added to the translation samples. The digestions were incubated at 0 °C for 30 min. The reaction was stopped by the addition of 5 µl of 0.2 M PMSF in ethanol and immediately transferred to boiling SDS-PAGE loading buffer. Under these conditions protection efficiencies of

translocated pαF varied form 70 to 90 %,: whereas unglycosylated pαF was not detectable (i.e. > 98% digested).

Sedimentation analysis.

Potassium acetate was added to 500 mM to 20 µl translation reactions. The samples were layered on top of a 100 µl cushion containing 350 mM sucrose, 500 mM potassium acetate and 2 mM magnesium acetate. Centrifugation was in a Beckman Airfuge at 30 psi for 5 min (A-110 rotor). The supernatant including the upper half of the cushion was carefully removed from the top and TCA-precipitated. The pellet fraction was directly solubilized in loading buffer.

Alkaline sodium carbonate extraction.

Translation reactions were diluted 100 fold with ice-cold 100 mM sodium carbonate pH 11.5. After a 30 min incubation at 4 ^{o}C, samples were centrifuged for 30 min at 100,000 rpm (360,000 x g, TLA-100 rotor) in a Beckman TL-100 ultracentrifuge. Seventy percent of the supernatant was carefully removed from the top, neutralized with acetic acid and TCA-precipitated. The remainder of the supernatant in the tube was discarded. The visible pellet containing ribosomes and membrane remnants were directly solubilized in SDS-PAGE sample buffer.

RESULTS

Our first goal in defining the molecular components involved in protein translocation across the RER membrane of Saccharomyces cerevisiae was to establish an assay which, in analogy to the systems described for higher eukaryotes, would faithfully reproduce this process in vitro. For this purpose yeast cells were fractionated to yield a high speed supernatant fraction that would promote protein synthesis directed from an exogenously added mRNA template and a membrane vesicle fraction that

would promote translocation of secretory proteins synthesized in vitro.

For the reasons outlined in the introduction, we chose α-factor as a model secretory protein. A plasmid containing the pαF gene cloned behind an SP6 RNA polymerase promoter (a generous gift from Dr. D. Julius) was linearized and then transcribed with SP6 RNA polymerase under conditions that produced transcripts terminating at the restriction site and containing a GpppG cap at their 5' ends (see Methods). The data in Figure 1 demonstrate that the synthetic mRNA directs the synthesis of pαF as a single protein band of the correct molecular weight (Fig. 1, lane 3) when translated in a yeast cell free extract. The identity of the primary translation product as pαF is further confirmed by immunoprecipitation with an rabbit antibody raised against authentic α-factor (Fig. 1, lane 10).

A simple procedure was devised to prepare microsomal membranes from Saccharomyces cerevisiae (see Methods). A post-mitochondrial supernatant was layered on top of a Percoll solution (a colloidal silica suspension) and centrifuged to generate a density gradient containing membranes banded at their equilibrium densities. After the brief centrifugation, two distinct turbid bands were visible in the gradient and are indicated in Figure 2 as shaded bars. We assayed NADPH-cytochrome c reductase activity (an ER marker enzyme) (Kubota et al., 1977) across the gradient (Fig. 2, solid squares). The bulk of this activity cosedimented with the band of lighter density which was collected. In this membrane fraction we recovered about 30% of the NADPH-cytochrome c reductase activity that was present in the crude yeast homogenate.

Addition of this fraction to the yeast translation system in the absence of exogenous mRNA frequently produced a considerable background of translation products. The extent of this cofractionating mRNA activity

Figure 1: In vitro translation of pαF.

Translations in a yeast cell-free extract (see Methods) were performed either in the absence (lanes 1, 2) or in the presence (lanes 3 - 11) of mRNA encoding pαF. yRM were present at 0.025 A_{280} units/20 µl (lanes 5, 8, 11; marked "++") or 0.0075 A_{280} units/20 µl (lanes 2, 4, 7; marked "+"), or omitted (lanes 1, 3, 6, 9 and 10; marked "-"). The products of 20 µl translation reactions (see Methods) were resolved on 10-15% SDS-PAGE (lanes 1 - 5). The samples shown in lanes 6 - 8 have been treated with endoglycosidase H prior to SDS-PAGE (see Methods). Lanes 10 and 11 show immunoprecipitations of translation products shown in lanes 3 and 5, respectively, using a rabbit serum raised against the secreted form of α-factor (M. Poritz and P.W., unpublished). Lane 9 is identical to lane 10, except that preimmune serum was used. The bands marked with dots are unidentified. They are not related to prepro-α-factor (see lanes 10 - 11), but rather are major Coomassie Blue staining bands in the translation system that become labeled even in the absence of ongoing protein synthesis (See chapter 3). Their presence is variable between different batches of translation extract.

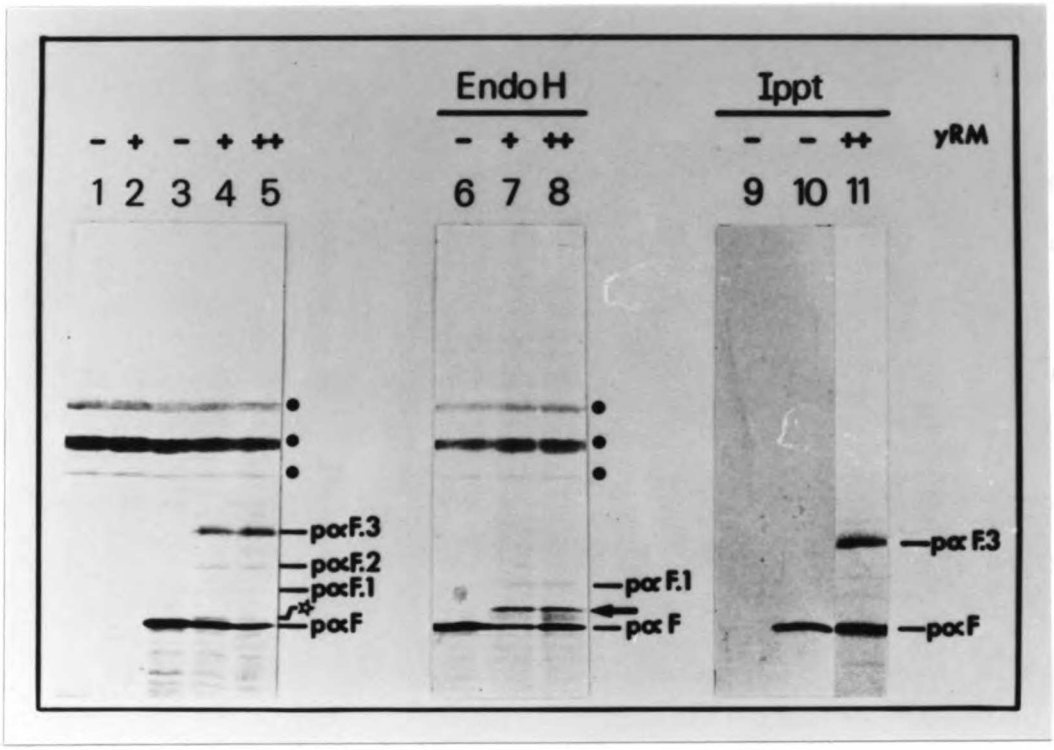
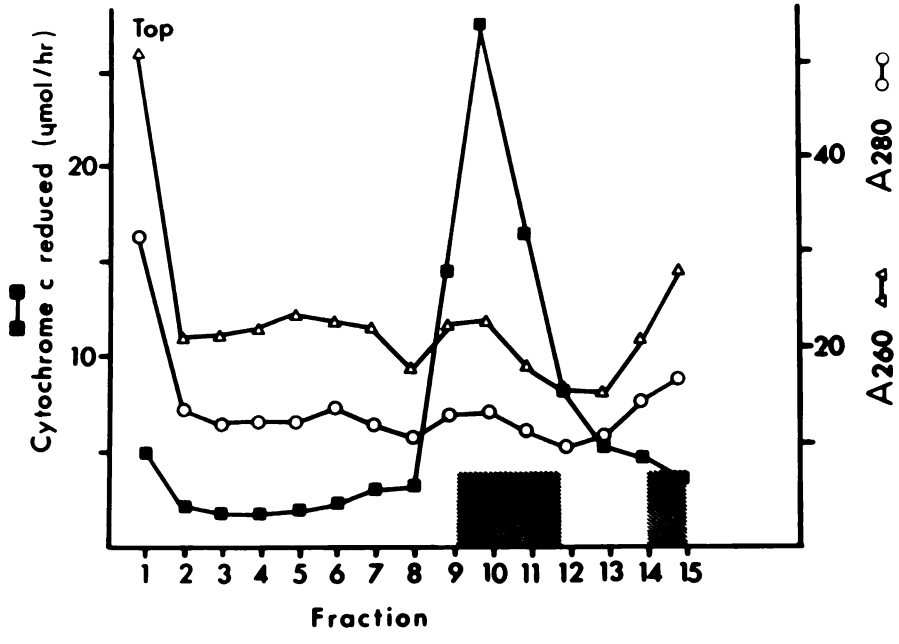

Endo H
Ippt
yRM
- + - + ++
1 2 3 4 5
- + ++
6 7 8
- - ++
9 10 11
pαF.3
pαF.2
pαF.1
pαF
pαF.1
pαF
pαF.3
pαF

Figure 2: Equilibrium density gradient fractionation of a yeast post-mitochondrial supernatant fraction.

Three milliliters of the S-10 fraction (derived from 1400 OD_{600} units of cells) were subfractionated on a Percoll density gradient as described in Methods. After centrifugation a volume corresponding to the load was removed from the top of the sample. The remainder was fractionated using a Buchler gradient fractionator; 15 fractions of 1.3 ml were collected. The fractions were assayed for NADPH cytochrome c reductase (solid squares) (Kubota et al., 1977) and the absorbances at 280 nm (open circles) and 260 nm (open triangles) were determined after dilution into 1% SDS. The approximate position of two visibly turbid bands is indicated by shaded bars.


Top
Cytochrome c reduced (μmol/hr)
20
10
40
20
A280
A260
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Fraction

was variable from one preparation to another (data not shown). We therefore proceeded to remove RNA by treatment with micrococcal nuclease, followed by EDTA extraction of the microsomes. We then further concentrated the membranes by banding them onto a high density Percoll cushion. The resulting membrane suspension contributed no detectable mRNA activity (Fig. 1, lane 2) and had lost over 70 % of the absorbance at 260 nm (most likely due loss of bound or adsorbed polysomes) that was originally present in the Percoll banded material. Henceforth, we will refer to this fraction of yeast nuclease-treated and EDTA-stripped rough microsomes as "yRM".

When pαF was translated in the presence of increasing concentrations of yRM, we observed translocation of the protein across the microsomal membrane as indicated by the attachment of core-oligosaccharides (Fig. 1, lanes 4 and 5). In particular three additional bands of larger molecular weight were visible that correspond to the addition of one, two or three core-oligosaccharide moieties, and are labelled pαF.1, pαF.2 and pαF.3, respectively. Note that increasing the membrane concentration caused a shift towards the fully glycosylated form (pαF.3). This may indicate that there is only a limited pool of assembled dolichol-linked core-oligosaccharides present in the microsome fraction that becomes depleted at the low membrane concentration. At higher yRM concentrations we also observed some general inhibition of protein synthesis.

Yeast glycoproteins are susceptible to digestion with endoglycosidase H, which removes all of the N-linked core-oligosaccharides or high mannose oligosaccharides, except for the N-acetylglucosamine directly bound to the asparagine residues (Chu et al., 1978). Digestion of the translation products produced in the presence of membranes with endoglycosidase H

resulted in the removal of the core-oligosaccharides and the production of correspondingly faster migrating bands (Fig. 1, lanes 7 and 8, marked with arrow). Presumably due to the residual N-acetylglucosamine residues that remain attached to the polypeptide after endoglycosidase H cleavage, these forms still show a reduced mobility compared to unglycosylated pαF (Julius et al., 1984). In addition to the three core-glycosylated forms of pαF we observed another minor species that was only slightly retarded in its mobility on SDS-PAGE (Fig. 1, lanes 4 and 5, marked with asterisk). This species corresponds to the signal peptide cleaved form of pαF (Waters et al, 1988) and, correspondingly, it is clearly translocated as indicated by resistance to proteases and cosedimentation with vesicles (see below). This form is more abundant in the reactions containing low yRM concentrations, indicating again that the microsomal vesicles contain a limited pool of the dolicol oligosaccharides.

We used two different criteria to confirm that the glycosylated forms of pαF are indeed translocated into the lumen of sealed microsomal vesicles. First, we found that the glycosylated and the signal peptide cleaved forms of pαF were protected from exogenously added protease (Fig. 3A, lanes 5 and 6), whereas unmodified pαF was completely degraded (Fig. 3A, lane 4). When the microsomal vesicles were dissolved by the inclusion of detergent during proteolysis, glycosylated pαF was also degraded (Fig. 3A, lanes 8 and 9). This indicates that these forms were not intrinsically resistant to protease but rather that this property was conferred to them due to their sequestration within closed vesicles.

The second criterion for proper translocation was to show that the glycosylated pαF can be selectively sedimented with the microsomal vesicles. Lanes 1 - 3 in Figure 3B show a series of translation reactions

Figure 3: Verification of pαF translocation.

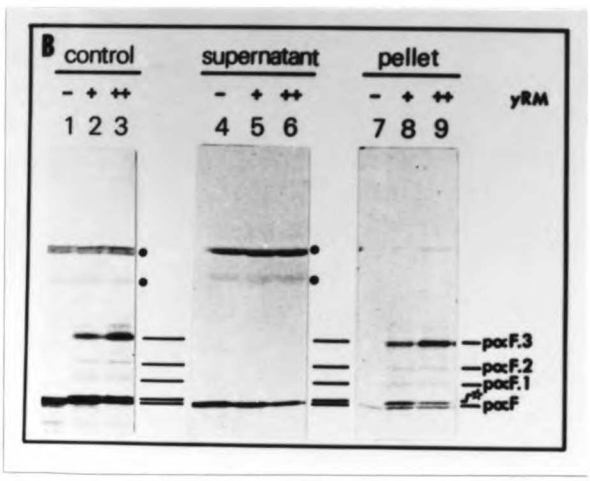
PANEL A: Proteinase K protection. pαF was translated in 20 µl reactions in the absence (lanes 1, 4, 7:, marked "-"), in the presence of 0.0075 A_{280} units (lanes 2, 5, 8:, marked "+") or in the presence of 0.025 A_{280} units yRM (lanes 3, 6, 9:, marked "++"). After one hour of translation the samples were either left untreated (lanes 1 - 3), or incubated with proteinase K in the absence (lanes 4 - 6) or presence (lanes 7 - 9) of 1% Triton X-100 (see Methods). The bands marked with dots are not related to pαF (see Figure 1).

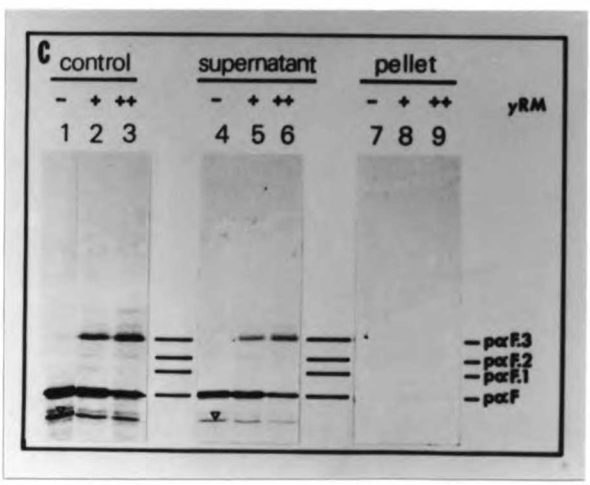
PANEL B: Sedimentation assay. Translation reactions were performed as in Panel A. They were then subjected to a brief centrifugation to generate a supernatant and a pellet fraction containing microsomal vesicles (see Methods). Shown are the total translation products (lanes 1 - 3), the supernatant fractions (lanes 4 - 5) and the translation products that pellet with the microsomal membranes (lane 7 - 9).

PANEL C: Alkaline carbonate extraction. Translations were performed as in Panel A. After translation 7 µl of translation products were analyzed directly by SDS-PAGE (lanes 1 - 3). Aliquots of 10 µl of the same translation reactions were carbonate extracted as described in Methods. The supernatants are shown in lanes 4 - 6, and the pellet fractions in lanes 7 - 9. The arrowheads indicate the position of globin included as a soluble protein control. The recovery is not complete due to losses in sample preparation from the relatively dilute supernatant fractions. Since, the pellets have been directly dissolved in sample buffer for SDS-PAGE, no losses could have occurred in the pellet fractions.

Depending on the translation extract used the translocation efficiency of in vitro synthesized pαF ranged from 10 - 40 % in a 1 hr incubation period. The reasons for this variability are presently unknown.


A
control
+ prot K
+prot K + Tx
yRM
1 2 3 4 5 6 7 8 9
pαF.3
pαF.2
pαF.1
pαF


B
control
supernatant
pellet
yRM
1 2 3 4 5 6 7 8 9
pαF.3
pαF.2
pαF.1
pαF


C
control
supernatant
pellet
yRM
1 2 3 4 5 6 7 8 9
pαF.3
pαF.2
pαF.1
pαF

with increasing yRM concentrations. After translation aliquots of these reactions were subjected to a brief high speed centrifugation to pellet the membranes. Comparison of the supernatant fractions (Fig. 3B, lanes 4 - 6) with the corresponding pellet fractions (Fig. 3B, lanes 7 - 9) revealed that the glycosylated and the signal peptide cleaved forms of pαF were quantitatively recovered in the pellet fractions; whereas, most of the unmodified pαF was recovered in the supernatant fractions. The small amount of pαF that was detected in the pellet fractions is likely to be an artifact of the fractionation procedure since it is found even in the samples where yRM were omitted (Fig. 3B, lane 7) and is degraded upon proteolytic digestion (Fig. 3A, lanes 5 and 6).

To test the possibility that glycosylated pαF remained associated with the lipid bilayer (as suggested by Julius et al, 1984), we performed an alkaline sodium carbonate extraction of the reaction products. Under these conditions the vesicles become converted into sheets, and only bona fide integral membrane proteins sediment with these membrane remnants (Fujiki et al., 1982, Davis and Model, 1985). The data shown in Figure 3C demonstrate that the glycosylated forms of pαF were only recovered in the supernatant fraction (Fig. 4C, lanes 5 and 6) indicating that they were not integrated into the lipid bilayer of yRM, but rather were released into the lumen of the vesicles.

DISCUSSION

We have established an *in vitro* assay for the translocation of prepro-α-factor (pαF) across the lipid bilayer of microsomal membranes of the yeast *Saccharomyces cerevisiae*. Similar assays have recently been developed by Waters and Blobel (1986) and Rothblatt and Meyer (1986a). This homologous assay requires two subcellular fractions: a soluble

cytoplasmic extract capable of in vitro protein synthesis prepared as previously described (Gasior et al, 1979) and a cytoplasmic membrane fraction that contains vesicles derived from the yeast endoplasmic reticulum or ER (described in methods). Our procedure for the isolation of yeast microsomes was derived from that of standard preparations of mammalian microsomes, which utilize canine pancreatic tissue as source. The method involves two steps (see methods for details): differential centrifugation to obtain a post-mithochondrial supernatant and a density gradient to purify and concentrate the microsomal vesicles. Although both procedures utilizes the same principles, the isolation of the yeast microsomal fraction presents two major problems relative to the preparation of mammalian microsomes. First, the endoplasmic reticulum is not a very extensive organelle in yeast and probably is mostly associated with the nuclear envelope (Schekman and Novick, 1982). Therefore, the yields of our preparations are comparatively low. Second, it appears that the density of membrane vesicles derived from the yeast ER is not markedly different from the density of other cellular membranes. Although we were capable of resolving the cytoplasmic membranes in two fractions on the percoll gradients, practically all the ER derived vesicles were recovered in the fraction of lower density (see fig. 2). The higher density membrane fraction contained no cytochrome c reductase (fig. 2) or protein translocation activity (data not shown). This fraction probably corresponds to plasma membrane associated with remnants of the cell wall and, therefore, is of higher density. Thus, the yeast ER vesicles could not be separated on the basis of a higher density due to bound ribosomes, as in the case for mammalian rough microsomal fraction (Walter and Blobel,

1983a). Therefore, our microsomal preparations are not as well enriched in rough ER vesicles as the mammalian microsomal fractions.

Despite these difficulties, the microsomal fraction obtained by our procedure was capable of faithfully reproducing in vitro the process of prepro-α-factor translocation across the ER membrane. To verify that translocation indeed occurred with fidelity in vitro we applied three independent criteria. First, the acquisition of endoglycosidase H-sensitive core-oligosaccharides on pαF, a modification that takes place exclusively on the ER of eukaryotic cells, was observed when yRM were present in the pαF translation reactions. This result indicates that the yRM prepared by our method fully retain the machinery required for protein glycosylation, although the pools of dolicol-oligosaccharides may be limiting as indicated. Second, the resistance of glycosylated and signal peptide cleaved forms of pαF, but not the precursor, to proteolysis indicates that these species have been sequestered inside the microsomal vesicles. Third, the co-sedimentation of the translocated products with the microsomal vesicles independently confirms their tight association with the translocated products. Thus, we have conclusively demonstrated that the yeast microsomal membranes obtained by our procedure are fully capable of in vitro reproducing the process of protein translocation across yeast ER and the early modifications to which secretory proteins are subject in the secretory pathway.

Finally, the efficiency of pαF translocation across yRM is comparable to that of its translocation or the translocation of mammalian secretory proteins across mammalian RM. This comes as no surprise considering that pαF possesses all the characteristics of a typical eukaryotic secretory protein (see chapter 1). However, other mammalian and yeast secretory

proteins are translocated at a more reduced efficiency across yRM (not shown, Hansen and Walter, 1988). At present the basis for this difference in translocation efficiency are unknown and probably a detailled description of the yeast translocation machinery will be required to fully understand this intriguing observation.

CHAPTER 3

ATP DEPENDENT POST-TRANSLATIONAL TRANSLOCATION OF PREPRO-α-FACTOR ACROSS MICROSOMAL MEMBRANES FROM THE YEAST *SACCHAROMYCES CEREVISIAE*

ABSTRACT

Using the experimental system described in chapter 2, we tested if translocation of pαF across yeast microsomal membranes can occur after protein synthesis has terminated. Our results showed that the process could still occur when yeast microsomes were added after protein synthesis was stopped by addition of cycloheximide or depletion of ribosomes. This is in striking contrast to the translocation of secretory proteins across mammalian microsomal membranes, where translation and translocation appear tightly coupled. The post-translational translocation reaction required protein components in the yRM fraction which could be inactivated by alkylation or proteolysis, was ATP-dependent and was insensitive to the presence of a variety of uncouplers and ionophores.

INTRODUCTION

In higher eukaryotic cells, secretory proteins are translocated across the ER membrane while they are being synthesized on ribosomes that are attached to the cytoplasmic side of the ER membrane (Palade, 1975). This fact and the observation that translocation can be reconstituted in vitro only when microsomal membranes are present during protein synthesis (Blobel and Dobberstein, 1975a&b), led to the conclusion that protein translocation across membranes was an strictly co-translational process. The signal recognition particle (SRP) and the SRP receptor are components of the translocation machinery in higher eukaryotes which are primarily involved in the targeting of ribosomes synthesizing secretory proteins to the ER membrane (see Walter et al, 1984; Walter and Lingappa, 1986). SRP binds with high affinity to ribosomes synthesizing secretory protein when the signal peptide emerges from the ribosome (Walter et al, 1981). This binding results in inhibition or a kinetic delay of the elongation of the

protein, a phenomena known as SRP dependent elongation arrest (Walter and Blobel, 1981b). Then SRP mediates the targeting of the ribosome to the ER membrane by binding to the SRP receptor (Walter and Blobel, 1981a; Gilmore et al, 1982; Meyer et al, 1982), a membraneous component of the translocation machinery. After reaching the membrane, SRP and SRP receptor recycle (Gilmore and Blobel, 1983), the elongation arrest is released (Walter and Blobel, 1981b) and a translocation competent ribosome-membrane junction is stablished. Translocation then proceeds while the secretory protein is being synthesized. Thus, the discovery of SRP, SRP receptor and their primary functions provided further evidence for co-translational mechanism of protein translocation.

In contrast protein translocation in other membranous systems can occur post-translationally. For example, some mitochondrial and chloroplast proteins are encoded by nuclear genes and, therefore, are synthesized on cytoplasmic ribosomes. These proteins are imported into the organelles and in this process they are translocated across membranes after they have been completely synthesized (Tzagollof and Myer, 1986; Schmidt and Mishkind, 1986). Also, bacterial secretion can occur post-translationally. β-lactamase can be translocated across the bacterial plasma membrane after it has been completely sinthesized, both in vivo (Koshland and Botstein, 1982) and in vitro (Muller and Blobel, 1984a). However, protein translocation across the ER membrane and the bacterial plasma membrane are conceptually analogous processes. Furtheremore, the nature of the signal sequence for both systems is remarkably conserved (Fraser and Bruce, 1978; Talmadge et al, 1980a&b; Muller et al, 1982), indicating that there is a common origin for both translocation machineries. Therefore, we were interested in determining if translocation

across the ER membrane of all eukaryotes, was a co-translational process and different from the bacterial translocation process. In the present work we have answered this question by using microsomal membranes from the yeast Saccharomyces cerevisiae that were prepared by our recently developed procedure (see chapter 1). We found that translocation of prepro-α-factor (pαF) across yeast microsomes can occur after its synthesis has been completed, at efficiencies comparable to those observed when the microsomes are present during protein synthesis.

MATERIAL AND METHODS

In vitro transcriptions and translations, preparation of yeast microsomes (yRM), protease protection and sedimentation assays were performed as described in chapter 2.

Alkylation of yRM.

yRM (1.5 units per µl) were incubated at 20 °C for 30 min in the presence of 5 mM N-ethylmaleimide followed by the addition of dithiothreitol to a concentration of 10 mM. Control membranes were prepared by addition of the dithiothreitol prior to N-ethylmaleimide addition. The translocation activity of these membranes was unaffected by this treatment.

Quantitation of translation products.

The bands corresponding to pαF and glycosylated pαF were quantitated by densitometry of the autoradiogram and compared to standards of known radioactivity. Absolute amounts of pαF were calculated based on an endogenous methionine concentration of 1 µM in the translation mix as determined by isotope dilution (Walter and Blobel, 1981).

Energy depletion.

In vitro protein synthesis was carried out for 60 min as described in chapter 2 followed by the addition of cycloheximide to a final concentration of 2 mM. The reaction was then centrifuged and desalted at 4 °C by two successive passages over ten sample volumes of Sephadex G-25 fine (3 min at 1600 x g) equilibrated in 150 mM ammonium acetate, 20 mM Hepes/KOH pH 7.4, 3.1 mM magnesium acetate, 2.4 mM dithiothreitol, 0.5% bovine serum albumin, 8% glycerol, 2.2 mM putrescine, 2 mM cycloheximide, 17 µg/ml aprotinin, and 2 µg/ml each of chymostatin, antipain, leupeptin, and pepstatin. Equivalent fractions of the centrifugal eluate were incubated for 60 min at 20 °C in the presence of yRM (0.05 A_{280} units per 15 µl) in the absence of energy substrates or after additions as described in the legend to Figure 2.

RESULTS

Post-translational translocation of pαF.

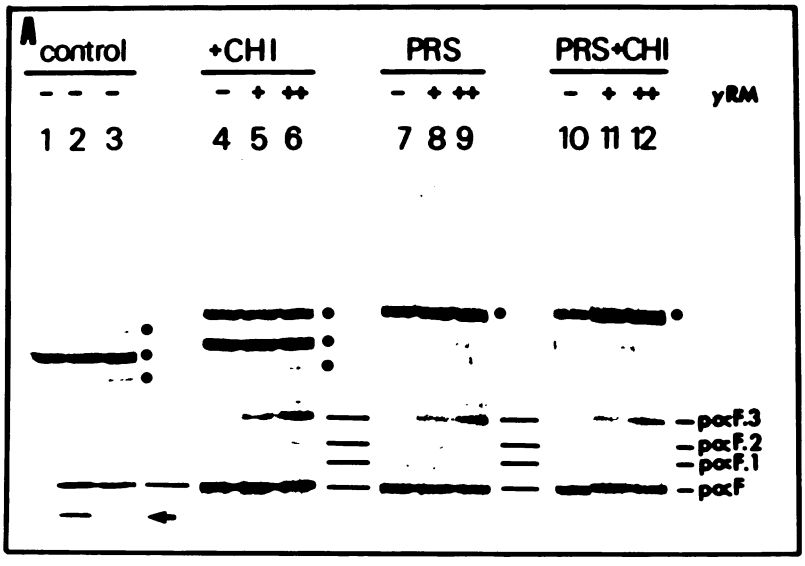
Post-translational translocation of pαF was demonstrated as follows. mRNA encoding pαF was translated in the yeast translation system for one hour (see methods of chapter 2). We then added mRNA coding for globin which was also translated upon further incubation (Fig. 1A, lane 2, arrow). However, when cycloheximide was added together with the globin mRNA, no globin was synthesized (Fig. 1A, lane 3 to 6), indicating that cycloheximide effectively inhibited protein synthesis. Addition of yRM to the cycloheximide - inhibited translation system resulted in the formation of fully glycosylated pαF (Fig. 15A, lanes 5 and 6). In order to ascertain that the ribosome is not participating in the observed translocation, we prepared a ribosome-depleted supernatant containing in vitro synthesized pαF (prepared by a one hour spin in a Beckman airfuge, see Methods). This

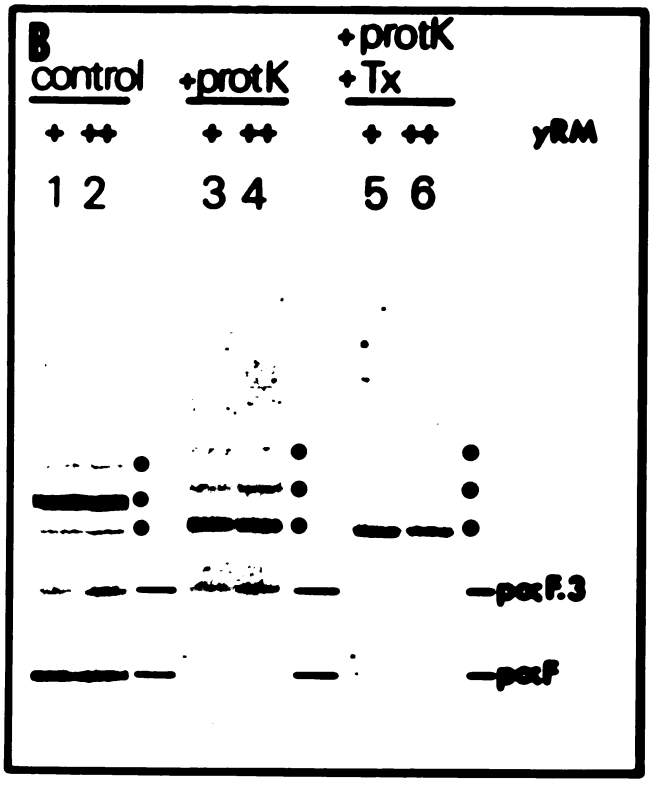
Figure 1: Post-translational translocation of pαF.

PANEL A: pαF mRNA was translated for one hour as described in Methods. After this time further translation was inhibited by the addition of cycloheximide to 2 mM (lanes 3 - 6, marked "CHI"), or by removing the ribosomes by centrifugation (lanes 7 - 9, marked post-ribosomal supernatant "PRS"), or both (lanes 10 - 11, marked "PRS + CHI"). After these treatments yRM were added to 0.025 A_{280} units/20 µl (lanes 6, 9, 12; marked "++"), or to 0.0075 A_{280} units/20 µl (lanes 5, 8, 11, marked "+"), or no yRM addition (lanes 3, 4, 7, 8:, marked "-"). At the same time globin mRNA was also added in order to ascertain that further protein synthesis was indeed inhibited. The reactions were then incubated for an additional hour at 20 °C.

Lanes 1-3 show control reactions in the absence of yRM. Lane 1: cycloheximide was added at the beginning of the translation showing that it completely abolishes pαF synthesis. In lane 2 translation was carried out for one hour, then globin mRNA was added and the incubation was continued for an additional hour. Translated globin is marked with an arrow. Lane 3 is identical to lane 2, except that cycloheximide was added together with globin mRNA. The bands marked with dots are not related to pαF (see Figure 1 of Chapter 2).

PANEL B: Protease protection of post-translationally translocated pro-α-factor. The reactions shown in lanes 1 and 2 correspond to reactions shown in Panel A, lanes 5 and 6. After the post-translational incubation the samples were treated with proteinase K under the same conditions as described in Figure 4, panel A either in the absence (lanes 3 and 4) or presence (lanes 5 and 6) of 1% Triton X-100.


A
control
+CHI
PRS
PRS+CHI
- - -
- + ++
- + ++
- + ++
yRM
1 2 3
4 5 6
7 8 9
10 11 12
pαF.3
pαF.2
pαF.1
pαF


B
control
+protK
+protK
+Tx
+ ++
+ ++
+ ++
yRM
1 2
3 4
5 6
pαF.3
pαF

supernatant no longer promoted protein synthesis (Fig. 1A, lane 7, note the absence of globin),: however, pαF was still translocated when yRM were added in the absence (Fig. 1A, lanes 8 and 9) or in the presence (Fig. A, lanes 11 and 12) of cycloheximide. Post-translationally translocated pαF was properly sequestered inside the yRM vesicles as shown by protease protection (Fig. 1B, lanes 3 and 4). Kinetic analysis (not shown) of the reaction showed that after a short (about 3 min) lag phase the post-translational translocation of pαF was linear with time for roughly 60 min. From a quantitative comparison of the data from Figures 3A of chapter 2 and 1A of this chapter, we conclude that the post-translational translocation reaction of pαF occurred with comparable efficiency as the co-translational process (3.2 fmoles pαF translocated per 0.025 A_{280} units of yRM co-translationally, compared to 2.8 fmoles post-translationally).

The experiments shown in Fig. 2 demonstrate that the post-translational translocation of pαF is not a spontaneous process, as suggested for membrane proteins (for review, see Wickner, 1979) or for secretory proteins (von Heijne, 1979), but rather requires the participation of membrane proteins, as well as the presence of an energy source. As shown in Figure 2 (lanes 1 - 3) alkylation of yRM with N-ethylmaleimide inhibited the reaction. Furthermore, yRM could be inactivated by trypsin digestion (not shown, digestions were for 30 min at 0 ^{o}C at 500 µg/ml trypsin), thereby providing additional support for the conjecture that cytoplasmically exposed membrane proteins are essential. We presently cannot rule out the possibility that core-oligosaccharide transferase contains a cytoplasmically exposed domain that renders the enzyme susceptible to alkylation or proteolytic inactivation. However, no translocated unglycosylated pαF was detected (not shown).

Our ability to uncouple translocation from protein systhesis allowed us to characterize directly the energy requirement of the reaction. We depleted a translation extract containing in vitro synthesized pαF of small molecules by gel filtration. Upon subsequent addition of yRM no translocation was observed (Fig. 2, compare lanes 4 and 5). Readdition of ATP and a regenerating system restored translocation (Fig. 2, lane 7), whereas non-hydrolysable ATP analogs do not support the reaction (Fig. 2, lane 6). This reaction was completely inhibited by the addition of E. Coli glycerol kinase (which is absolutely ATP-specific; Hayashi and Lin, 1967; Thorner and Paulus, 1973) (Fig. 2, lane 8), demonstrating conclusively that ATP is essential. Addition of the regenerating system alone, or addition of GTP (1 mM) also promoted translocation, albeit at reduced efficiency (not shown). This is likely to be due to the regeneration of ATP from residual protein-bound ATP or ADP, since inclusion of E.Coli glycerol kinase also abolished these reactions.

In contrast to post-translational protein translocation across the prokaryotic plasma membrane or the mitochondrial envelope, we found that a variety of uncouplers and ionophores has no effect on the translocation reaction. Specifically, translocation was not inhibited by the proton ionophores SF6847 and FCCP, the potassium ionophore valinomycin and proton/potassium ionophore nigericin alone or in combinations (Fig. 2, lane 9 and 10 and legend).

Translocation of pαF in heterologous systems.

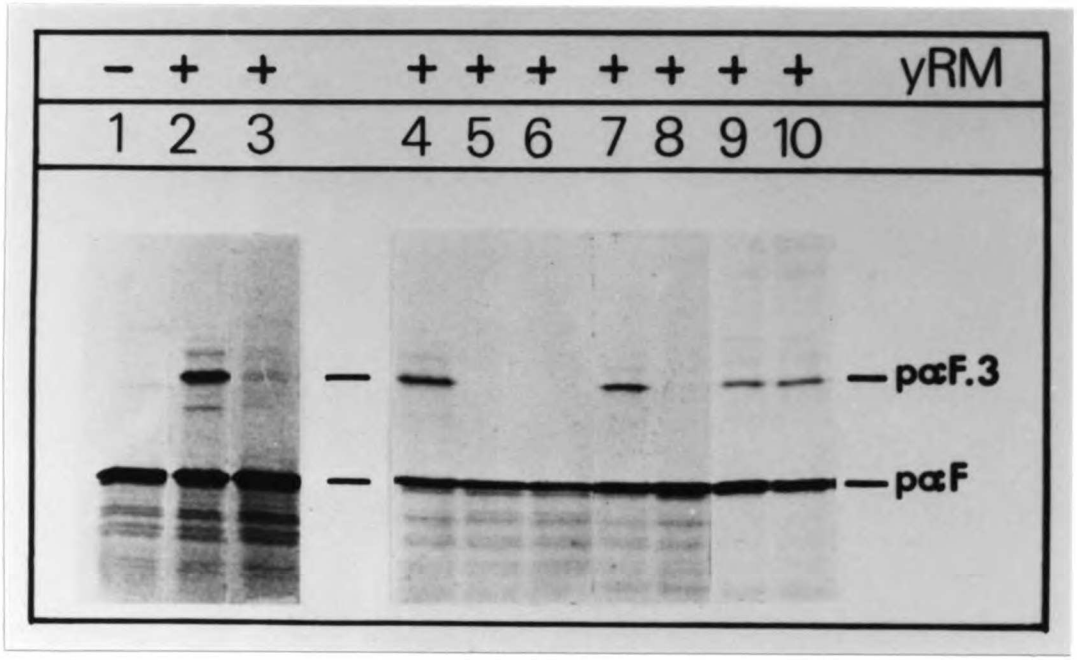
Translocation of secretory proteins across mammalian microsomal membranes occurs co-translationally. Our finding of a post-translational mechanism for pαF translocation therefore raises the question whether this apparent difference results from a special property of pαF or whether it is

Figure 2: Requirements for post-translational translocation.

Pro-α-factor was synthesized in a yeast translation extract at 20 °C for 60 minutes followed by inhibition of protein synthesis with 2 mM cycloheximide. Ten microliter aliquots were removed and incubated an additional 60 minutes in a final volume of 15 µl in the absence (lane 1) or presence (lanes 2-10) of 0.05 A_{280} units of yRM with the following variations: lanes 1 and 2, control incubations, lane 3, yRM were alkylated with N-ethylmaleimide (see Methods): lane 4, control incubation. For the experiments shown in lanes 5 - 10 the translation extract was desalted on Sephadex G-25 (see Methods) prior to post-translational incubation in the presence of 0.05 A_{280} units of yRM and the following additions: lane 5: no additions, lane 6: 2 mM of the non-hydrolyzable ATP analog adenosine-5'-[β,γ-imido]-triphosphate was included (Identical results were obtained when the [β,γ-methylene] or the [γ-thio] deriviatives of ATP were included at 2 mM (not shown)), lane 7: 1 mM ATP and 17.5 mM creatine phosphate

were included, lane 8: same as lane 7 except that 2.5 micrograms of E. coli glycerol kinase were included, lane 9: control incubation containing 0.4 % dimethylsulfoxide, lane 10: as lane 9 except that 20 µM SF6847 (a benzylidenemalononitrile proton ionophore), 10 µM nigericin, 10 µM valinomycin and 25 mM potassium acetate were included (each of the listed ionophores were also ineffective to inhibit pαF translocation when assayed by themselves or in pairwise combinations. Likewise, the addition of 50 µM FCCP had no effect (not shown)).

Quantitations for lanes 4 - 10 (amount pαF translocated in fmoles): lane 4: 2.5, lane 7: 2.3, lane 9: 1.8, lane 10: 1.8, lanes 5, 6, 8: < 0.2.


- + + + + + + + + yRM
1 2 3 4 5 6 7 8 9 10
pαF.3
pαF

due to inherent differences in the translocation machineries of yeast and mammalian ER membranes. We therefore tested pαF as a substrate in a translocation assay containing canine SRP and salt-extracted (i.e. SRP-depleted) microsomes (cKRM). pαF mRNA was translated in a wheat germ extract (Figure 3A, lane 2). As for other secretory proteins, translation was arrested if canine SRP was present co-translationally (Figure 3A, lane 3). Translation of mRNA encoding the cytoplasmic protein globin was not affected (not shown). Addition of cKRM in the presence of SRP resulted in release of the elongation arrest and translocation of the synthesized pαF across the membrane. The resulting glycosylated form of pαF (Fig. 3A, lane 4) migrated slightly slower on the gel than glycosylated pαF produced in the presence of yRM (see legend of fig.3). The translocation was dependent on the presence of SRP, since cKRM in the absence of SRP were not sufficient to effect translocation (Fig. 3A, lane 5). If yRM were present co-translationally, pαF synthesized in the wheat germ system was efficiently translocated (Fig. 3A, lane 6). However, when translocation across yRM was assayed in the presence of SRP, the yRM fraction was apparently not able to release the SRP-induced elongation-arrest and as a consequence correspondingly less translocated pαF was obtained (Fig. 3A, lane 7). Thus the mechanism of pαF translocation across the mammalian microsomal membrane seems to be indistinguishable from that of other secretory proteins. yRM can function in the heterologous wheat germ system, yet are unable to interact productively with mammalian SRP.

If cKRM alone were added post-translationally to the wheat germ extract containing pαF, no translocation was detected (Fig. 3B, lanes 1 and 2). However if canine SRP was present during this incubation, a trace amount of glycosylated pαF was observed (Fig. 3B, lane 3, arrow). The

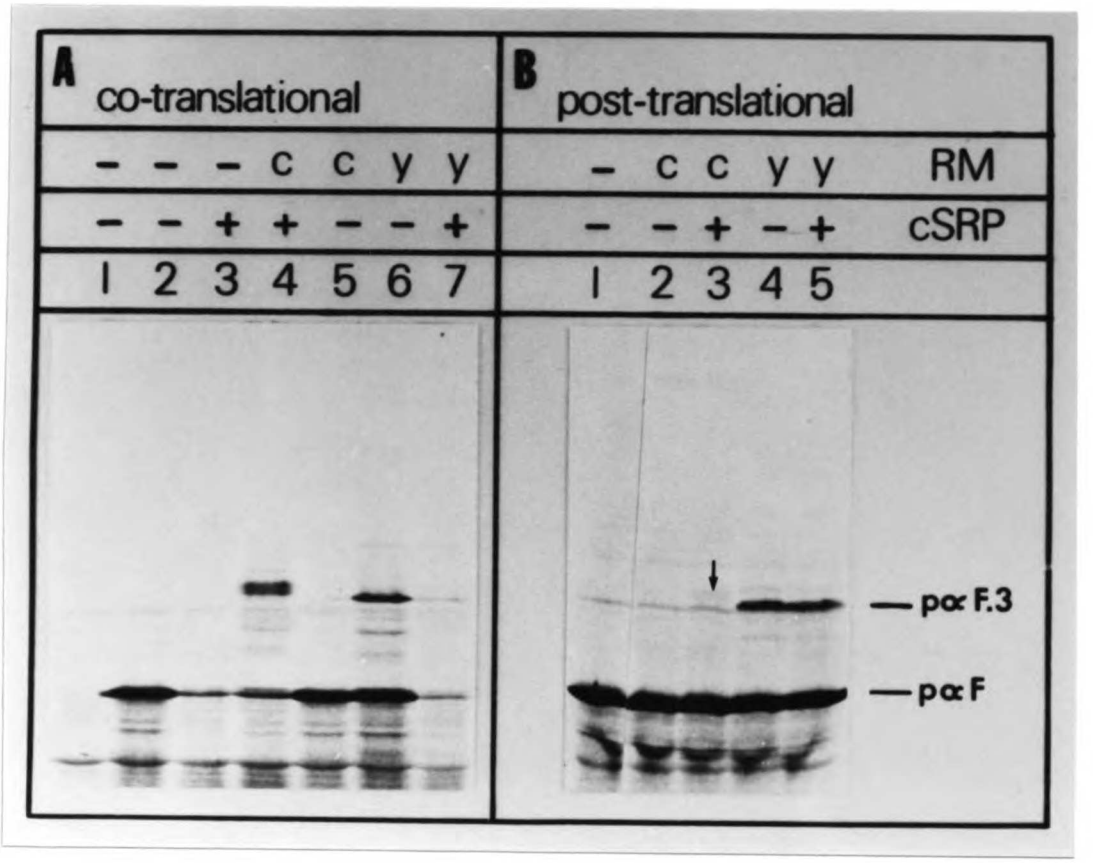
Figure 3: Translation and translocation in heterologous systems.

Translations in a wheat germ cell-free extract (Walter and Blobel, 1980) were performed for 60 min at 20 °C in the absence (Panel A, lane 1) or in the presence (Panel A, lane 2-7, panel B, lanes 1-5) of mRNA encoding pαF. PANEL A: At the beginning of translation, 10 µl reactions were supplemented with 45 nM canine SRP (cSRP, lanes 3, 4, 7), 2 equivalents (Walter and Blobel, 1980) of canine salt- and EDTA-extracted, i.e. SRP- depleted microsomes (cKRM, denoted: "c") (lanes 4 and 5) and 0.025 A_{280} units of yRM (denoted: "y")(lanes 6 and 7), respectively. PANEL B: Translation was terminated by the addition of 2 mM cycloheximide. Aliquots of 10 µl were further incubated for 60 min at 20 °C with the following post-translational additions: 45 nM cSRP (lanes 3 and 5), 2 equivalents of cKRM (lanes 2 and 3) and 0.025 A_{280} units of yRM (lanes 4 and 5), respectively.

Note that the autoradiogram shown in panel B was exposed four-fold longer. The absolute amounts of pαF translocated across yRM

(0.025 A_{280} units) co- versus post-translationally were: Panel A, lane 6: 15 fmoles, panel B, lane 4: 2.7 fmoles. In panel B, lane 3 the glycosylated form of pαF is marked with an arrow.

The difference in electrophoretic mobility of the glycosylated pαF factor produced by canine membranes compared to that produced by yeast membranes could result from the absence of glucose residues from the core-oligosaccharides either due to trimming of the glucose residues after transfer, or alternatively due to transfer of non or partially glucosylated oligosaccharide. Yeast core-oligosaccharide transferase is less strict than the mammalian enzyme in its requirement for the presence of glucose residues on the dolichol-linked oligosaccharide (Trimble et al., 1980). A minor species migrating slightly above glycosylated pF with similar mobility as that produced by cKRM is also visible in most other figures.


A
co-translational
B
post-translational
– – – c c y y
– – + + – – +
1 2 3 4 5 6 7
– c c y y
– – + – +
1 2 3 4 5
RM
cSRP
pαF.3
pαF

identity of this band was confirmed by immunoprecipitation and its cosedimentation with the membrane vesicles (not shown). In contrast, yRM were able to translocate pαF more efficiently, when added post-translationally to the wheat germ extract both in the presence or absence of canine SRP (with SRP added post-translationally) (Fig. 3B, lanes 4 and 5), although the absolute amount of translocated pαF was reduced when compared to the co-translational incubation (Fig. 3A, lane 6). We conclude that the post-translational translocation of pαF appears to occur efficiently only across yeast microsomal membranes and therefore reflects a special (or at least significantly enhanced) property of the yeast translocation system.

DISCUSSION

Prepro-α-factor (pαF) has a very well characterized biosynthetic pathway (Julius et al., 1984, Schekman, 1985) that resembles very much that of "classical" mammalian secretory proteins. The translocation of secretory proteins across mammalian ER membranes, occurs only when the protein is being synthesized. We were therefore surprised to discover that the translocation of pαF across the yeast ER membrane can occur post-translationally and thus does not require the strict coupling between protein synthesis and membrane translocation that is obligatory for mammalian secretory proteins. To determine whether these differences are due to a special property of pαF per se or whether the translocation machineries in yeast and mammalian microsomal membranes function differently, we performed a series of heterologous cross mixing experiments using a wheat germ translation system co-translationally supplemented with mammalian SRP and/or mammalian microsomal vesicles (Fig. 3A). Like other presecretory proteins, co-translational translocation of pαF across this

membrane system strictly required targeting of pαF by the SRP SRP receptor system. In contrast to other presecretory proteins, however, we observed that pαF can also cross mammalian microsomal membranes in an SRP-dependent reaction post-translationally. In spite of the very low efficiency of this reaction, we have to conclude that pαF differs from other presecretory proteins in this respect.

A variety of proteins can be secreted post-translationally across the plasma membrane of prokaryotic cells. For example, this has been documented for β-lactamase both in vivo (Koshland and Botstein, 1982) and in vitro (Muller and Blobel, 1984). However, as for pαF, efficient translocation of β-lactamase across mammalian microsomal membranes required SRP and micrososmal vesicles to be present during translation; no translocated β-lactamase was detected when translocation was assayed post-translationally (Muller et al. 1982). Thus to work efficiently, the mammalian translocation machinery requires translation of both proteins to be coupled to translocation. In contrast, pαF could be translocated across yRM post-translationally with an efficiency comparable to that observed co-translationally. Post-translational translocation also was independent of whether pαF was synthesized in the homologous yeast system or in the wheat germ extract. Therefore it is unlikely that the yeast translation system contributes specialized, soluble factors required for this process. yRM, however, have the ability to catalyze this process very efficiently.

It has been demonstrated that the "pro"-region of pαF is sufficient to cause other, unrelated proteins to become translocated. DNA encoding the "prepro"-peptide of pαF has been fused to genes encoding many secretory (for a recent review see Smith et al, 1985), as well as cytoplasmic proteins (e.g superoxide dismutase (P. Valenzuela, personal

communication)), and shown to cause secretion of the resulting fusion proteins by yeast *in vivo*. This strongly suggests that the machinery used for pαF translocation is not specific for this particular protein, but rather is sufficiently pliable to accept completely heterologous, even cytoplasmic proteins. We need to keep in mind, however, that the demonstration that translocation of pαF can occur post-translationally *in vitro* does not necessarily imply that this translocation mode is operating *in vivo* (or even *in vitro* if yRM are present during translation). Rather the degree of coupling between the protein synthesis and translocation may depend on the relative *in vivo* rates of the two respective processes and, if translocation is rapid, may result in a strictly co-translational translocation. Our *in vitro* assay conditions, where the system is artificially deprived of microsomal membranes, may have allowed us to uncouple the two processes. Our finding that pF can be translocated post-translationally does therefore not necessarily suggest that the above mentioned fusion proteins (or other yeast secretory proteins that have not yet been tested) will show the same property.

This ability to uncouple protein synthesis from translocation allowed us to characterize the translocation reaction of pαF across yRM in more detail. For example, the ribosome itself is not directly involved in this process. It follows directly that it cannot be energy from the elongation process itself that drives the nascent chain across the membrane. Rather, the post-translational translocation reaction is an ATP dependent process (Fig. 2). Also, it is noteworthy that so far no electrochemical potential across microsomal membranes has been detected, and that various ionophores have no effect on in vitro protein translocation in yeast (Fig. 2) or higher eukaryotic systems (P. Walter, unpublished). This is in contrast to

the prokaryotic plasma membrane (Bakker and Randall, 1984) and the mitochondrial envelope (Gasser, et al., 1982, Pfanner and Neupert, 1985), where post-translational protein translocation is driven by a protonmotive force and a membrane potential, respectively, but seems to resemble protein import into chloroplasts which also is an ATP-dependent process (Grossman et al., 1980).

Recently other evidence has been provided that mammalian microsomal membranes in principle are capable of accept and translocate proteins post-translationally. It was shown that the cytoplasmic protein globin fused to signal peptide could be translocated across canine rough microsomal membranes post-translationally (Perara and Lingappa, 1985, Perara et al. 1986). This reaction was shown to be efficient, but required that the nascent chain was still associated with the ribosome for proper targeting. Mueckler and Lodish (1986a) demonstrated that the amino-terminal domain of the glucose transporter (an integral plasma membrane protein) can also translocate across mammalian microsomal membranes post-translationally, albeit very inefficiently. In these cases, as well as for pαF, the way in which a completely synthesized protein crosses a membrane remains obscure. pαF contains no significantly hydrophobic regions (other than a typical signal sequence) or amphipathic stretches that might suggest a direct interaction with the lipid bilayer. The protein may either become actively unfolded (or somehow be prevented from folding during synthesis) and then be translocated as a linear polypeptide chain. Alternatively, the translocation machinery may be able to accept whole domains of prefolded proteins. The latter case would imply that the signal sequence causes a large but selective pore to form in the membrane that facilitates protein translocation.

In summary, it is unlikely that protein translocation in yeast is fundamentally different from that in mammalian cells. The apparent differences may result from the degree of coupling between translation, targeting and translocation. In addition, certain proteins may retain the ability to be translocated after they have been completely synthesized, while others may strictly require translocation before their synthesis has advanced beyond some critical point. Co-translational translocation could be necessary if, for example, a particular folding or oligomerization renders a protein incompatible with subsequent translocation. Alternatively, complete synthesis of certain proteins (such as potentially harmful nucleases, proteases, etc) in the cytoplasmic compartment could be detrimental for the cell. Thus different proteins for a variety of reasons may have a more or less stringent requirement for a coupling of translation to translocation. We speculate that the translocation machinery in the ER membrane has evolved means (such as the postulated SRP-mediated elongation arrest) to cope with such particular requirements.

CHAPTER 4

FULL-LENGTH PREPRO-α-FACTOR CAN BE TRANSLOCATED ACROSS THE MAMMALIAN MICROSOMAL MEMBRANE ONLY IF TRANSLATION HAS NOT TERMINATED.

ABSTRACT

We have previously shown that fully synthesized prepro-α-factor (ppαF), the precursor for the yeast pheromone α-factor, can be translocated post-translationally across yeast rough microsomal (RM) membranes from a soluble, ribosome-free pool. We show here that this is not the case for translocation of ppαF across mammalian RM. Rather we found that a small amount of translocation of full-length ppαF is observed, but is solely due to polypeptide chains that were still ribosome-bound and covalently attached to tRNA, i.e. not terminated. In addition, both SRP and SRP receptor are required, i.e. the same targeting machinery that is normally responsible for the coupling between protein synthesis and translocation. Thus, the molecular requirements for targeting are distinct from post-translational translocation across yeast RM. As termination is generally regarded as part of translation, the translocation of full-length ppαF across mammalian RM does not occur "post-translationally", albeit independent of elongation. Most other proteins for which post-translational translocation across mammalian RM was previously claimed fall into the same category in that ribosome attachment as peptidyl-tRNA is required. To clearly separate these two distinct processes, we suggest that the term "post-translational" be reserved for those processes that occur in the complete absence of the translational machinery. We propose the term "ribosome-coupled translocation" for the events described here.

INTRODUCTION

In higher eukaryotes, secretory and some integral membrane proteins are synthesized on ribosomes attached to the endoplasmic reticulum (ER) membrane (Palade, 1975). This observation, together with the finding that efficient translocation could be obtained *in vitro* only for nascent

proteins during their synthesis (Blobel and Dobberstein, 1975), led to the conclusion that translocation across mammalian RM is a strictly "co-translational" process. The signal recognition particle (SRP) has a high affinity for ribosomes synthesizing secretory proteins (Walter et al, 1981), and in conjunction with the SRP receptor (Walter and Blobel, 1981; Gilmore et al, 1982a and b; Meyer et al, 1982) was determined to function as the adapter between the translation and translocation machinery, thus providing further support for this hypothesis.

In contrast, it has recently been reported that translocation can occur, albeit at reduced efficiency, for several fully synthesized proteins across mammalian RM after further translation has been inhibited with cycloheximide (Hansen et al, 1986; Caulfield et al, 1986; Mueckler and Lodish, 1986a and b; Perara et al, 1986; Chao et al, 1987). These findings led to the conclusion that translocation of proteins across mammalian ER membranes is not necessarily coupled to translation and, thus, would resemble the process of translocation across yeast ER membranes which can occur efficiently post-translationally (Hansen et al, 1986; Waters and Blobel, 1986; Rothblatt and Meyer, 1986). Paradoxically, in control experiments it was noted that SRP, thought to function to target the ribosomes synthesizing secretory proteins to the ER, appeared to be required for this elongation-independent translocation process across mammalian membranes (Hansen et al, 1986; Mueckler and Lodish, 1986a). In contrast, SRP, SRP receptor or ribosomes do not appear to be required for post-translational translocation across yeast RM. This prompted us to further analyze in molecular detail the translocation of full-lengh proteins across mammalian RM to address these apparent differences. To compare directly the requirements for translocation across the yeast and

mammalian RM, we used ppαF as substrate since this preprotein retains its translocation competency after its termination when assayed for post-translational translocation across yeast RM.

MATERIALS AND METHODS

Rough microsomal membranes (RM; Walter and Blobel, 1983a), salt extracted RM (K-RM; Walter and Blobel, 1983b), trypsin treated RM (T-RM; Gilmore et al, 1982a), SRP (Walter and Blobel, 1983b) and the 52 kD cytoplasmic fragment of the SRP receptor α-subunit (SRαf; Siegel and Walter, 1985) were prepared as previously described. Synthetic ppαF mRNA (Hansen et al, 1986) was translated in a wheat germ extract (Erickson and Blobel, 1983). After 30 min, cycloheximide was added to 1 mM to inhibit further elongation. Translocation reactions were initiated by the addition of 5 equivalents (Walter and Blobel, 1980) of microsomal membranes (either RM, K-RM or T-RM) and SRP and/or SRαf, and the incubation was continued for 30 min at 26 °C. The total volume of each reaction was 50 µl, containing 40 µl of translation extract. The ionic conditions of the translocation reactions were kept constant in all cases. After the second incubation, the microsomal vesicles were collected by centrifugation through a 50 µl 0.5 M sucrose cushion as previously described (Hansen et al, 1986). The pellets (containing the RM fraction) were dissolved directly in sample buffer and subjected to SDS-PAGE on 10-15 % gradient gels. The gels were exposed to X-Omat AR Kodak film after fluorography with 2,5-diphenyloxazole.

Precipitations of peptidyl-tRNA with the cationic detergent hexadecyltrimethylammonium bromide (CTABr) were carried out as previously described (Gilmore and Blobel, 1985). Deacylation of nascent chains was carried out by the addition of 0.1 N KOH and incubation at 37 °C for 15

min.; the solution was then neutralized with acetic acid prior to CTABr precipitation. The post-ribosomal supernatant (PRS) and ribosomal pellet (RP) fractions were prepared as described (Hansen et al, 1986).

Removal of the low molecular weight molecules for the energy requirement experiments was performed as follows. After synthesis of ppαF was inhibited by the addition of cycloheximide, a 0.5 ml translation reaction was chromatographed on a 5 ml Sephadex G25 column equilibrated in translation buffer without ATP, GTP and creatine phosphate. New cycloheximide was added to the eluate, which was then aliquoted for translocation reactions.

RESULTS

Translocation of Full-Length ppαF Across Mammalian Microsomes Requires SRP and SRP Receptor.

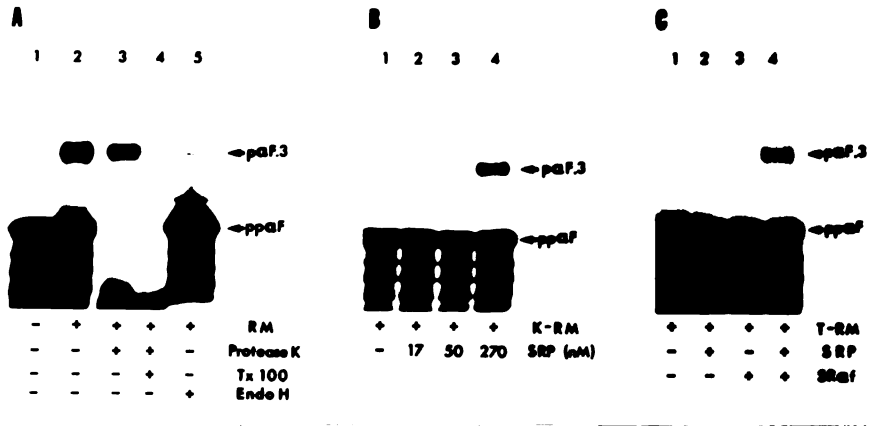
To determine the SRP dependence of the translocation of full-length ppαF, we chose the wheat germ translation system which lacks endogenous SRP (Walter and Blobel, 1980; Meyer et al, 1982). Following translation of ppαF mRNA for 30 min at 26 ^{o}C, further protein elongation was inhibited by the addition of 1 mM cycloheximide. After a second 30 min incubation in the presence of canine pancreatic RM (containing endogenous SRP), the membranes were sedimented by centrifugation and the pellet fraction subjected to SDS-PAGE. In the presence of RM (Fig. 1A, lane 2) we observed that a small fraction (about 1-2 %) of ppαF sedimented as the glycosylated form (termed pαF.3; note that in addition to glycosylation the signal sequence of translocated ppαF is cleaved by signal peptidase (Waters et al, 1988), indicating that it had been translocated across the lipid bilayer. Translocation was confirmed by the resistance of pαF.3, but not cosedimenting ppαF, to externally added proteases (Fig. 1A, lane 3). As

FIGURE 1: Tranlocation of full-length ppαF across mammalian ER membranes in the absence of protein elongation requires SRP and SRP receptor.

PANEL A: Translocation of full-length ppαF across mammalian ER membranes can occur in the absence of elongation. Translocation reactions (see below) were carried out in the absence (lane 1) or presence of 5 eq of RM (lanes 2 to 5). Microsomal vesicles were then collected by centrifugation (see below) and subjected to SDS-PAGE. The samples in lanes 3 and 4 were treated with protease K prior to RM sedimentation (Hansen et al, 1986). Triton X-100 (Tx 100, 0.4 %) was added together with the protease to the sample in lane 4. The sample in lane 5 was treated with endoglycosidase H (Endo H; Hansen et al, 1986) after RM sedimentation.

PANEL B: Translocation of full-length ppαF in the absence of elongation is dependent on SRP. The reactions were carried out as in panel A with the exception that K-RM (depleted of SRP) were added instead of RM (lanes 1 to 4). Purified SRP at 17 nM (lane 2), 50 nM (lane 3) or 270 nM (lane 4) was added together with K-RM.

PANEL C: Translocation of full-length ppαF in the absence of elongation is dependent on SRP receptor. The reactions were carried out as in panel A with the exception that T-RM (depleted of SRP and SRαf) were added (lanes 1 to 4). SRP was added (225 nM) to the reactions in lanes 2 and 4. Purified SRαf (100 nM) was added to reactions in lanes 3 and 4.


A
1 2 3 4 5
◄paF.3
◄ppaF
- + + + + RM
- - + + - Protease K
- - - + - Tx 100
- - - - + Endo H
B
1 2 3 4
◄paF.3
◄ppaF
+ + + + K-RM
- 17 50 270 SRP (nM)
C
1 2 3 4
◄paF.3
◄ppaF
+ + + + T-RM
- + - + SRP
- - + + SRaf

expected, pαF.3 was completely digested by protease if the permeability barrier of the membrane was disrupted by the addition of detergent (Fig. 1A, lane 4). The identity of pαF.3 was further verified by demonstrating its sensitivity to endoglycosidase H (Fig. 1A, lane 5). The presence of ppαF in the pellet fractions (about 5% of the total ppαF synthesized is sedimented) was likely due to non-specific aggregation: it was observed even in the absence of added RM (Fig. 1A, lane 1) and the sedimented ppαF was completely susceptible to proteolytic degradation (Fig. 1A, lane 3). Thus, the results presented in Figure 1A demonstrate that translocation of full-length ppαF across mammalian ER can occur (though only at 1-2 % efficiency) in the absence of elongation as we previously noted (Hansen et al, 1986), although others failed to detect any translocation (Rothblatt and Meter, 1986). It is important to note that if yeast RM instead of mammalian RM are added during the second incubation, ppαF is efficiently (30 - 50%) translocated as was previously described (Hansen et al, 1986; Waters and Blobel, 1986; Rothblatt and Meyer, 1986). Hence, no factors that may be required for the post-translational process were limiting in the extract.

We tested if this elongation independent translocation of ppαF across mammalian RM is dependent on the known components of the translocation machinery, such as SRP and SRP-receptor. The results shown in Figure 1B indicate that the process is SRP-dependent. When salt-extracted RM (K-RM), which are thus depleted of SRP, were added in the second incubation, no pαF.3 was obtained (Fig. 1B, lane 1). If, in addition to K-RM, the reactions were supplemented with increasing concentrations of purified SRP (Fig. 1B, lanes 3-4) translocation was restored and correspondingly increasing amounts of pαF.3 were obtained. As would be expected from this

result, we demonstrate in Figure 1C that SRP receptor is also required. For this purpose, the 52 kD cytoplasmic domain of the SRP receptor α-subunit (SRαf, Tajima et al, 1986) was removed by mild proteolysis of K-RM with trypsin yielding inactive K-RM (T-RM, Fig. 1C, lane 4). The activity of T-RM was recovered by reconstitution of SRP-receptor accomplished by addition of the purified SRαf (Fig. 1C, lane 4). Thus, we can conclude that the translocation of full-length ppαF across mammalian RM requires both SRP and its receptor.

SRP is thought to bind to the signal peptide of a nascent protein after it has emerged from the ribosome (Walter et al, 1981). The SRP-ribosome-nascent chain complex is then targeted to the ER membrane by the specific interaction of SRP with SRP receptor (Walter and Blobel, 1981; Gilmore et al, 1982a and b; Meyer et al, 1982) and translocation is initiated. Signal recognition by SRP has been shown by direct crosslinking experiments but could only be demonstrated on nascent chains emerging from ribosomes and not after their release (Krieg et al, 1986; Kurzchalia et al, 1986). We were therefore interested in determining if the SRP and SRP receptor-dependent translocation of full-length ppαF also requires the functional involvement of the ribosome, even though elongation was no longer taking place.

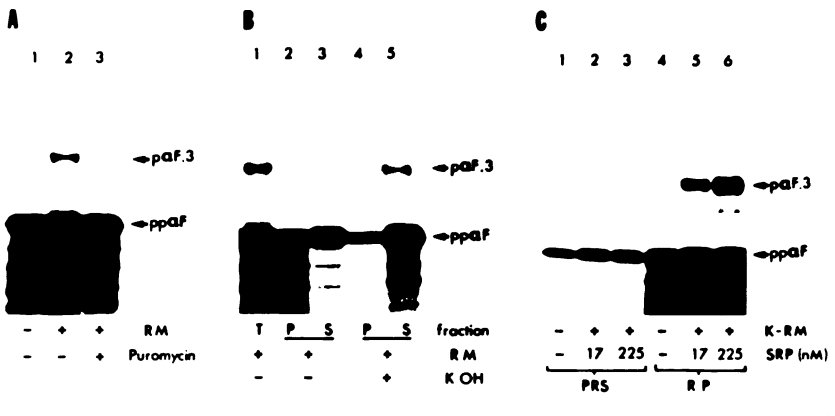
Translocation of Full-Length ppαF Across Mammalian Microsomes Occurs Only if Translation Has Not Terminated.

We tested if the ribosome was required for translocation of full-lengh ppαF by three distinct criteria (Figure 2). First, we found that preincubation of the translation extract containing ppαF with puromycin, an antibiotic that inhibits translation by releasing nascent chains from ribosomes, abolishes translocation (Fig. 2A, lane 3). This suggested that

FIGURE 2: Translocation of full-length ppαF across the mammalian ER membrane occurs only if translation has not terminated.

PANEL A: Translocation of full-length ppαF cannot occur if translation is inhibited with puromycin. Translocation reactions were carried out as in figure 1 in the absence (lane 1) or presence of RM (lanes 2 and 3). Puromycin (1 mM) was added instead of cycloheximide to the reaction in lane 3. To this reaction, RM was added after an additional 10 min incubation at 26 ^{o}C in the presence of puromycin.
PANEL B: CTABr precipitation of the products of translocation in the absence of elongation. Reactions carried out as in figure 1A were fractionated by CTABr precipitation. The total (T) products of reactions in the presence (lane 1) of RM are shown. The CTABr pellets (P, lanes 2 and 4) and supernatants (S, lanes 3 and 5) of reactions identical to the one in lane 1 are shown. The samples shown in lanes 4 and 5 were deacylated by treatment with base prior to CTABr precipitation.
PANEL C: Translocation of ppαF in the absence of elongation is associated with the ribosomal fraction. After translation of ppαF the

sample was fractionated into post-ribosomal supernatant (PRS) and ribosomal pellet (RP) fractions. The RP was resuspended in the same buffer and cycloheximide was added to both fractions. Translocation reactions were carried out as in figure 1A. The reactions were adjusted such that the same amount of ppαF was present in each assay. K-RM at (5 eq/50µl) was included in the reactions in lanes 2, 3, 5 and 6. SRP at 17 nM (lanes 2 and 5) and at 225 nM (lanes 3 and 6) was included. Control experiments in which no K-RM were added to PRS or RP are shown in lanes 1 and 4, respectively. Note that only small amounts of ppαF are sedimented in the absence of RM from the PRS, since aggregated ppαF was largely recovered in the RP fraction.


A
1 2 3
pαF.3
ppαF
- + + RM
- - + Puromycin
B
1 2 3 4 5
pαF.3
ppαF
T P S P S fraction
+ + + RM
- - + KOH
C
1 2 3 4 5 6
pαF.3
ppαF
- + + - + + K-RM
- 17 225 - 17 225 SRP (nM)
PRS
RP

the fraction of ppαF which could be translocated was ribosome associated as non-terminated peptidyl tRNA. We therefore asked if we could detect glycosylated pαF.3 that still retained the linkage to tRNA. For this purpose we precipitated the products after translocation with the cationic detergent hexadecyltrimethylammonium bromide (CTABr), which at low pH will precipitate those polypeptides which are covalently attached to RNA (Hobden and Cundliffe, 1978). As shown in Figure 2B, about 50 % of the pαF.3 was recovered in the pellet fraction (Fig. 2B, compare lanes 2 (pellet) and 3 (supernatant) with the total reaction products in lane 1). When the products were deacylated by treatment with base prior to CTABr precipitation, no pαF.3 was found in the pellet fraction (Fig. 2B, compare lanes 4 and 5), indicating that precipitation was indeed due to the presence of the covalently attached tRNA on these polypeptides. Thus a large fraction of the glycosylated pαF.3 remains attached to tRNA and presumably the ribosome. We envision these chains to be spanning the membrane such that the glycosylation sites are exposed on the lumenal side, yet the carboxy terminal ends are still within (and protected from protease by) ribosomes on the cytoplasmic face of the membrane. We assume that the fraction of ppαF.3 that was not CTABr precipitable has become deacylated during the incubation or subsequent manipulations.

A direct demonstration that all ppαF chains have to be ribosome associated to be translocation competent is shown in Figure 2C. We fractionated the translation reactions into a ribosomal pellet (Fig. 2C, RP) and a post-ribosomal supernatant (Fig. 2C, PRS) prior to the addition of K-RM and SRP. We observed translocation only when RM were incubated with the ribosome pellet fraction (Fig. 2C, lanes 5 and 6). No pαF.3 was observed when the post-ribosomal supernatant fraction was used, even at

high SRP concentrations (Fig. 2C, lanes 2 and 3). Thus, the translocation of full-length ppαF across mammalian ER membranes must be dependent on the continued participation of the translation machinery. We can thus explain the apparent low efficiency of translocation under these conditions by the fact that the competent substrate (non-terminated ppαF) was present as a minor fraction of the translation products (about 5 % after 30 min of translation as determined by CTABr precipitation, data not shown). Given that 1-2 % of the synthesized full-length ppαF was translocated, the reaction is in fact 20-40 % efficient, and thus, comparable to other in vitro translocation systems. We found that prolonged incubation times will reduce the amount of non-terminated ppαF present in the translation extract. Thus, after a one hour translation reduced or no translocation of full-lengh ppαF was observed (not shown). This may explain why Rothblatt and Meyer (1986) failed to detect translocation of full-length ppαF in their assays.

Energy Requirement for Translocation of Full-Length ppαF Across Mammalian Microsomes.

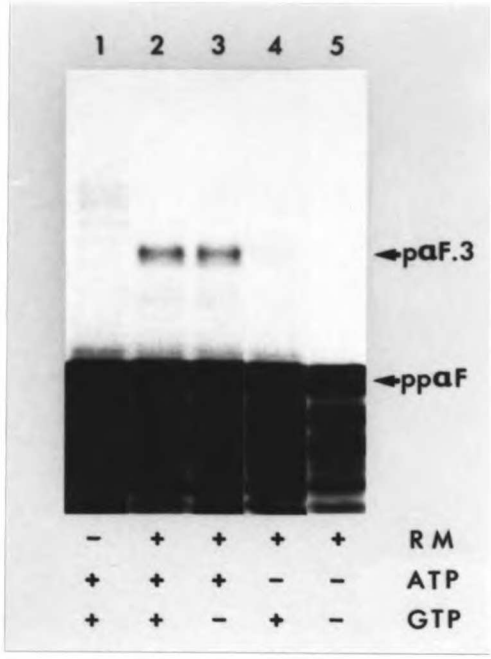
Lastly, we were interested in characterizing the energy requirements of the translocation reaction. After ppαF synthesis, translation was inhibited by cycloheximide as described above and small molecules (i.e. ATP, GTP and creatine phosphate) were removed by gel filtration. No translocation of ppαF was observed upon addition of RM to the desalted fraction in the absence (not shown) or presence (Fig. 3A, lane 5) of an energy regenerating system. Translocation could be restored if ATP (1 mM, Fig. 3A, lane 3) was added back to the system. In addition, we found that the non-hydrolyzable ATP analogue, ATPγS, competed with ATP, causing half-maximal inhibition at 5 mM in the presence of 1 mM ATP (not shown).

FIGURE 3: ATP and GTP hydrolysis is required for the ribosome-coupled translocation of full-length ppαF.

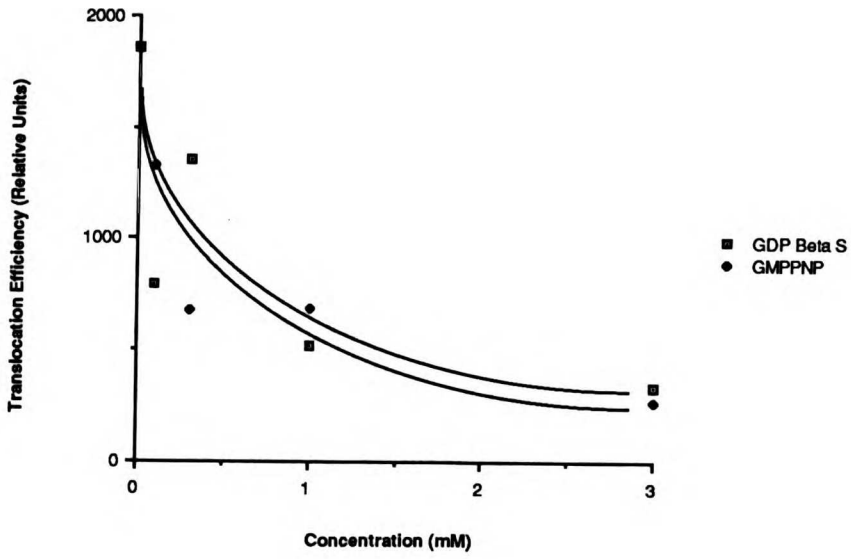
PANEL A: Reactions were carried out as in figure 1 with the exception that small molecules were removed from the translation mixture by gel filtration (see methods). K-RM (5 eq/50 µl) and SRP (200 nM) were added to each reaction. A control reaction with no K-RM added is shown in lane 1. ATP (1 mM, lanes 1, 2 and 3), GTP (100µM, lanes 1, 2 and 4) and creatine phosphate (8 mM to all reactions) were included. No translocation activity was observed when creatine phosphate was omitted (not shown). The microsomal vesicles were collected and analyzed as before.

PANEL B: Translocation reaction were carried out as in figure 3A (1 mM ATP, 100 µM GTP and 8 mM Creatine Phosphate), but in the presence of increasing concentration of the guanosine nucleotide analogs GMPPNP and GDPβS (the analogs were purified by preparative TLC before use). After SDS-PAGE and autoradiography, the intensity of the pαF.3 band was determined by scanning of the film in BioRad densitometer and these values were plotted versus the analog concentration.

A.


1 2 3 4 5
pαF.3
ppαF
– + + + + RM
+ + + – – ATP
+ + – + – GTP

B.


2000
1000
0
0
1
2
3
Translocation Efficiency (Relative Units)
Concentration (mM)
GDP Beta S
GMPPNP

Thus translocation of full-length ppαF across mammalian RM requires ATP hydrolysis. In contrast, no translocation was observed if GTP (100 μM, Fig. 3A, lane 4 or 1 mM, not shown) was added in the absence of ATP, and no stimulation of translocation was observed if GTP was added in combination with ATP. While GTP by itself was not sufficient to promote translocation, it may still be required in addition to ATP, since a small amount of GTP could be present as a contaminant in the ATP solution or could be generated from residual GDP in the desalted extract. In fact, inhibitor studies with guanosine nucleotide analogs hint at an additional requirement for GTP binding proteins (Fig. 3B). Competition experiments with increasing concentration of a non-hydrolyzable analog of GTP (GMPPNP) and with a GDP analog (GDPβS) that cannot be kinased to triphosphate were carried out (fig. 3B). The translocation products of these reactions were resolved by SDS-PAGE and the intensity of the pαF.3 band was determined by densitometic scanning of the autoradiography. In the presence of 1 mM ATP and 100 μM GTP, half-maximal inhibition of translocation was observed at about 500 μM GMPPNP and 500 μM GDPβS (fig. 3B). Since GMPPNP inhibits translocation we conclude that either an additional GTPase is required (which is not needed for short truncated products, Connolly and Gilmore, 1986) or that GMPPNP inhibits the ATPase described. The latter case is unlikely, since the corresponding adenine analogue, AMPPNP, showed only minor inhibition, even at 10 mM (not shown). The effect of GDPβS could then be explained by either inhibition of this GTPase, or by inhibition at the stage of the GTP binding protein described (Connolly and Gilmore, 1986). It is clear from these studies that the energy requirements are complex and that their complete understanding may have to await the biochemical description of the enzymes involved.

DISCUSSION

We have shown here that full-length ppαF can be efficiently translocated across mammalian RM membranes as long as the polypeptide chain is retained as peptidyl-tRNA on the ribosome. Thus, this reaction is distinct in its molecular requirements from the post-translational translocation of ppαF across yeast RM from both the yeast translation system (Hansen et al, 1986; Waters and Blobel, 1986; Rothblatt and Meyer, 1986) or the wheat germ translation system (Hansen et al, 1986), which can occur in the absence of SRP, SRP receptor and ribosomes. Yeast RM appear to be more flexible in which forms of presecretory proteins are acceptable as translocation substrates. In molecular terms one can envision that the yeast analogue of the recently identified signal sequence receptor (SSR; Wiedmann et al, 1987) in the RM membrane can functionally engage with signal sequences on soluble preproteins, thereby bypassing a requirement for the ribosome, SRP and SRP receptor (Walter, 1987). During translocation across mammalian RM, the signal sequence appears to be handed from SRP to the SSR, once that the SRP-ribosome-nascent chain complex has been targeted via SRP receptor. Therefore, it appears that the mammalian SSR is more stringent that its yeast counterpart, since it can functionally interact with signal peptides only when these have been properly "delivered" by the action of other components of the mammalian targeting machinery.

Our results indicate that signal recognition and targeting to the mammalian RM membrane by SRP occur only if the preprotein is seen in the context of the ribosome. Indeed, while SRP can be directly crosslinked to signal sequences that are part of the nascent polypeptide emerging from the ribosome (Kurzchalia et al, 1986; Krieg et al, 1986), no affinity of SRP

for isolated signal peptides or preproteins released from the ribosome has yet been demonstrated. Since terminated ppαF is an efficient translocation substrate across yeast RM, our results rule out that the ribosome merely acts to hold the nascent polypeptide in a translocation competent state by sequestering the carboxy terminal forty amino acids within the ribosome and, thus, interfering with protein folding. Rather, the ribosome seems to be directly involved as a ligand required for signal recognition by SRP and is possibly required later for the formation of the ribosome-membrane junction.

It was previously suggested that the formation of a ribosome membrane junction requires GTP and involves a GTP binding protein, but that no additional energy input is required to translocate small (86 amino acids) nascent preprolactin polypeptide chains (Connolly and Gilmore, 1986). Although we have not been able to demonstrate unambiguously a GTP requirement for the translocation of full-length ppαF, our data are not in disagreement. However, we clearly demonstrated that ATP hydrolysis is required, as was previously found for post-translational translocation of soluble ppαF across yeast RM (Hansen et al, 1986; Waters and Blobel, 1986; Rothblatt and Meyer, 1986) and suggested for the insertion of a fragment of the glucose transporter protein into mammalian RM (Mueckler and Lodish, 1986b). We speculate that longer polypeptide chains have a tendency to fold, thereby making the signal peptide inaccessible. Consequently, additional energy may be necessary to unfold the substrate prior to translocation. This notion is further supported by the finding that optimal translocation occurs at high SRP concentrations, which is consistent with the idea that the nascent chain is in equilibrium between having a buried or an exposed signal peptide, and that high SRP

concentration drives this equilibrium to the exposed state. A correlation between nascent chain length and the concentration of SRP required for efficient targeting has also been directly demonstrated for bovine preprolactin (Siegel & Walter, 1988c).

Finally we wish to comment on the nomenclature currently used. We have demonstrated here that the mammalian translocation machinery requires that nascent secretory proteins be attached to the ribosome as peptidyl-tRNA. Previously, elongation independent processes have been collectively referred to as "post-translational". Thus, while targeting and translocation of full-length preproteins across mammalian RM are independent of ongoing elongation, they are not "post-translational" events. No translocation of these chains would occur if the final step in translation, termination, had already taken place. This is in contrast to the translocation of ppαF and other yeast secretory proteins (Hansen et al, 1986; Hansen and Walter, 1988) which can be translocated across yeast RM from a soluble pool in a truly post-translational mode. We therefore wish to distinguish between these two processes, fundamentally different in their molecular requirements, and propose the term "ribosome-coupled translocation" for the events described here for mammalian RM. We suggest that SRP, which is required in this reaction, has evolved primarily as an adapter between the ribosome and the membrane. Most proteins that have been described to be translocated across mammalian RM in the absence of protein synthesis fall into this category. In all cases a ribosome-dependence has been noted and their translocation has been improperly referred to as "post-translational" (Caulfield et al, 1986; Mueckler and Lodish, 1986a and b; Perara et al, 1986; Chao et al, 1987; see also figure 7 in Hansen et al, 1986). The only known exceptions are a few

small peptides, prepromelittin (Zimmermann and Mollay, 1986), m13 precoat protein (Watts et al, 1983) and GLa peptide (Schenstedt and Zimmermann, 1987). These peptides appear to be substrates for post-translational translocation across mammalian RM with no ribosome, SRP and SRP receptor requirement. Due to their small size and/or particular structure, it is possible that they use a different translocation mechanism with different molecular requirements.

CHAPTER 5

PROTEIN TRANSLOCATION ACROSS MAMMALIAN MICROSOMAL MEMBRANE REQUIRES ATP AND GTP HYDROLYSIS AFTER SIGNAL RECOGNITION BY SRP.

ABSTRACT

Previously it has been demonstrated that ATP and GTP are required for protein translocation across the endoplasmic reticulum membrane. To test for these requirements in the signal recognition step, we have developed a simple and fast assay to measure the binding of the signal recognition particle (SRP) to ribosomes that are synthesizing secretory proteins. The assay uses gel filtration in mini-columns to resolve ribosome-bound from free SRP, as an alternative to sucrose gradients. Using this assay, we have found that GTP, ATP or their hydrolysis are not required for signal recognition by SRP. Removal of nucleotides by gel filtration or enzymatic hydrolysis prior to the assay does not inhibit binding of radiolabeled SRP to ribosomes containing nascent chains of the yeast Saccharomyces cerevisiae prepro-α-factor protein. Also, no inhibition of binding was observed by the addition of non-hydrolyzable analogs of ATP, GTP or a non-phosphorylable analog of GTP. Since all these treatments completely abolish protein translocation across the microsomal membranes, we conclude that the nucleotides are required for steps after signal recognition, probably during targeting and/or the actual translocation across to the mammalian endoplasmic reticulum membrane.

INTRODUCTION

Translocation of proteins across the membrane of the endoplasmic reticulum (ER) of higher eukaryotes requires the signal recognition particle or "SRP" (Walter and Blobel, 1980), a cytoplasmic ribonucleoprotein that serves as an adaptor between the translation and translocation machinery. SRP binds to ribosomes with two different affinities, depending on the nature of the protein that is being translated. The low affinity binding (K_D of about 5×10^{-5} M) occurs when the ribosome is not engaged in

translation or when it is translating cytoplasmic proteins (Walter et al, 1981). In contrast, SRP binds with high affinity (K_D of about 8×10^{-9} M) when the signal sequence of a secretory protein emerges from the ribosome (Walter et al, 1981; Walter and Blobel, 1981b). The high affinity binding results in a transient inhibition or a kinetic delay in the elongation of the secretory polypeptide, a phenomenon known as SRP-dependent elongation arrest (Walter and Blobel, 1981b). Then SRP mediates the targeting of the ribosome to the ER membrane (Walter and Blobel, 1981a) through its interaction with the SRP receptor (Gilmore et al, 1982a&b; Meyer et al, 1982), a component of the ER membrane (Hortsch and Meyer, 1985; Tajima et al, 1986). Binding of SRP receptor to SRP results in the release of the elongation arrest (Walter and Blobel, 1981b; Gilmore et al, 1982a). This interaction also results in the release of SRP and SRP receptor from the ribosome. They are presumably then free to participate in a new cycle of ribosome targeting (Gilmore and Blobel, 1983). Once targeted, the ribosome binds to the ER membrane in a manner competent for translocation; translocation of the protein then occurs concomitantly with its elongation. Thus, the known functions of SRP and SRP receptor are primarily related to events that occur before the secretory protein is fully synthesized.

The discovery that protein translocation across the ER membrane of the yeast Saccharomyces cerevisiae can occur post-translationally led us and others to test for energy requirements in protein translocation. This was possible since for the first time translocation reactions could be carried out independently of ongoing protein synthesis (Chapter 3; Hansen et al, 1986; Waters and Blobel, 1986; Rothblatt and Meyer, 1986b). Thus it was demonstrated that ATP hydrolysis provided the energy required for translocation in this system. Testing for the energy requirements in

protein translocation across the ER membrane of higher eukaryotes was possible after development of a ribosome-dependent and elongation-independent translocation assay (Chapter 4; Perara et al, 1986; Mueckler and Lodish, 1986a; Connoly and Gilmore, 1096; Garcia and Walter 1988). As in the yeast system, translocation of long nascent chains was found to require the energy provided by the hydrolysis of ATP (Chapter 4; Mueckler and Lodish, 1986b; Garcia and Walter, 1988). Moreover, it was demonstrated that after SRP recognition, the presence of GTP (but not its hydrolysis) was required for targeting and translocation of short nascent chains across mammalian microsomes (Connolly and Gilmore, 1986). Furthermore, we found that if added simultaneously with SRP, the presence of non-hydrolyzable analogs of GTP inhibit (in a competitive manner with GTP) the translocation of long nascent chains in the mammalian system (Chapter 4, Garcia and Walter, 1988). Thus, a relatively complex picture emerges for the nucleotide requirements. A full understanding of the mechanism of protein translocation will require the description of the precise processes in which these nucleotides are necessary. Translocation across mammalian microsomal membranes can be separated experimentally into two steps: a) signal recognition by SRP, and b) targeting to the ER and translocation across the membrane. In the present work we have assigned the nucleotide requirements to one of these two steps.

MATERIAL AND METHODS

Plasmids:

Plasmids containing the coding sequences for the bovine preprolactin (pSP-BP4) and the yeast Saccharomyces cerevisiae prepro-α-factor (pDJ100 and p64T-ppαF) proteins downstream of the bacteriophage SP6 promoter were used in the present work. The relevant characteristics of pDJ100

(constructed by Dr. D. Julius) and pSP-BP4 (constructed by Drs. W.B. Hansen and L. Lauffer), have been described previously (Chapter 2; Hansen et al, 1986 and Siegel and Walter, 1988a). The plasmid p64T-ppαF was constructed by cloning of the BamHI insert of pDJ100 into the BglII site of the pSP64T vector (a generous gift of Drs. E. Perara and V. Lingappa). The relevant characteristic of this plasmid is that no NciI site is present between the SP6 promotor and the coding sequences of ppαF. Since an NciI site is present 3 codons before the termination codon of the ppαF (Kurjan and Herskowittz, 1982), in vitro transcription of NciI-digested p64T-ppαF results in a ppαF mRNA that lacks its termination codon. Therefore, the translation products from this mRNA cannot be released from the ribosomes. Thus, in vitro translation of this mRNA results in an accumulation of ribosomes containing the almost full-length ppαF as a nascent chain.

In Vitro Transcription and Translations:

All the in vitro transcriptions were carried out using the above plasmids linearized with the respective restriction enzymes as substrates and the commercially available SP6 RNA polymerase (Promega Biotec). The exact procedure for in vitro transcription has been described in detail elsewhere (Chapter 2; Hansen et al 1986). Translation of the synthetic transcripts or total rabbit reticulocyte RNA was performed in wheat germ extracts as described (Erickson and Blobel, 1983; Chapter 4), with the exception that no radioactive amino acid was included. Instead, 125 μM of each of the 20 amino acids were present. All translation reactions were incubated for 15 min. at 26 ^{o}C, and then stoped by transfering to an ice-water bath or the addition of cycloheximide (2 mM final) in the cases in which post-synthesis incubation were necessary.

Gel Filtration on Sephadex G-25 Columns:

Translation reactions or purified SRP were desalted by gel filtration in Sephadex G-25 columns. All the manipulations were carried out at 4 °C. The 0.5 ml mini-columns were packed in tuberculin syringes and equilibrated with the desired buffer. Samples of 50 µl were loaded into each column and eight 50 µl fractions were collected. The excluded macromolecules eluted in fractions 5 and 6. The fraction with the higher concentration of proteins was used for further experiments.

SRP Purification and Labeling with ^{125}I-Bolton-Hunter Reagent:

SRP was purified from canine pancreatic microsomes as described previously (Walter and Blobel, 1983b). Labeling of SRP with Bolton-Hunter reagent was performed as previously described (Siegel and Walter, 1988a). Before labeling, the triethanolamine buffer of the SRP solution was removed by gel filtration on a Sephadex G-25 column equilibrated with SRP buffer, which contain 20 mM Hepes pH 7.5, 500 mM kOAc. pH 7.5, 5 mM $Mg(OAc.)_2$, 1 mM DTT and 0.01 % Nikkol (octa-ethyleneglycol-n-dodecylether). Labeling of this SRP with ^{125}I-Bolton-Hunter reagent (Amersham) was performed as follows. Two hundred and fifty µCi of the reagent was dried under a nitrogen atmosphere and dissolved into 25 µl of SRP buffer. Five picomoles of SRP were added to this solution and the mixture was incubated at 4 °C for two hours. The reaction was stopped by the addition of 2 µl of 1 M Tris-HCl pH 8.0. Labeled SRP was purified from unincorporated label in a preformed sucrose step-gradient as published (Siegel and Walter, 1988a). The fractions containing labeled SRP were detected by resolving small aliquots of each fraction in SDS-PAGE followed by autoradiography.

Assay for SRP Binding to Ribosomes:

Labeled SRP was incubated with ribosomes during or after synthesis of

proteins. Typically, 20 µl translation reactions were carried out as indicated above. If ^{125}I-SRP (about 10,000 cpm) was present during translation, the reaction was stopped by transferring to an ice-water bath. Then, 10 µl of equilibration buffer (see bellow) was added and the mix was loaded onto the Sephacryl S-400 columns (see bellow). In the binding assays after protein synthesis, translation was terminated by the addition of 2 mM cycloheximide. Then, the same amount of ^{125}I-SRP was added and the final volume was adjusted to 30 µl, keeping the ionic conditions constant. After a second incubation at 26 °C for 15 min, the samples were transferred to an ice-water bath and then loaded into the Sephacryl S-400 columns.

Gel Filtration on Sephacryl S-400 to Determine Binding to Ribosomes:

All the procedures were carried out at 4 °C. 1.2 ml Sephacryl S-400 columns were packed into tuberculin syringes. Before running the samples, the columns were equilibrated with 5 ml of a buffer containing 8 mM Hepes pH 7.5, 150 mM KOAc, 3.5 mM $Mg(OAc.)_2$ and 1 mM DTT. The columns were run with the same buffer after 30 µl samples were loaded. 30 fractions of 40 µl each were collected directly into scintillation vials and counted in a Beckman 4000 gamma counter. The columns were recycled by: a) a 2 ml wash with distilled water; b) followed by a wash with 2 ml of 5 M NaCl; c) followed by a wash with 5 ml of 0.02 % NaN_3; and d) storage in this solution at 4 °C until equilibration for the next assay.

RESULTS

Assay for SRP binding to Polysomes:

To assign the nucleotide requirements to specific steps in the process of protein translocation across the ER membrane, we began our analysis with the first step of protein translocation: signal recognition by SRP as

the signal sequence emerges from the ribosome. This step can be directly measured by the high affinity binding of SRP to ribosomes synthesizing secretory proteins (Walter et al, 1981). Binding of SRP to ribosomes has been traditionally measured by briefly incubating radiolabeled SRP in an in vitro translation system, followed by resolution of the assembled SRP-ribosome complexes in sucrose gradients (Walter et al, 1981). Although this procedure is extremely accurate and reproducible, the number of assays that can be simultaneously carried out is limited by the ultracentrifuge capacity. Since testing for the nucleotide requirements will require a large number of independent, simultaneous assays, we have developed a simpler and faster SRP-ribosome binding assay. The key to simplifying the assay was the replacement of the sucrose gradients by a gel filtration step. Several gel filtration media were tested for their ability to resolve ribosomes from free SRP. Sephacryl S-400 was found to be the most appropriate (not shown). In brief, the procedure involves the addition of radiolabeled SRP in a translation reaction, followed by fractionation of the products in a gel filtration mini-column (see methods for details). By collecting the eluted fractions directly into scintillation vials, we have eliminated all unnecessary manipulations. Using this assay, up to 16-20 independent tests can be carried out simultaneously. The procedure takes less than one hour from the loading of the columns to putting the samples into the scintillation counter.

Before studying the requirements for signal recognition, we wanted to test the reliability of our procedure by comparing the results obtained with it with those obtained previously using sucrose gradients (Walter et al, 1981). For this purpose, we measured the binding of SRP to ribosomes in translation reactions carried out in the absence of added mRNA (fig. 1A)

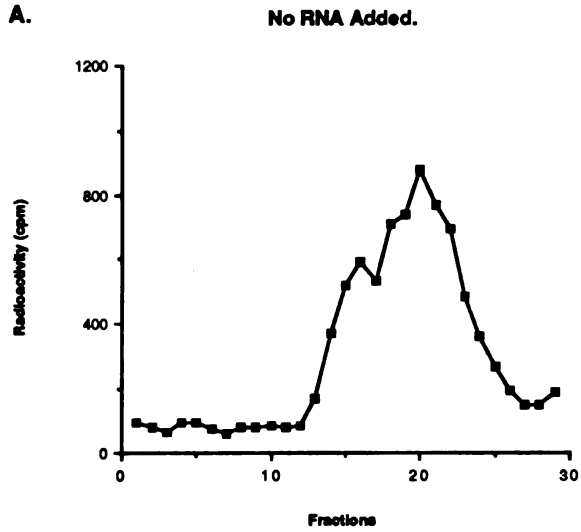
and when messages for the cytoplasmic protein globin (fig. 1B) or the secretory protein preprolactin (fig. 1C) were included. Total rabbit reticulocytes was used as source of α and β globin mRNAs and an in vitro transcript was used for preprolactin mRNA (see methods). Translation reactions were carried out in wheat germ extract in the presence of ^{125}I-SRP labeled with the Bolton-Hunter reagent under conditions identical to those published (Walter et al, 1981; see methods for details). The reactions were transferred to an ice-water bath and loaded onto 1.2 ml Sephacryl S-400 columns (see methods for details). Thirty fractions of 40 µl were collected and their radioactivity content was determined. Figure 1 shows the elution profiles of these experiments. In all these cases a peak of radioactivity can be observed between fractions 18 to 23 (fig. 1). This corresponds to the elution peak of free SRP, as its elution position is that expected for molecules the size of SRP (about 233 kDa). Also, this was the position in which both the SRP polypeptides and RNA were eluted if pure SRP was fractionated under identical conditions (data not shown).

When no RNA was included in the translation reaction, a small peak was also observed between fractions 15 to 17 (fig. 1A). The position of this peak corresponds to the elution volume for particles of the size of a monosome (about 4,500 kDa) and therefore reflects the low affinity binding of SRP to ribosomes not engaged in translation (Walter et al, 1981). This conclusion was confirmed by the disappearance of the peak when SRP was incubated in an extract in which the ribosomes were removed by centrifugation (data not shown). When preprolactin mRNA was translated, a large peak between fractions 12 to 14 was observed (fig. 1C). The position of this peak correspond to the void volumen of the column in which

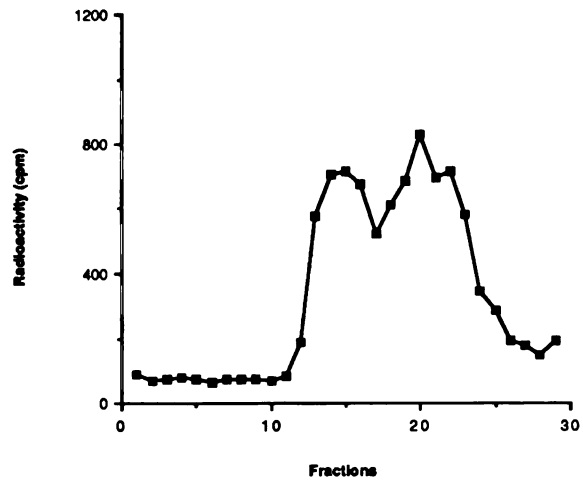
FIGURE 1: SRP binding to monosomes and polysomes.

Shown are the elution profiles of ^{125}I-SRP from Sephacryl S-400 columns after incubation of translation reactions programmed in the absence of exogenous mRNA (panel A), with 10 µg of total rabbit reticulocyte RNA (panel B), and with synthetic preprolactin mRNA (panel C). Virtually identical results were obtained in three independent experiments.

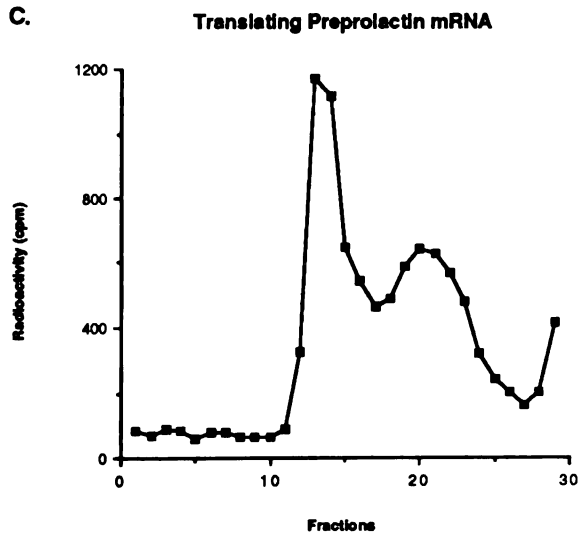
A.


No RNA Added.
1200
800
400
0
Radioactivity (cpm)
0
10
20
30
Fractions

B.


Translating Globin mRNA
1200
800
400
0
Radioactivity (cpm)
0
10
20
30
Fractions

C.


Translating Preprolactin mRNA
1200
800
400
0
Radioactivity (cpm)
0
10
20
30
Fractions

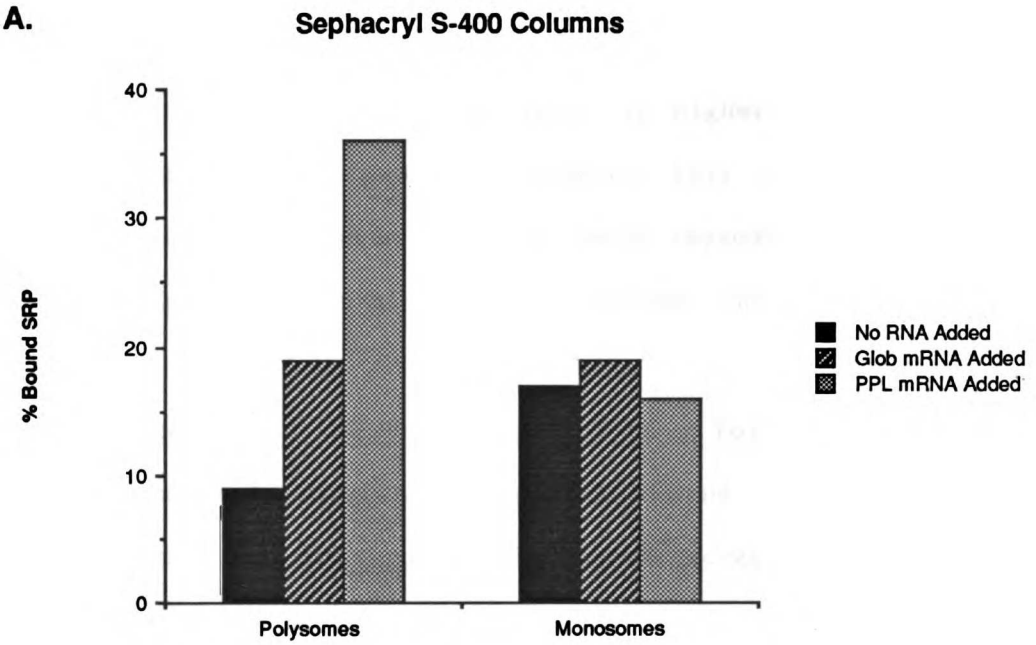
particles larger than 8,000 kDa in molecular mass elute. Thus, this peak corresponds to the SRP that is binding to polysomes engaged in translation of preprolactin and therefore reflects its high affinity binding to ribosomes (Walter et al, 1981). Globin translation does not result in high affinity binding as reflected by the absence of a peak in the polysome fractions (fig. 1B). However, some radioactivity elutes in the void volume fractions resulting in a broad peak of low affinity binding (fig. 1B). This broader peak represents low affinity binding of SRP to both monosomes and polysomes synthesizing cytoplasmic proteins (Walter et al, 1981).

We have quantitatively compared these data with the sucrose gradients data (Walter et al, 1981) by determining the percentage of SRP that was bound to monosomes and polysomes in both cases. Figure 2 shows a graphic representation of results using the gel filtration method in panel A and using the sucrose gradient method in panel B. Both methods gave the same general results. The percentages of SRP binding to both monosomes and polysomes were slightly higher in the gel filtration method than in the sucrose gradient method. Since during its purification the SRP-ribosome complex can dissociate, this difference could be due to the fewer and shorter manipulations of the gel filtration method. Both methods show that SRP binding to polysomes synthesizing preprolaction is markedly increased with respect to polysomes in which globin were being synthesized. We observed, however, that SRP binding to polysomes programmed with total reticulocyte RNA was higher when measured by the gel filtration assay (compare panels A and B of fig. 2). At present we do not know the reason for this difference. However, in this assay we used ten times more reticulocyte RNA than preprolactin mRNA, relative to the intensity of the

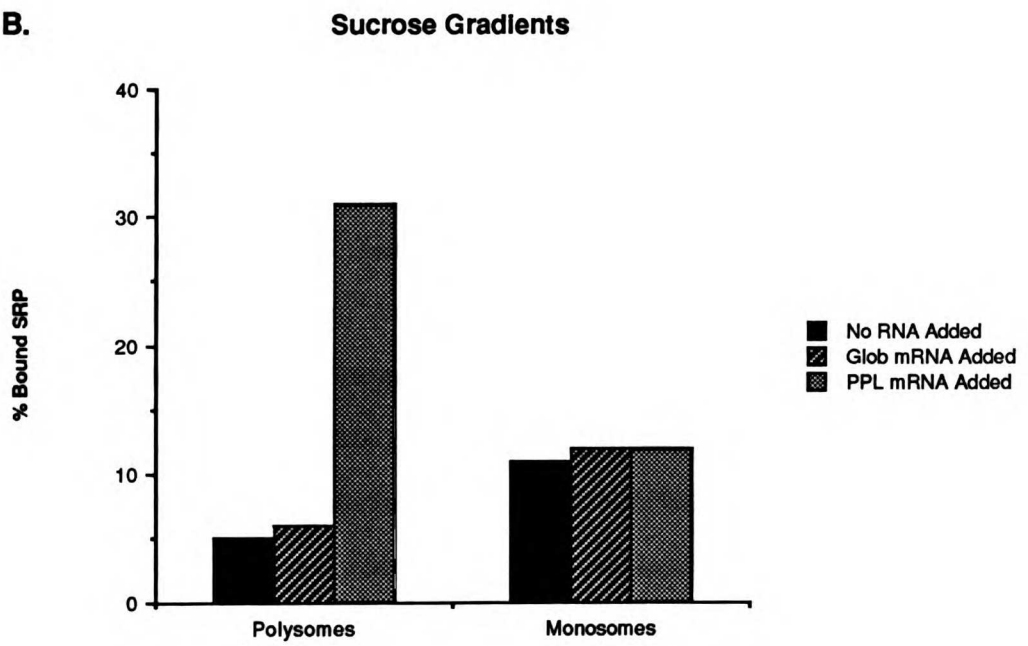
FIGURE 2: Quantitation of SRP binding to monosomes and polysomes.

The percentage of SRP binding to monosomes and polysomes in the experiment shown in figure 1 (panel A) was determined as follows. The total cpm present in each peak was determined by adding the cpm of each fraction in the peak. Fractions 12 to 14 correspond to the polysome peak, fractions 15 to 17 to the monosome peak and fractions 18 to 23 to free SRP. The extent of binding was determined by calculating the percentage of radioactivity in each peak relative to the radioactivity contained in all three peaks. For comparison purposes, panel B shows the equivalent data obtained previously (Walter et al, 1981), using sucrose gradients instead of gel filtration on Sephacryl S-400 columns.

A.


Sephacryl S-400 Columns
% Bound SRP
40
30
20
10
0
Polysomes
Monosomes
No RNA Added
Glob mRNA Added
PPL mRNA Added

B.


Sucrose Gradients
% Bound SRP
40
30
20
10
0
Polysomes
Monosomes
No RNA Added
Glob mRNA Added
PPL mRNA Added

respective protein bands in SDS-PAGE (not shown). Although the rabbit reticulocyte RNA primarily encodes the cytoplasmic proteins α and β globin, it also contains mRNA other cellular proteins. Therefore, it is possible that this difference was due to the presence of higher concentrations of some mRNA encoding secretory proteins. Despite this difference, the gel filtration method has been proven to be extremely reproducible and reliable to measure the high affinity binding to ribosomes that are synthesizing secretory proteins.

SRP Does Not Requires ATP or GTP Hydrolysis to Bind to Polysomes:

The SRP-ribosome binding assays described and tested above were performed by carrying out in vitro translation of secretory proteins in the presence of radiolabeled SRP. Testing for the involvement of nucleotides in signal recognition by SRP, however, requires first the removal of the nucleotide triphosphates necessary for protein synthesis from the extracts. Therefore, the assays must be performed by measuring SRP binding to ribosomes containing already made nascent chains. We have shown previously that SRP can functionally interact with such ribosome-nascent chain complexes (Chapter 4; Garcia and Walter, 1988; Siegel and Walter, 1988c). However, this conclusion was reached indirectly by determining that these nascent chains were capable of being translocated across the mammalian ER membrane in the absence of elongation (Chapter 4; Garcia and Walter, 1988; Siegel and Walter, 1988c). Therefore, we wanted first to confirm this conclusion by directly measuring the high affinity binding of SRP to ribosomes containing nascent chains in the absence of their elongation. For this purpose, synthesis of the secretory proteins were carried out first and then stopped by the addition of cycloheximide. Labeled SRP was then added and a second incubation was performed before subjecting the

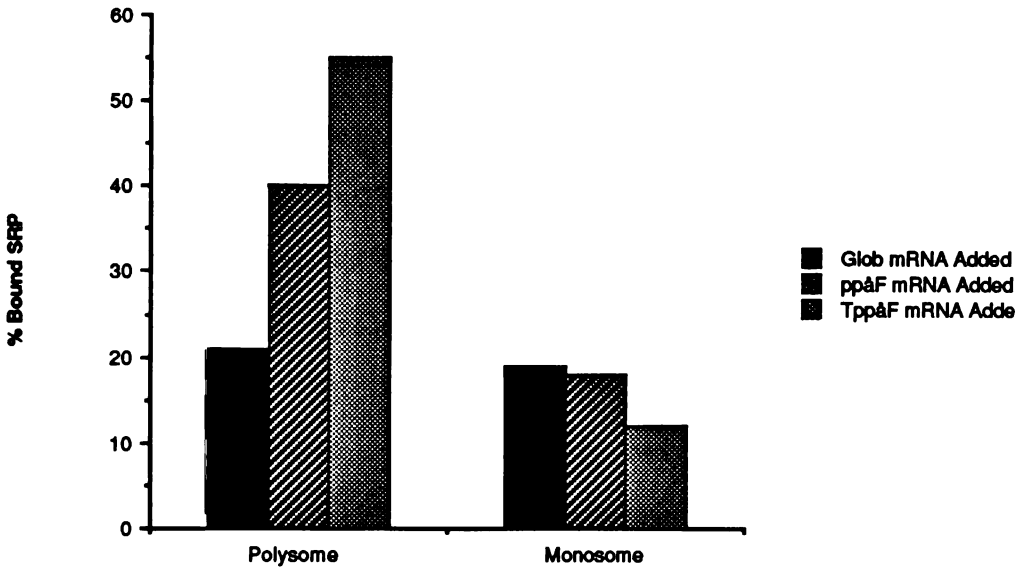
samples to analysis on gel filtration columns.

As in the previous chapters, the yeast Saccharomyces cerevisiae prepro-α-factor protein was used in these experiments as a model secretory protein. These results are presented in figure 3 in terms of the percentage of binding to polysomes and monosomes. Two mRNA species for this protein were used: one contained the complete coding sequences for the protein (labeled as ppαF in fig. 3), and the other lacked the termination codon (labeled TppαF in figure 3; see methods for details). Since it lacks the termination codon, translation of the second mRNA results in a protein that cannot be released from the ribosome; therefore, a larger number of ribosome-nascent chain complexes accumulate. The percentage binding of SRP to polysomes after inhibition of synthesis by cycloheximide was about the same as when SRP was present during protein synthesis, for both total reticulocyte RNA and secretory proteins (compare fig. 3 with fig. 2A). As expected, however, the binding was higher to polysomes translating mRNAs that lack the termination codon (40% and 55% binding for ppαF and TppαF mRNAs, respectively), due to the higher concentrations of ribosome-nascent chain complexes in the extracts. Therefore, in these experiments we have directly demonstrated that SRP can bind with high affinity to ribosomes containing already made nascent secretory proteins, thereby independently confirming our previous conclusions (Chapter 4; Garcia and Walter, 1988; Siegel and Walter, 1988). More importantly, these results indicate that using our binding assay, we can directly test for the nucleotide requirements in signal recognition by SRP.

As indicated above, we needed to remove nucleotide triphosphates required for in vitro translation in order to test their involvement in signal sequence recognition by SRP. We accomplished this by subjecting the

FIGURE 3: SRP binding after inhibition of polypeptide elongation.

Binding of SRP to ribosomes was allowed to occur after synthesis of proteins was inhibited by the addition of cycloheximide (see text for details), and the percentage of binding was determined as in figure 2. As for figure 1, the data shown are representative of three independent experiments.

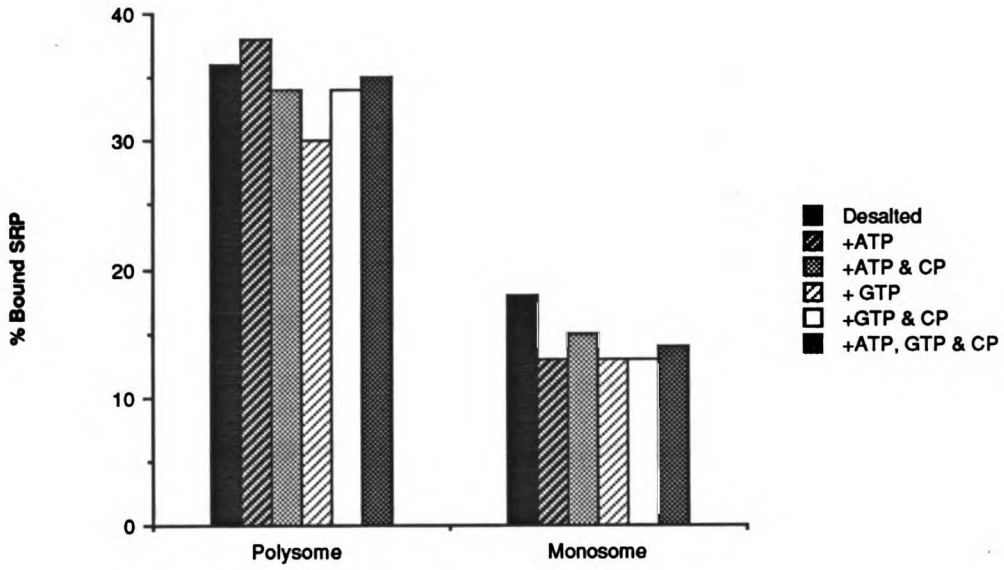

% Bound SRP
60
50
40
30
20
10
0
Polysome
Monosome
Glob mRNA Added
ppâF mRNA Added
TppâF mRNA Adde

extracts to a gel filtration step after synthesis of the nascent secretory protein. Translation of the TppαF mRNA was performed in wheat germ extract as before, followed by fractionation of the sample on a Sephadex G-25 column (see methods for details). This desalted extract was then incubated with labeled SRP under the same conditions as the in vitro translation, with the exception that they were performed in the absence or presence of ATP (1 mM), GTP (100 μM) and the nucleotide triphosphate regenerating substrate creatine phosphate ("CP", at 8 mM). The samples were then subjected to analysis on Sephacryl S-400 columns to determine the SRP fraction bound to monosomes and polysomes. The results of these experiments are shown in figure 4. The desalted sample containing the TppαF products shows a 36% level of SRP binding to polysomes. Although the binding level was somewhat reduced (compared to 55% binding in fig. 3), it was well above background levels or the binding to polysomes programmed with rabbit reticulocyte RNA (compared to data in figures 2 and 3). Therefore, this reduced level of SRP binding was probably due to sample dilution that results from the gel filtration procedure (almost a two-fold dilution in this particular case; data not shown). More importantly, readdition of ATP, GTP and CP individually or in combinations (see figure legend for details), did not result in increased SRP binding (fig. 4). Therefore, these results indicate in principle that nucleotide triphosphates are not required for signal sequence recognition by SRP.

This conclusion, however, should be limited only to the requirement of high concentration of ATP for protein translocation across mammalian ER membranes (see Chapter 4 and Garcia and Walter, 1988). Because the gel filtration procedure cannot completely remove all nucleotides (especially those tightly bound to proteins), desalted extracts will show full

FIGURE 4: SRP binding to ribosomes in the absence and presence of nucleotide triphosphate.

Prior to the addition of SRP, nucleotides were removed by gel filtration from extract containing ribosome-nascent secretory proteins complexes (see text for details). Then the percentage of SRP binding was determined as in figure 2. These results are representative of three independent assays.


40
30
20
10
0
% Bound SRP
Polysome
Monosome
Desalted
+ATP
+ATP & CP
+ GTP
+GTP & CP
+ATP, GTP & CP

activity if only low concentration of them is required. For example, when translocation across the mammalian ER membrane was assayed from a similar desalted extract, only an ATP effect was observed (Chapter 4; Garcia and Walter, 1988). The addition of ATP and CP was sufficient to restore protein translocation; GTP did not restore translocation by itself, nor did stimulate the process when added together with ATP (Chapter 4; Garcia and Walter, 1988). However, the requirement of GTP hydrolysis was evident when a non-hydrolyzable analog of the nucleotide was included. Addition of the analog in sufficient amounts to compete with endogenous GTP resulted in inhibition of protein translocation (Chapter 4; Garcia and Walter, 1988). Therefore, to conclusively determine the involvement of small amounts of nucleotides on signal recognition by SRP, similar competition experiments should be carried out. Such competition experiments were carried out using non-hydrolyzable analogs of ATP (ATPγS) and GTP (GMPPNP and GTPγS), and a non-kinasable analog of GDP (GDPβS). Extracts containing the translation products from the TppαF mRNA were desalted as above and used in second incubations performed in the presence of labeled SRP and each of the nucleotide analogs indicated. The percentages of SRP binding were determined as before and are presented in figure 5. The concentration of the nucleotide analogs used (2 mM) was in a vast excess to any residual nucleotide left after gel filtration on the Sephadex G-25 column. Therefore, if small amounts of ATP or GTP hydrolysis are required for signal sequence recognition by SRP, the binding of SRP should be completely reduced to background levels. The results shown in figure 5 indicate that none of the analogs significantly reduce SRP binding to polysomes containing nascent secretory proteins. Therefore, our results conclusively demonstrate that all the nucleotide requirements for protein translocation

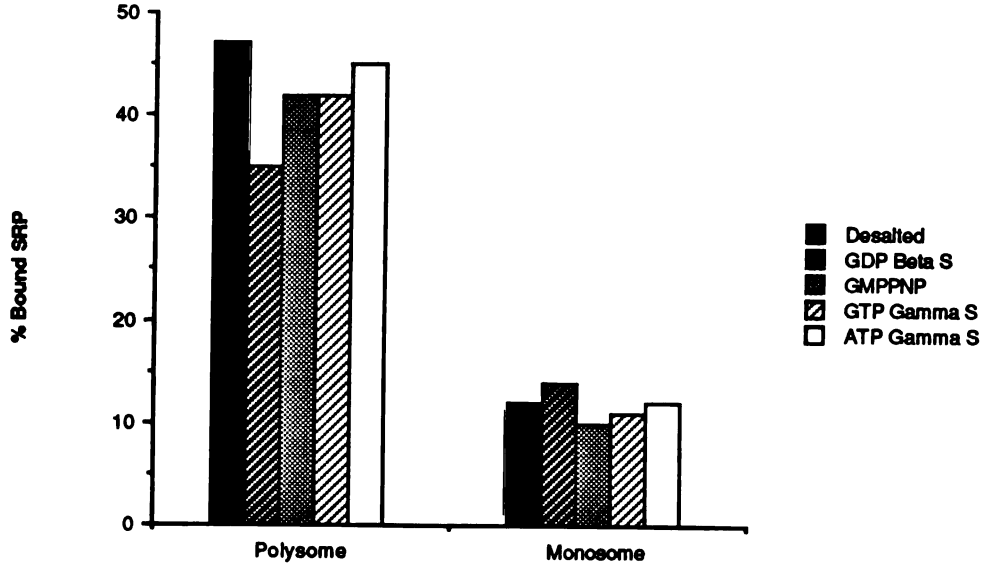
across the mammalian ER membrane must occur at steps after signal recognition.

DISCUSSION

In the present work we have determined that the requirements for ATP and GTP in protein translocation across the mammalian ER membrane must occur at a step (or steps) after signal recognition by SRP on ribosomes that are synthesizing secretory proteins. Understanding the mechanism of protein translocation has been the object of intensive research in the last ten years. The best undertood system is that of translocation across mammalian ER membranes. However, although a detailed understanding of the mechanism of signal recognition by SRP and targeting to the ER membrane has emerged, the actual mechanism by which proteins cross the lipid bilayer remains largely unknown (Walter et al, 1984; Walter and Lingappa, 1986). From the little that is known about the actual translocation process, at least the following steps at the ER membrane can be inferred: a) binding of the SRP-ribosome-nascent chain complex to the SRP receptor, b) attachment of the ribosomes to the membrane, c) binding of the signal sequence to a signal sequence receptor (Wiedman et al, 1987), d) assembly of the putative pore through which the protein crosses the membrane, and e) the actual passage of the protein through this pore. In principle, nucleotide hydrolysis could by required in any of these steps or in any other(s) that remain to be described. To precisely determine the steps at which the nucleotides are required, experimental assays to measure individually each one of these activities must be developed. Accomplishing this task has proven to be extremelly difficult, and most likely will have to wait for the identification and functional reconstitution in lipid bilayers of all the components of the translocation machinery.

Figure 5: SRP binding in the presence of nucleotide analogs.

Nucleotides were removed from the extracts as in figure 4. SRP was added after the addition of 2 mM of the nucleotide analogs indicated (see text for details). The percentage of binding was determined as in figure 2. These results are representative of three independent experiments.


% Bound SRP
50
40
30
20
10
0
Polysome
Monosome
Desalted
GDP Beta S
GMPPNP
GTP Gamma S
ATP Gamma S

Since the discovery that ATP was required for translocation across the ER membrane in both yeast (Chapter 3, Hansen et al, 1986; Waters and Blobel, 1986; Rothblatt and Meyer, 1986b) and mammalian (Mueckler and Lodish, 1986b; Chapter 4; Garcia and Walter, 1988) ER membranes, it has been specifically assumed that its hydrolysis somehow is involved in the unfolding of the protein to be translocated. Alternatively, it is possible that the translocation machinery could require ATP hydrolysis for the active transport of the polypeptide across the membrane, or for the assembly or disassembly of a protein channel. Interestingly, no requirement of ATP or its hydrolysis was observed when short nascent chains (about 80 amino acids long) were transferred across the mammalian ER membrane (Connolly and Gilmore, 1986). Therefore, since short polypeptides can be translocated without the paticipation of ATP, the assumption is that ATP is required for unfolding of large proteins prior or during translocation. Our data indicate that if ATP hydrolysis is involved in unfolding long nascent chains for translocation across mammalian ER membrane, then the unfolding step must occur at the membrane level and probably during translocation.

Recently, it has been shown that a heat-shock cognate protein (HSP70) stimulates the post-translational translocation of proteins across the yeast microsomal membrane, both in vivo and in vitro (Deshaies et al, 1988; Chirico et al, 1988). Proteins of the HSP70 family are known to bind tightly to ATP and to denatured or unfolded proteins (Pelham, 1986). By extrapolation, it has been suggested that they may bind to precursor secretory proteins in the cytoplasm (perhaps requiring ATP hydrolysis) and prevent them from folding. Thus, HSP70 proteins could keep precursors in a translocation competent state until they are targetted to the yeast ER

membrane. This suggestion has not been tested; hence the role of HSP70 proteins in translocation across yeast ER membranes remains to be determined. A similar "maintenance of translocation competence" activity has recently been reported for SRP. It appears that purified SRP can maintain (in the absence of any other cytoplasmic factor) the translocation competent state of precursor proteins, when assayed in the Escherichia coli plasma membrane or yeast microsomal membranes systems (Crooke et al, 1988; Sanz and Meyer, 1988). Given this potential overlap of activities between SRP and HSP70, we have tested if the presence of the HSP70 proteins has any effect on protein translocation across mammalian ER membranes. We found that SRP was absolutely required for translocation across mammalian ER membranes of long nascent secretory proteins contained in partially purified ribosomes (Chapter 4; Garcia and Walter, 1988), even in the presence of high concentrations of yeast or wheat germ HSP 70 proteins (Garcia and Walter, unpublished results). Furthermore, we found that the HSP70 proteins have no effect on the efficiency of translocation of long nascent chains at a wide range of SRP concentrations (Garcia and Walter, unpublished results). Furthermore, we have shown here that SRP does not require ATP for the recognition and binding of precursor secretory proteins contained in ribosomes. Therefore, although it is possible that some of their activities appear to be similar, the functions of SRP and HSP70 are not interchangeable and their molecular roles on protein translocation is completely different.

Perhaps the most surprising of our results is that the previously characterized GTP hydrolysis (Chapter 4; Garcia and Walter, 1988) requirement for translocation of long nascent chains was assigned to events occurring after signal recognition. This conclusion sharply contrasts the

results of Connoly and Gilmore (1986). They have conclusively demonstrated that for translocation of short nascent chains across mammalian ER membranes, only the presence of GTP (and not its hydrolysis) was required. Although, at present we do not understand the basis for this difference, we would like to consider some possibilities. Both long and short nascent chains must go through the same processes to be translocated, perhaps with the exception of the unfolding step. Therefore, an obvious possibility is that in addition to the required presence of GTP for targeting or translocation, its hydrolysis is also required during protein unfolding, perhaps for the regulation or recycling of some of the molecules involved in this process. A more provocative possibility is that the translocation machinery could work by a saltatory mechanism, requiring GTP hydrolysis for the regulation or accomplishment of the cycles involved. For example, in such a model one or two structural domains (α-helixes for example) can be translocated as a unit and reactivation of the translocation machinery (regulated or produced by GTP hydrolysis) will be required for the translocation of the following domains. Thus, the presence of GTP, and not its hydrolysis, will be sufficient to translocate small nascent chains that at the most contain one or two such domains. However, long nascent chains will require more than one translocation cycle and, therefore, no translocation will be observed if GTP hydrolysis is blocked. One interesting implication of this model is that more than one domain of the secretory protein could be simultaneously interacting with the translocation machinery. This raises the possibility that simultaneous interaction of more than one topological domain of the secretory protein could regulate and determine the ultimate fate of the process in cases in which more than one final destination is possible. Examples of such cases

have been described for the hepatitis B virus precore protein (Appendix 2; Garcia et al, 1988) or the scrapie protein (V. Lingappa, personal communication). In conclusion, although all these speculations need to be explored, our results provided new insights of the mechanism by which protein translocation across membranes occurs.

APPENDIX 1

WILD TYPE AND MUTANT SIGNAL PEPTIDES OF *ESCHERICHIA COLI* OUTER MEMBRANE LIPOPROTEIN INTERACT WITH EQUAL EFFICIENCY WITH MAMMALIAN SIGNAL RECOGNITION PARTICLE.

ABSTRACT

The signal peptide of the outer membrane lipoprotein (OMLP) of E.coli was shown to be capable of promoting protein translocation across mammalian microsomal membranes in vitro. We assayed translocation of a fusion protein containing the OMLP signal peptide and nine amino acids of OMLP fused in frame to β-lactamase. The efficiency with which the mammalian translocation machinery recognizes and accepts the OMLP signal peptide as substrate is indistinguishable from that of mammalian secretory proteins. Upon translocation mammalian signal peptidase (SPase) processes the pre-OMLP-β-Lactamase protein at different sites than are utilized in vivo by E.coli OMLP signal peptidase (SPaseII), but that can be predicted as mammalian SPase cleavage sites.

Mutants in the OMLP signal peptide were tested for their ability to promote translocation of the fusion protein in this assay system. It has been shown previously that mutants in the positively charged amino acids at the amino terminus of the signal peptide severely delay the translocation of OMLP in vivo in E.coli. However, these mutants had no detectable effect either on signal recognition by mammalian signal recognition particle or on the efficiency of translocation itself.

INTRODUCTION

Secretory, lysosomal and most integral membrane proteins contain peptide sequences that act as signals for their specific translocation across the membrane of the endoplasmic reticulum or ER (Walter et al, 1984; Walter and Lingappa, 1986). Usually, the signal peptides are cleaved by signal peptidase (SPase) located in the luminal side of the ER membrane during or immediately after translocation (Blobel and Dobberstein, 1975a). Biochemical dissection of canine ER fractions capable of in vitro protein

translocation has led to the characterization of the signal recognition particle or "SRP" (Walter and Blobel, 1980) and the SRP receptor (Meyer and Dobberstein, 1980a&b; Meyer et al, 1982; Gilmore et al, 1982a&b). These two components function to target nascent secretory proteins to the ER membrane (Walter et al, 1984; Walter and Lingappa, 1986) and to initiate the translocation process. SRP has high affinity for ribosomes engaged in synthesis of secretory proteins (Walter et al, 1981) and upon binding causes inhibition of protein synthesis or "elongation arrest" (Walter and Blobel, 1981b). Upon interaction of the SRP/ribosome/nascent chain complex with the SRP receptor in the ER membrane, the SRP dependent elongation arrest is released and the ribosomes engage in a functional ribosome-membrane junction (Walter and Blobel, 1981a; Gilmore et al, 1982a&b). Subsequent translocation of the protein across the membrane proceeds - most likely coupled to translation - by an essentially unknown mechanism.

Several observations indicate that the prokaryotic protein translocation machinery may function by a mechanism similar to that described for the translocation of secretory proteins across the mammalian endoplasmic reticulum membrane: a) Prokaryotic secretory and some integral membrane proteins contain signal peptides that have similar features to that of eukaryotic proteins (Watson, 1984; Briggs and Gierasch, 1986). b) The signal peptide of β-lactamase (a periplasmic enzyme) requires SRP and SRP receptor to be translocated across canine ER membranes *in vitro*, and the translocated protein is correctly processed by the mammalian SPase (Muller et al, 1982). c) Expression of β-lactamase in Xenopus oocytes results in secretion of the correctly processed enzyme (Wiedmann et al, 1984), indicating that the signal peptide of this bacterial protein is recognized *in vivo* by the eukaryotic translocation machinery. d) The

expression in E.coli of eukaryotic secretory proteins containing their natural signal peptides results in many cases in localization of the mature protein into the periplasmic space (Fraser and Bruce, 1978; Talmadge et al, 1980a&b). e) In vitro translocation systems have been developed recently using inverted plasma membrane vesicles from E.coli (Muller and Blobel, 1984a; Rhoads et al 1984). Such a system has been used to show that a soluble factor that can be separated from the membranes is required for their translocation activity (Muller and Blobel, 1984b). Such a factor may act similarly to SRP in that it may somehow prevent precursor proteins from assuming a tertiary structure that would then be incompatible with translocation (Randall and Hardy, 1986). In spite of these similarities, prokaryotic and eukaryotic protein secretion appears to differ in the degree of coupling between translation and translocation (Walter and Lingappa, 1986; Randall and Hardy, 1986).

The evolutionary functional conservation of signal peptides raises the question about the molecular nature of the information contained within them, since no primary sequence homology is apparent (Watson, 1984). Compilations of known signal sequences reveal features that appear to be conserved (Watson, 1984; Briggs and Gierasch, 1986; vonHeijne, 1984 and 1985): i) One to three basic amino acids are usually found at the amino terminus of the signal peptide, ii) 10 to 15 hydrophobic amino acids follow these basic amino acids, and iii) amino acids of small side chain (alanine or glycine) are preferentially found at positions -1 and -3 of the SPase cleavage site. Recently it has been shown that the a remarkable number of random amino acid sequences can function as signal peptides in vivo, albeit most at reduced efficiency (Kaiser et al, 1987). Nevertheless, it appears that the specificity with which these peptides are recognized must be

surprisingly low and seems to correlate primary to the overall hydrophobicity (Kaiser et al, 1987). Thus, it would seem that other conserved features of signal peptides may indeed be dispensable and may at most add fidelity to the process.

To test specifically which of the conserved features of the signal peptides are relevant to their function, mutations in the signal peptide of the E.coli outer membrane lipoprotein (OMLP) have been constructed and their in vivo effects have been determined (Inouye et al, 1982, 1983a&b, 1984; Vlasuk et al, 1983, 1984 Pollitt et al 1984). Replacement of the positively charged amino acids of the signal peptide with acidic amino acids, although not completely abolishing translocation, results in accumulation of the precursor in the cytoplasm (Inouye et al, 1982; Vlasuk et al, 1983). This cytoplasmic pre-OMLP can be post-translationally translocated across the plasma membrane with a considerable delay relative to the wild type protein (Inouye et al, 1982; Vlasuk et al, 1983). Most of the eukaryotic signal peptides contain positively charged amino acids in their amino terminus. However, in contrast to the prokaryotic signal peptides, some of them contain acidic amino acids resulting in a signal peptide with a negatively charged amino terminus (vonHeijne, 1984). This fact raises questions about the functional importance of these charges for protein translocation across eukaryotic ER membranes. To address this question, we have characterized and quantitated the efficiency by which the eukaryotic translocation machinery recognizes OMLP signal peptide mutants.

MATERIALS AND METHODS

Plasmid constructions: The construction of a plasmid containing the coding sequences for the signal peptide and the first nine amino acids of the OMLP fused in frame to the coding sequences for mature β-lactamase has been

described elsewhere (Ghrayeb and Inouye, 1984). An XbaI-BamHI fragment of plasmid pJG300 (Ghrayeb and Inouye, 1984) that contains the coding sequences for the wild type or alternatively mutations in the signal peptide of this fusion protein were inserted between the XbaI and BamHI site of the pSP64 vector (Krieg and Melton, 1987). The resulting plasmids contain the fusion gene in the correct orientation for transcription from the SP6 phage promoter.

Transcription by the SP6 phage RNA polymerase: The plasmids were linearized with BamHI and transcribed in 20 µl reactions containing 40 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 0.5 mM each ATP, CTP and UTP, 0.1 mM GTP, 0.5 mM G(5')ppp(5')G, 10 mM dithiothreitol, 1,000 U/ml of human placental ribonuclease inhibitor, 0.1 mg/ml of linearized plasmid and 500 U/ml of SP6 RNA polymerase. The reactions were incubated at 40 ^{o}C for 60 min, and were terminated by phenol-chloroform extraction. Nucleic acids were ethanol precipitated and the resulting pellet was dissolved in 40 µl of water.

In vitro translation and translocation assays: Wheat germ translation extracts were prepared as described previously (Erickson and Blobel, 1983). Pancreatic microsomal vesicles (K-RM) were depleted of SRP and ribosomes by EDTA and salt extraction as described (Walter and Blobel, 1983a). SRP was prepared from canine pancreas as previously described (Walter and Blobel, 1983b). Translation were performed at 26 ^{o}C for 1 hour as reported (Erickson and Blobel, 1983), except that the magnesium concentration was found optimal at 3.5 mM and that 0.002 % Nikkol detergent (octa-ethyleneglycol-n-dodecylether) was included to stabilize SRP activity (Walter and Blobel, 1980). RNA transcripts of 50 ng of plasmid (contained in 1 µl) were translated in a 25 µl reaction containing 25 µCi of ^{35}S-Methionine. Translation products were visualized after overnight

exposure after SDS polyacrylamide gel electrophoresis.

Microsome sedimentation assay: Translocation of the processed form of OMLP-β-lactamase was assayed by cosedimentation of this protein with the microsomal membranes. After translation the reactions were transferred to an ice-water bath and KOAc was added to a concentration of 500 mM. Ten equivalents of K-RM (Walter and Blobel, 1983a) were added as carrier membranes. The reactions were layered on a 100 µl cushion containing 0.5 M sucrose, 0.5 M KOAc and 2 mM $Mg(OAc)_2$. After centrifugation at 30 psi in a Beckman Airfuge for 5 min, the proteins from the supernatant and the pellet were TCA precipitated and subjected to SDS-PAGE and autoradiography. This sedimentation assay was preferable over proteolytic protection assays, due to the intrinsic protease resistance of mature β-lactamase (Muller et al, 1982).

SRP arresting and translocation efficiency assays: For quantitative interpretation of the SRP dependent elongation arrest and translocation efficiency, globin mRNA was included as an internal standard of a protein whose translation is not affected by SRP (Walter and Blobel 1981b). Bands corresponding to the precursor and processed forms of the OMLP-β-lactamase protein and globin were quantified by densitometric scanning of the preflashed autoradiograms. To measure elongation arrest activity, the percentage of synthesis at an SRP concentration equal to (A) was determined by:

$$\% \text{ synthesis} = \frac{\text{pre-OMLP-}\beta\text{-lactamase(A)} \times \text{globin(0)}}{\text{pre-OMLP-}\beta\text{-lactamase(0)} \times \text{globin(A)}} \times 100$$

pre-OMLP-β-lactamase(A) and globin(A) correspond to the intensities of the bands of these proteins made at a SRP concentration equal to A, and pre-OMLP-β-lactamase(0) and globin(0) correspond to the intensities of the bands of these proteins made in the absence of SRP. To quantitate translocation efficiency, the percentage of signal peptide processing at a given SRP concentration (A) was determined relative to the total pre-OMLP-β-lactamase synthesis when no SRP was added. The factor 10/9 was included because one of the ten methionine residues of pre-OMLP-β-lactamase is removed upon processing. For this calculation the following equation was used:

$$\% \text{ translocation} = \frac{10/9\ \text{OMLP-}\beta\text{-lactamase(A)} \times \text{globin(0)} \times 100}{[\text{pre-OMLP-}\beta\text{-lactamase(0)} + 10/9\ \text{OMLP-}\beta\text{-lactamase(0)}] \times \text{globin(A)}}$$

Note, that this term is different from the one previously used to measure the efficiency of translocation (Siegel and Walter, 1985). Because we determine the percentage of translocated protein relative to the total amount of protein synthesized in the absence of SRP (rather than the total protein made in each reaction, i.e. in the presence of various SRP concentrations), this new term reflects more accurately the translocation activity of a given amount of membranes at different SRP concentrations. The distortion of the measurements produced by the translational arrest of the precursor protein observed at high SRP concentration is thereby eliminated.

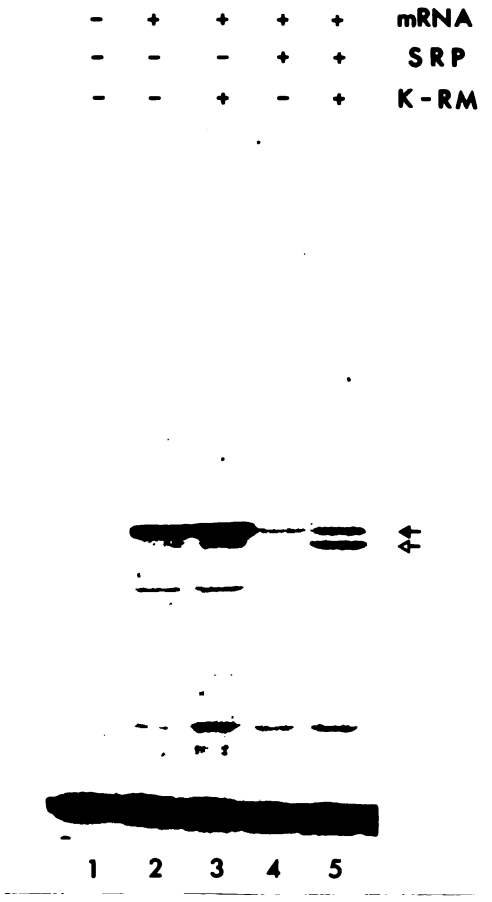
RESULTS

The signal peptide of the E.coli OMLP has all the features commonly found in both eukaryotic and prokaryotic signal sequences (Watson, 1984; Briggs and Gierasch, 1986). Using a standard in vitro protein translocation assay, we tested if this prokaryotic signal peptide can be properly recognized by the eukaryotic translocation machinery. In order to avoid complications due to the small size of authentic pre-OMLP (58 amino acids plus the 20 amino acid signal peptide, i.e. the signal peptide would just be barely exposed outside the eukaryotic ribosome by the time protein synthesis is terminated), we chose to study the function of the OMLP signal peptide as part of a larger fusion protein. An in vitro synthesized mRNA (Krieg and Melton, 1987) encoding a fusion protein of the signal peptide and the first 9 amino acids of OMLP fused to β-lactamase (pre-OMLP-β-lactamase, see Ghrayeb and Inouye, 1984) was translated in a wheat germ cell free extract (Fig.1, lane 2). Addition of salt extracted microsomal membranes (K-RM) in the presence (Fig.1, lane 5), but not in the absence (Fig.1, lane 3), of SRP led to the formation of an additional band of lower molecular weight. Both the primary translation product and the lower molecular weight band can be immunoprecipitated by anti-β-lactamase antibodies (data not shown), indicating that the latter corresponds to a processed form of pre-OMLP-β-lactamase protein. Addition of SRP in the absence of microsomal membranes (Fig.1, lane 4) led to a substantial decrease in pre-OMLP-β-lactamase synthesis due to elongation arrest by SRP.

To verify that the processed form of OMLP-β-lactamase is indeed translocated across the membrane of the microsomal vesicles, the translocation reactions were fractionated by sedimentation prior to SDS-PAGE analysis. Translation reactions performed in the absence or presence of SRP

FIGURE 1: SRP dependent processing of the OMLP-β-lactamase protein by mammalian microsomes.

The translation reactions were carried out in a 25 µl volume. Transcripts from 50 ng of the plasmid were added per reaction. SRP (about 7 nM and K-RM (one equivalent) were added in the indicated reactions. Lane 1, no exogenous RNA was included. Lane 2, only RNA was added. Lane 3, RNA and K-RM were added. Lane 4, RNA and SRP were added. Lane 5, RNA, SRP and K-RM were added. The full and open arrows indicates the bands corresponding to the precursor (32 kD) and the processed form (30 kD) of the OMLP-β-lactamase protein, respectively.


- + + + + mRNA
- - - + + SRP
- - + - + K-RM
1 2 3 4 5

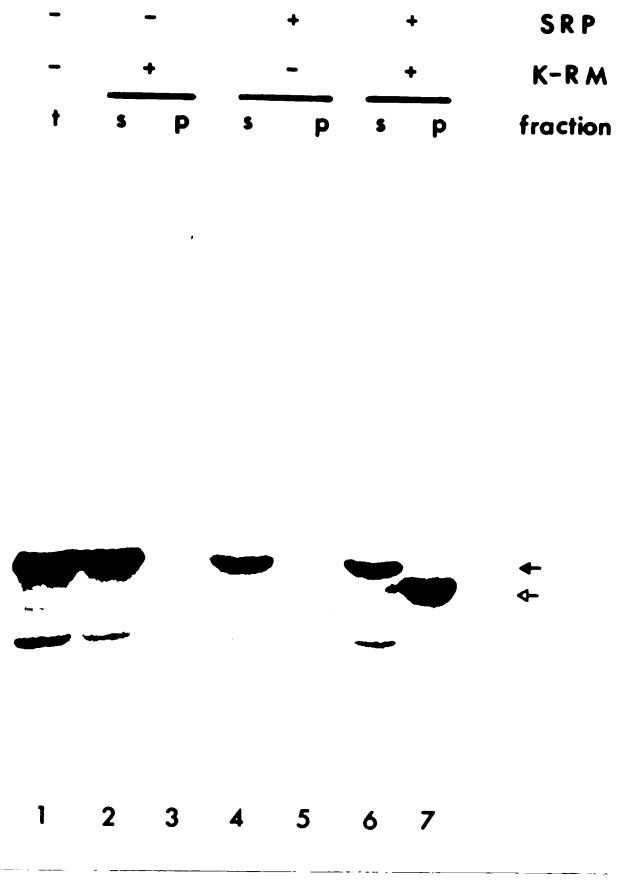
and/or K-RM were centrifuged as described in methods. Figure 2 shows an analysis of the pellets and supernatants by SDS-PAGE. When both SRP and K-RM were included in the translation, the processed form of the fusion protein pelleted quantitatively with the microsomal membranes (Fig.2, lane 7), while, as expected, the precursor protein remained in the supernatant (Fig.2, lane 6). Pre-OMLP-β-lactamase also remained in the supernatant fractions if K-RM (Fig.2, lanes 2 and 3) or SRP (Fig.2, lanes 4 and 5) were added independently. Taken together the results of Figure 1 and 2 demonstrate that i) the signal peptide of pre-OMLP is recognized by mammalian SRP and ii) that the OMLP-β-lactamase fusion protein is properly targeted to and translocated into the lumen of the microsomal vesicles, where it appears proteolytically processed by SPase.

Most E.coli periplasmic and membrane proteins are processed by a signal peptidase (SPaseI) that is very similar in its specificity to the mammalian counterpart (Benson et al, 1985). In contrast, processing of pre-OMLP is coupled to the addition of a fatty acid moiety to OMLP (Hussain et al, 1980) and the proteolytic cleavage step is performed by a specialized signal peptidase (SPaseII, see Benson et al, 1985). This peptidase is sensitive to the peptide antibiotic globomycin (Dev et al, 1985). To test whether a mammalian counterpart of SPaseII exists we added globomycin to the in vitro translocation assay. Even at high concentrations (30 µg/ml) of globomycin, no inhibition of the processing of pre-OMLP-β-lactamase was observed (data not shown; 1 µg/ml globomycin completely inactivate E.coli SPaseII (Dev et al, 1985)).

To determine the position of the cleavage site in OMLP-β-lactamase by the mammalian SPase, we sequenced a sample of the processed protein. Translation products labeled with ^{3}H-proline in the presence of SRP and

FIGURE 2: Cosedimentation assay of the translation products with the microsomal fraction.

The translation reactions were performed as indicated in figure 1. After translation, the microsomal and soluble fractions were obtained as indicated in materials and methods. "t" indicates the total translation products, "s" indicates the supernatant (soluble fraction) and "p" indicates the pellet (microsomal fraction). The presence of small quantities of pre-OMLP-β-lactamase in the pellet fractions (lanes 3 and 5), is not reproducible and probably corresponds to unspecific sticking to the walls of the centrifuge tubes.


- - + + SRP
- + - + K-RM
t s p s p s p fraction
1 2 3 4 5 6 7

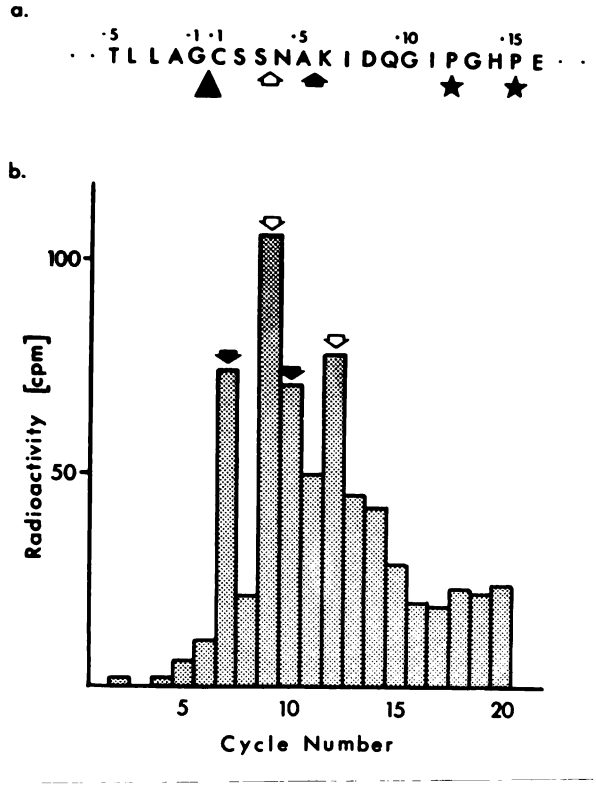
K-RM were resolved by SDS-PAGE. The processed form of OMLP-β-lactamase was electroeluted from the gel (Hunkapillar et al, 1983) and subjected to sequential Edman degradation in a gas phase sequenator. Figure 3b shows that peaks of released radioactivity were obtained at cycles 7, 9, 10 and 12. Peaks 7 and 10 (labeled with close arrows in Figure 3) and peaks 9 and 12 (labeled with open arrows) can be aligned with prolines +12 and +15 in OMLP-β-lactamase (labeled with stars in Figure 3a). These results indicate that mammalian SPase cleaves at two positions in the OMLP-β-lactamase protein: between serine +3 and asparagine +4, and between alanine +5 and lysine +6 (see Figure 3a). The first cleavage position is somewhat preferred (note that the peak in cycle 9 is larger than that in cycle 7). The processed form of OMLP-β-lactamase is therefore a mixture of two proteins differing by two amino acids at their amino terminus. Both cleavage positions differ from that used by E.coli SPaseII which cleaves between position -1 and +1 (Fig. 3a, triangle).

One of the "consensus features" of signal sequences is the presence of basic amino acids at their amino terminal end (Watson, 1984; Briggs and Gierasch, 1986). It has been argued that these positive charges play a role in the translocation process across the membrane (Briggs and Gierasch, 1986; Inouye et al, 1977; vonHeijne, 1984). Mutations that alter these amino acids in the OMLP signal peptide have been constructed by site directed mutagenesis (Inouye et al, 1982; Vlasuk et al, 1983) and have been shown to affect OMLP translocation when assayed in vivo in E.coli. In particular, mutations that change the positive charge to a negative net charge cause a delay in translocation of OMLP (Vlasuk et al, 1983). To test if such mutations also have an effect on the translocation of the OMLP-β-lactamase across mammalian microsomes membranes, we assayed the properties

FIGURE 3: Localization of the signal peptidase cleavage site.

A. Sequence of the OMLP-β-lactamase protein around the signal peptidase cleavage site. The first amino acid of the mature OMLP is indicated as +1. The full triangle shows the positions of the cleavage by the OMLP signal peptidase (SPaseII). The full and empty arrows indicate the two cleavage sites by the mammalian signal peptidase. The stars show the positions of the two radiolabeled prolines detected in the Edman degradation cycles (figure 3b).

B. Edman degradation analysis of the processed form of the OMLP-β-lactamase protein. The ^{3}H-proline radiolabeled processed form of the OMLP-β-lactamase protein was applied to a gas phase sequenator (see results for details). The products of each reaction cycle were diluted in 10 ml of Aquasol (New England Nuclear) and counted for 5 minutes two times. The radioactivity levels indicated in the figure represent the average cpm. Background levels (that represent 20 % of the total counts of the major peaks) are subtracted from the numbers indicated. The background levels of radioactivity for these experiments, were determined by averaging the radioactive contents in the products of two degradation cycles containing no radiolabeled proteins. Similar results were obtained in two other independent Edman degradation experiments (data not shown). We estimate that about 10 % of the radioactivity incorporated in each proline residue was recovered in cycle 7.


a.
-5 -1 -1 -5 -10 -15
· · T L L A G C S S N A K I D Q G I P G H P E · ·
b.
100
50
Radioactivity [cpm]
5
10
15
20
Cycle Number

of three of these mutant signal peptides both for signal recognition by SRP (observed as SRP-dependent elongation arrest) and for translocation itself. In the signal peptide of two of these mutants (I-4 and I-7; see Table 1) negative charges have been introduced, whereas the third mutant (I-6) contains only neutral amino acid residues in its signal peptide (Table 1).

The relative efficiency of SRP to recognize wild type and mutant pre-OMLP signal peptides was measured by determining elongation arrest at different SRP concentrations. The data in Figure 4 show that the synthesis of pre-OMLP-β-lactamase containing wild type or mutant signal sequences is inhibited by SRP to very similar degrees, which are about the same as for an authentic eukaryotic secretory protein, bovine preprolactin (data not shown). This indicates that a net positive charge is not essential for an efficient signal peptide - SRP interaction in vitro. There are some slight, yet reproducible, variabilities in the relative inhibition of synthesis at low SRP concentrations for the different mutations. This effect does not correlate with the net charge at the amino terminus of the signal peptide and therefore may reflect other structural differences of unknown nature that affect signal recognition. In a similar series of experiments we addressed the question whether the efficiency of membrane translocation is dependent on the terminal charge of the signal peptide. Figure 5 demonstrates that mutant and wild type proteins are translocated to an indistinguishable extent at all the SRP concentrations assayed. We conclude that neither signal recognition by SRP nor the subsequent targeting to and translocation across the microsomal membrane is measurably affected by a variety of drastic changes in the amino terminal charge of this signal peptide in vitro.

TABLE 1: AMINO TERMINAL SIGNAL PEPTIDE MUTANTS OF THE OMLP.

MUTANT	AMINO TERMINAL SEQUENCE	NET CHARGE IN THE AMINO TERMINUS
	+ +	
WILD TYPE	+ Met Lys Ala Thr Lys Leu ...	+ 3
	- - +	
I-4	+ Met Glu Asp Thr Lys Leu ...	0
I-6	+ Met ... Ala Thr Asn Leu ...	+ 1
	- -	
I-7	+ Met Glu Asp Thr Asn Leu ...	- 1

FIGURE 4: SRP dependent translation arrest of the OMLP-β-lactamase protein containing the wild type and mutant signal peptides.

Translation reactions were performed as indicated in methods, in the presence of varying concentrations of SRP. Rabbit reticulocyte total RNA (coding primarily for the cytoplasmic protein globin) was included in the reactions simultaneously with the mRNA for the wild type or the signal peptide mutants of the OMLP-β-lactamase protein. The products of each translation reaction were resolved in a 10-15 % gradient SDS-PAGE. After electrophoresis, the gels were dried and exposed on preflashed Kodak XAR-5 film. The intensities of the bands corresponding to each protein were determined in an LKB scanning densitometer. The percentage of synthesis of the OMLP-β-lactamase in each reaction was determined as indicated in materials and methods. The symbols are: closed circles, wild type protein; open triangles, I-4; open circles, I-6; and closed triangles, I-7 mutant proteins respectively.

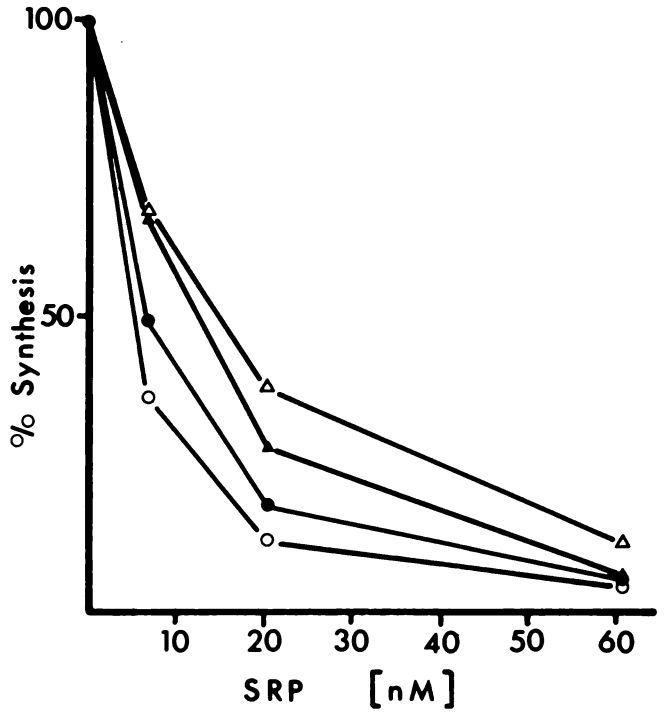
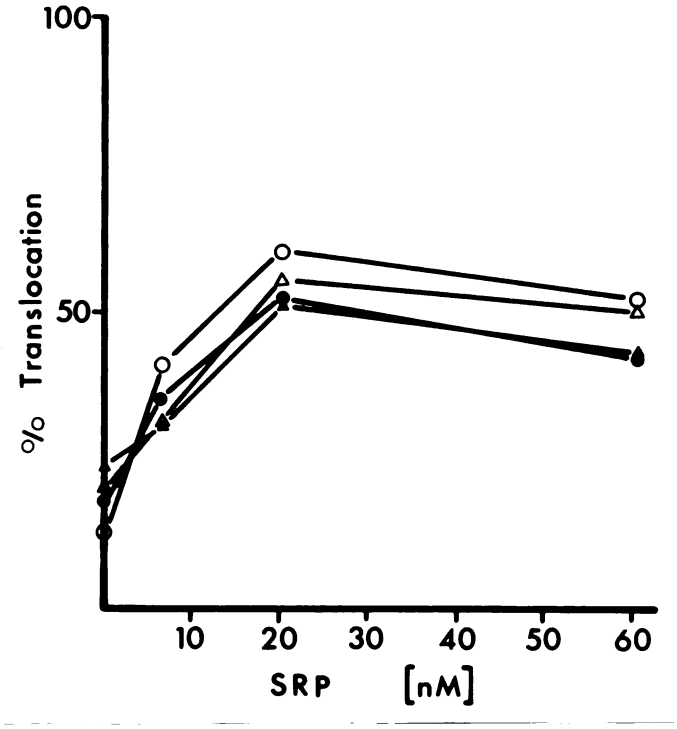

100
50
% Synthesis
10
20
30
40
50
60
SRP [nM]

FIGURE 5: SRP dependent translocation of the OMLP-β-lactamase protein containing the wild type and mutants signal peptides.

Translation reactions were performed as indicated in figure 4, with the exception that K-RM (2 equivalents in a 25 µl reaction) were included in all reactions. The translation products were analyzed as for figure 4. The percentage of translocation was determined as indicated in materials and methods. The symbols are as for figure 4.


100
50
% Translocation
10
20
30
40
50
60
SRP [nM]

DISCUSSION

We have shown that the signal peptide of the OMLP can direct the translocation of OMLP-β-lactamase across the canine microsomal membrane in vitro. Due to the small size of the OMLP (58 amino acids, i.e. it would barely span the large subunit of a eukaryotic ribosome) we chose to study the functions of this signal peptide as part of a fusion protein with β-lactamase. The efficiency by which this prokaryotic signal peptide promotes translocation across mammalian microsomes is indistinguishable from that of mammalian signal peptides, both at the level of signal recognition by SRP and at the level of subsequent translocation across the lipid bilayer. It follows that the OMLP signal peptide contains all the information necessary for efficient protein translocation across the mammalian ER membrane. This confirms the notion that signal sequences directing proteins to the prokaryotic plasma membrane and the mammalian ER are functionally conserved and evolutionarily related.

Similar results were previously obtained for β-lactamase, a periplasmic protein of E.coli. Its signal sequence is also efficiently recognized by the eukaryotic translocation machinery (Muller et al, 1982). Translation of the mRNA for this protein in the presence of canine pancreatic microsomes results in translocation and correct processing of its signal peptide. Thus the mammalian SPase will cleave pre-β-lactamase at the same position as cleaved in E.coli. This is in contrast with the processing observed for the OMLP signal peptide, which is cleaved at positions by the mammalian microsomes that are cryptic in E.coli (Fig.3). This difference can be reconciled considering that the localization and processing pathway for the OMLPs in E.coli is different from that of other membrane and periplasmic proteins. In particular, the signal peptide of the

OMLP is removed by a specialized signal peptidase (SPaseII) that is specific for OMLPs, whereas SPaseI removes the signal peptides of the other membrane and periplasmic proteins (like β-lactamase). SPaseII requires a covalent modification (the addition of a glyceride moiety) on the cysteine residue at the cleavage site of the OMLP (position +1 in Fig.3), as a prerequisite for processing (Inouye et al, 1983a&b). If this modification is prevented (either by site-directed mutagenesis of Cysteine (+1) into a Glycine residue, see Inouye et al, 1983a, or by inhibition of the SPaseII with globomycin, see Ghrayeb et al, 1985), then signal peptide cleavage will not take place. If the same mutation is introduced in the pre-OMLP-β-lactamase fusion protein, the E.coli SPaseI will now cleave at a previously cryptic position (between residues +5 and +6 in Fig. 3a, see Ghrayeb et al, 1985). This cleavage is no longer sensitive to the SPaseII inhibitor globomycin (Ghrayeb et al, 1985), and therefore is performed by SPaseI. In the mammalian system, cleavage is observed at the position where SPaseI cleaves the Glycine (+1) substitution in E.coli (see Fig. 3b), as well as an alternative site that is less frequently used (between residues +3 and +4 in Fig. 3a). As expected, in the mammalian in vitro system the Glycine (+1) substitution behaves indistinguishably from the wild type (data not shown). These results indicate that the mammalian microsomes lack an activity equivalent to SPaseII and that, as for the cleavage of the β-lactamase signal peptide, the E.coli SPaseI and the mammalian SPase have the same specificity for this particular site. The cryptic cleavage site(s) that get utilized by both prokaryotic SPaseI and eukaryotic SPase agree well with the rules described by vonHeijne for signal peptide cleavage (vonHeijne, 1984).

Mutations that change the positively charged amino acids in the amino terminus of the OMLP signal peptide have no effect on signal recognition by SRP or translocation across mammalian microsomal membranes. These results indicate that a net positive charge in the amino terminus of the signal peptide is not required nor does it increase the efficiency of the process in vitro. Functional interactions of the conserved positive charges with the membrane have been suggested (Inouye et al, 1977; vonHeijna, 1984) to be essential for the translocation of the protein. However, some eukaryotic signal sequences deviate from this "consensus" and have acidic amino terminus (Watson, 1984; vonHeijne, 1984), demonstrating that a requirement for an amino-terminal positive net charge is not absolute. Some of the mutants assayed in this study show kinetically delayed translocation in E.coli in vivo, both in OMLP (Inouye et al, 1982; Vlasuk et al, 1983) or in the fusion protein used here (Lunn and Inouye, 1978). It may be possible that the mammalian translocation machinery overcomes the requirement for a net positive charge by coupling translation to translocation more tightly (by targeting the nascent chain via the SRP/SRP receptor) than it is observed in E.coli, where at least the mutant proteins of pre-OMLP were clearly shown to be translocated post-translationally (Inouye et al, 1982; Vlasuk et al, 1983). Alternatively, the α-amino group of the initiating Methionine in eukaryotic cells carries an additional positive charge that is not present when the proteins are synthesized in prokaryotic systems (due to the formylated amino terminus) and which, at least in principle, could compensate for effects that would otherwise be induced by the mutations. Since we cannot construct mutations that completely lack basic groups, our interpretation has to be limited to the effects of the net charges on the amino end of signal sequences.

The question remains, what constitutes a "minimal signal sequence" and how can signal sequences, diverse as they are, be efficiently recognized in a protein/protein interaction by mammalian SRP and/or additional signal receptors within the membrane. For the signal sequence/SRP interaction it was clearly shown by crosslinking experiments that the recognition involves a direct binding of the signal sequence to the 54 kD polypeptide of SRP (Krieg et al, 1986; Kurzchalia et al, 1986). Similarly, the subsequent interactions of signal sequences with membrane components also seem to involve protein/protein interactions, although this has only been demonstarted indirectly (Gilmore and Blobel, 1985; Connolly and Gilmore 1986). Molecular models that account for such interactions must take into account the considerable diversity of signal sequences. Thus, it seems reasonable to consider a model in which some features of the signal peptides, such as a certain amount of hydrophobicity together with the ability to assume a particular secondary structure, may constitute a structural moiety that is recognized in the context of its interactions with other components. Interestingly, an analogous model has been proposed to explain the related problem of how a class II major histocompatibility antigen can bind to different peptide antigens (Guillet et al, 1986). Class II major histocompatibility antigens appear to contain a single binding site for different peptides which, once bound, may adopt a similar general structure that is reinforced by the binding site (Guillet et al, 1986). For signal recognition one could envision a hydrophobic patch or grove on the surface of the receptors (SRP or the yet putative membrane receptor) that binds to signal sequences forcing them to assume an α-helical or β-sheet configuration. Thus, signal recognition and antigen presentation may have evolved similar mechanisms to solve a related problem

and it will be interesting to compare the two systems once more detailed structural information about the receptor/ligand interactions becomes available.

APPENDIX 2

TARGETING OF THE HEPATITIS B VIRUS PRECORE PROTEIN TO THE ENDOPLASMIC RETICULUM MEMBRANE: AFTER SIGNAL PEPTIDE CLEAVAGE TRANSLOCATION CAN BE ABORTED AND THE PRODUCTS RELEASED INTO THE CYTOPLASM

ABSTRACT

The major Hepatitis B Virus (HBV) core protein is a viral structural protein involved in nucleic acid binding. Its coding sequence contains an extension of 29 codons (the "precore" region) at the amino terminus of the protein which is present in a fraction of the viral transcripts. This region is evolutionarily conserved among mammalian and avian HBVs, suggesting it has functional importance, although at least for duck HBV it has been shown to be nonessential for replication of infectious virions.

Using in vitro assays for protein translocation across the endoplasmic reticulum (ER) membrane, we found that the precore region of the HBV genome encodes a signal sequence. This signal sequence was recognized by signal recognition particle (SRP), which targeted the nascent precore protein to the ER membrane with efficiencies comparable to those of other mammalian secretory proteins. A 19 amino acid signal peptide was removed by signal peptidase on the lumenal side of the microsomal membrane, generating a protein similar to the HBV major core protein, but containing 10 additional amino acids from the precore region at its amino terminus. Surprisingly, we found that 70 - 80 % of this signal peptidase cleaved product was localized on the cytoplasmic side of the microsomal vesicles and was not associated with the membranes. We conclude that translocation was aborted by an unknown mechanism, then the protein disengaged from the translocation machinery and was released back into the cytoplasm. Thus, a cytoplasmically disposed protein was created whose amino terminus resulted from signal peptidase cleavage. The remaining 20 - 30 % appeared to be completely translocated into the lumen of the microsomes. A deletion mutant lacking the carboxy terminal nucleic acid binding domain of the precore protein was similarly partitioned between the lumen of the

microsomes and the cytoplasmic compartment, indicating that this highly charged domain is not responsible for the aborted translocation. We discuss the implications of our findings for the protein translocation process and suggest a possible role in the virus life cycle.

INTRODUCTION

Human Hepatitis B Virus (HBV) is a member of a group of enveloped DNA viruses (Hepadna viruses) that use reverse transcription as part of their life cycle (for review see Tiollais et al, 1985; Standring and Rutter, 1986). The HBV virion genome is a circular partially double stranded DNA of 3.2 kb. Due to its small size, the coding information in the DNA is densely packed into four partially overlapping reading frames. The products of two of these open reading frames are found as the major structural components in the viral particles, the core protein and the surface protein. The core protein assembles with pregenomic viral RNA into a core particle, where reverse transcription takes place in the cytoplasm of the infected cells. Core particles are then thought to interact with the cytoplasmic domains of the surface protein, a membrane protein integrated into the endoplasmic reticulum (ER) membrane. Presumably, the surface protein forms patches which bud into the ER lumen enveloping the core particles in the process (Eble et al, 1986). The virion then leaves the cell by passing through its normal secretory pathway. Studies on the biogenesis of HBV have been limited due to the lack, until very recently, of a tissue culture system in which the virus can be replicated. Thus, there is no detailed biochemical knowledge of the steps involved in the HBV assembly process.

The studies described in this paper concern some functional properties of the viral core protein and their potential significance in the viral

life cycle. The major protein found in the HBV core is 21 kD in size and contains at its carboxy terminus a stretch of 36 amino acids which are predominantly arginines (47 %) thought to be involved in nucleic acid binding (Valenzuela et al, 1980). The open reading frame encoding the core protein contains an amino terminal extension (the "precore" region) of 29 amino acids starting with an AUG codon that precedes the initiating methionine used for the translation of the major core protein (p21, Valenzuela et al, 1980). Mapping of the pregenomic RNA transcripts of the ground squirrel hepatitis virus (closely related to the human HBV), revealed that transcription is initiated at three sites around the first AUG codon of the core open reading frame (Enders et al, 1985). Thus, some of the transcripts initiate between the two AUG codons and, therefore, give rise to a translation product initiating at the second AUG corresponding to the major core protein (p21). Some transcripts initiate upstream of the first AUG codon of the core open reading frame and, therefore, are translated to yield a protein of approximately 25 kD (p25). Both transcripts are recruited into polysomes in infected cells (Enders et al, 1987), however p25 protein has not yet been detected in vivo. Since the precore region is conserved in the genome of hepadna viruses, it is likely to be of importance, yet it is not essential for viral replication. This was shown by Chang et al. (1987), who demonstrated that the introduction of a frameshift mutation into the precore region did not adversely affect infectivity of a related duck HBV.

The sequence of part of the precore region (Valenzuela et al, 1980; Ou et al, 1986), resembles that of signal sequences which direct proteins across the membrane of the ER (Watson, 1984). In particular, it contains a positively charged amino acid at its amino terminus, followed by a cluster

of about 10 hydrophobic residues. Furthermore, the expression of p25 (but not p21) in mammalian cells (Ou et al, 1986; Roossinck et al, 1986; McLachlan et al, 1987) or Xenopus oocytes (Standring et al, 1987) results in secretion of core related peptides. While our studies confirm that the precore region functions as an efficient signal peptide in targeting nascent precore protein to the ER membrane and engaging it with the translocation machinery, we observed the unprecedented property that a large proportion of the protein did not complete the translocation process, but was released back into the cytoplasmic compartment.

METHODS

Plasmid constructions: To facilitate the insertion of the HBV core coding sequences into plasmids containing the promoter for the bacteriophage SP6 RNA polymerase, convenient restriction sites were introduced into the HBV genome by site directed mutagenesis using the double primer method (Zoller, M.J., and Smith, M., 1984). The viral sequences TAGGTT and AACTTT (at positions 1757 and 1821 from the unique EcoRI site; Valenzuela et al, 1980) were mutated to the HindIII recognition sequence (AAGCTT) for the construction of pHBVc/p25 and pHBVc/p21 respectively. Double-strand DNA from M13 phage clones containing these mutant HBV genomes were digested with HindIII and HincII, and the DNA fragments containing the HBV core sequences were cloned into the HindIII-HincII sites in the pSP64 vector polylinker (Krieg and Melton, 1986). For constructing plasmid pHBVc/p25δ, the arginine codons 179 and 180 (the first positively charged amino acids of the carboxy terminal nucleic acid binding domain) were changed to the termination codon UGA by site directed mutagenesis (Zoller, M. J., and Smith, M., 1984). A DraI(position 2185)-EcoRI(position 1) viral DNA fragment containing the mutated HBV core coding sequences was transferred

to pHBVc/p25 digested with EcoRI and partially digested with DraI. The recombinant pHBVc/p25δ plasmid was selected according to both its restriction map and its capacity to express the mutated protein p25δ. The introduction of these changes was confirmed by sequencing of the mutated DNA.

Transcription with SP6 phage polymerase: The plasmid DNAs were linearized with EcoRI and transcribed in 20 µl reactions containing 40 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 0.5 mM each ATP, CTP and UTP, 0.1 mM GTP, 0.5 mM G(5')ppp(5')G, 10 mM dithiothreitol, 1,000 U/ml of human placental ribonuclease inhibitor, 0.1 mg/ml of linearized plasmid and 500 U/ml of SP6 RNA polymerase. The reactions were incubated at 40 °C for 60 min, and were terminated by phenol-chloroform extraction. Nucleic acids were ethanol precipitated and the resulting pellet was dissolved in 40 µl of water.

Translation and translocation assays: Wheat germ translation extracts were prepared as previously described (Erickson and Blobel, 1983). Microsomal vesicles (RM) were prepared from canine pancreas as described (Walter and Blobel, 1983a). Microsomal vesicles depleted of SRP and ribosomes (K-RM) were prepared by EDTA and salt extraction (Walter and Blobel, 1983a and 1983b). SRP was purified from RM as previously described (Walter and Blobel, 1983b). Translations were performed at 26 °C for 1 hour (unless indicated otherwise) as reported (Erickson and Blobel, 1983), except that the magnesium concentration was found optimal at 3.5 mM and that 0.002 % Nikkol detergent (octa-ethyleneglycol-n-dodecylether) was included to stabilize SRP activity (Walter and Blobel, 1980). RNA transcripts from 50 ng of plasmid (contained in 1 µl) were translated in 10 µl reactions containing 25 µCi of ^{35}S-Methionine. Translation products were visualized

after overnight exposure to X-Omat AR Kodak film of 10-15 % gradient SDS polyacrylamide gel electrophoresis (SDS-PAGE).

Synchronized translation assays: Translation reactions of the desired volume were prepared as described above. The reaction was prewarmed at 26 ^{o}C for 2 min prior to the addition of the mRNA at time 0 min. To synchronize translation, the initiation inhibitors 7-methylguanosine-5'-monophosphate (at 4 mM final) and edeine (at 5 µM final) were added at time 2 min. A 10 µl sample of the reactions was taken at the times indicated in the figures, and the proteins were resolved by SDS-PAGE. The gels were fluorographed with 2,5-diphenyloxazole and exposed to X-Omat AR Kodak film.

Protease protection assay: After translation, 10 µl reactions were transferred to an ice-water bath. One µl of 10 mg/ml trypsin or protease K was added and incubated at 0 ^{o}C for 30 min. Ten units of aprotinin or 1 µl of 100 mM phenylmethylsulphonyl fluoride (in ethanol), was added to inhibit trypsin or protease K, respectively. The products were precipitated by addition of one volume of 20 % trichloroacetic acid and resuspended in sample buffer for SDS-PAGE. To the reactions indicated, 1 µl of 4 % Triton X-100 was added before the addition of the proteases.

Microsome sedimentation assay: After translation, the reactions were transferred to an ice-water bath and potassium acetate was added to a concentration of 500 mM. Ten equivalents of K-RM were added as carrier membranes. The reactions were layered on a 100 µl cushion containing 500 mM sucrose, 500 mM potassium acetate and 2 mM magnesium acetate. After centrifugation at 30 psi in a Beckman Airfuge for 5 min, the proteins from the supernatant and the pellet were trichloroacetic acid precipitated and subjected to SDS-PAGE and autoradiography.

RESULTS

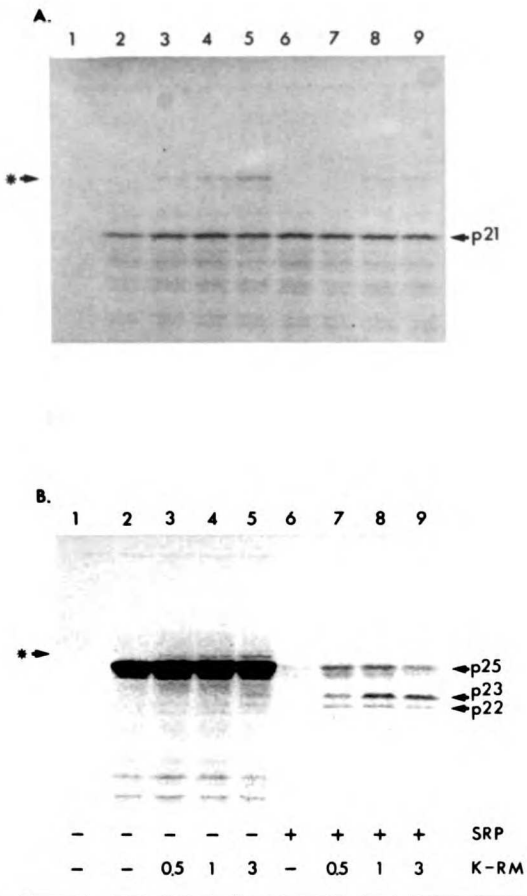
The precore region of the HBV core protein encodes a signal peptide.

In order to address questions concerning the function of the precore region of the HBV core protein, we decided to synthesize mRNAs which either include the full core protein open reading frame (p25 mRNA) or the open reading frame starting with the second AUG (p21 mRNA). For this purpose, plasmids (pHBVc/p21 and pHBVc/p25, see methods for details) were constructed which contain the coding sequences downstream of the bacteriophage SP6 RNA polymerase promoter (Krieg & Melton, 1987). These plasmids were transcribed *in vitro* as indicated in the methods, and the resulting transcripts were translated in a cell free wheat germ extract. Figure 1 shows the translation products of both the p21 mRNA (panel A, lane 2) and p25 mRNA (panel B, lane 2) transcripts. The respective molecular weights of both translation products are in agreement with those predicted from the coding sequences, although, as it will be shown below, the products obtained after the one hour incubation in the translation extract carried a covalent modification which increased their respective apparent molecular weights slightly (about 1 kD). Both p21 and p25 were immunoprecipitated by anti-HBV core protein antibodies (data not shown), confirming that the products were indeed derived from HBV core protein coding sequences. When the p25 mRNA was translated (Figure 1B) hardly any translation products comigrating with p21 were obtained due to the lack of internal initiation at the second AUG codon, i.e. translation is efficiently initiated at the first AUG codon.

To test whether the precore region will function as a signal peptide as suggested (see Introduction), we supplemented the translation reactions with salt-extracted rough microsomal membranes (K-RM) (Fig. 1, panels A and

FIGURE 1: Effect of SRP and K-RM on the translation of p21 and p25.

In vitro transcripts from plasmids pHBVc/p21 (panel A) and pHBVc/p25 (panel B) were translated by wheat germ extracts in 10 µl reactions for one hour at 26 ^{o}C. The translations were performed with increasing concentrations (0.5 to 3.0 equivalents per reaction) of salt-extracted microsomal membranes (K-RM, see methods), both in the absence (lanes 3-5) or presence (lanes 7-9) of 50 nM SRP. Reactions without RNA (lanes 1) and with mRNA alone (lanes 2) were included as controls. In one reaction only SRP (50 nM) was added (lane 6). The asterisk indicates the product of translation of a minor mRNA contaminant of our K-RM preparation. This protein is not immunoprecipitated with anti-HBV core antibodies (data not shown).


A.
1 2 3 4 5 6 7 8 9
p21
B.
1 2 3 4 5 6 7 8 9
p25
p23
p22
– – – – – + + + + SRP
– – 0.5 1 3 – 0.5 1 3 K-RM

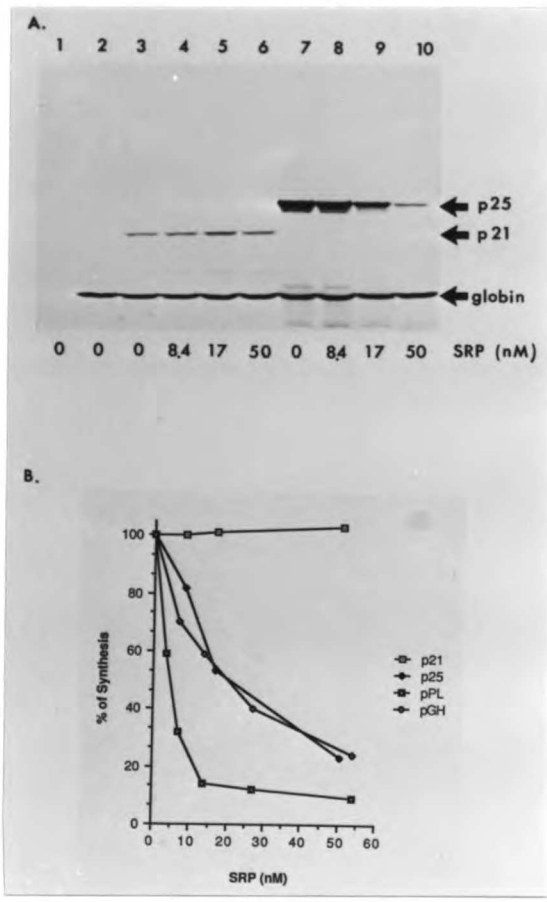
B, lanes 3-5). These membranes have been depleted of signal recognition particle (SRP) by the salt treatment (Walter and Blobel, 1980) and thus, as expected, had no effect on the translation of either p21 or p25. However, when the translation reactions were supplemented with purified SRP in addition to K-RM, processing of p25 was observed (Fig. 1B, lanes 7-9), whereas p21 was not affected (Fig. 1A, lanes 7-9). Unexpectedly, we found that the SRP-dependent processing of p25 by K-RM yielded a heterogeneous product; two distinct bands with molecular weights of approximately 22 and 23 kD (p22 and p23) were consistently obtained (Fig.1B, lanes 7-9). As it is shown below, p23 is derived from p22 by a secondary modification.

Further confirmation that the precore region contains a bona fide signal peptide and is directly recognized by SRP, is given by the result that SRP in the absence of K-RM causes inhibition of p25 synthesis. This effect is due to the ability of SRP to cause an arrest or pausing in the elongation of proteins after a signal sequence has been exposed outside the ribosome (Walter and Blobel, 1981). This elongation arrest is released once the SRP/ribosome/nascent chain complex interacts with the SRP receptor on the surface of the microsomal vesicles (Walter & Blobel, 1981). Although the in vivo significance of the elongation arrest reaction still remains to be determined, it has proven to be a valuable measure of the relative affinity (and thus efficiency) with which a signal sequence is recognized by SRP. Figure 1 (panels A and B, lanes 6) shows the qualitative effect of SRP addition on the translation of p21 and p25, respectively. Note, that the synthesis of p25 is severely inhibited, while that of p21 is unaffected. A quantitative comparison (Fig. 2) confirms these results over a range of SRP concentrations. Here, we included globin mRNA as an internal control of a cytoplasmic protein that, lacking a signal

FIGURE 2: Quantification of SRP-dependent elongation arrest on the translation of p21 and p25.

PANEL A: Translations of pHBVc/p21 (lanes 3-6) and pHBVc/p25 (lanes 7-10) mRNAs were performed for one hour at 26 ^{o}C, in the presence of increasing concentrations of SRP (as indicated) in 10 µl reactions. Total rabbit reticulocyte RNA (0.2 OD_{260} per reaction, primarily encoding the cytoplasmic protein globin) was included (lanes 2-10) as an internal control of a protein whose translation is not affected by SRP (Walter et al, 1981). A reaction with no RNA added is shown in lane 1.

PANEL B: The relative radioactivity in the bands in panel A were determined by densitometric scanning of the autoradiograms using an LKB scanner (model Ultroscan XL), under conditions in which the intensities measured were linear with respect to the radioactivity incorporated. The percentage of synthesis indicated was determined as described elsewhere (Garcia et al, 1987). The results of experiments for preprolactin (pPL) and pregrowth hormone (pGH), two secretory proteins, are included for comparison.

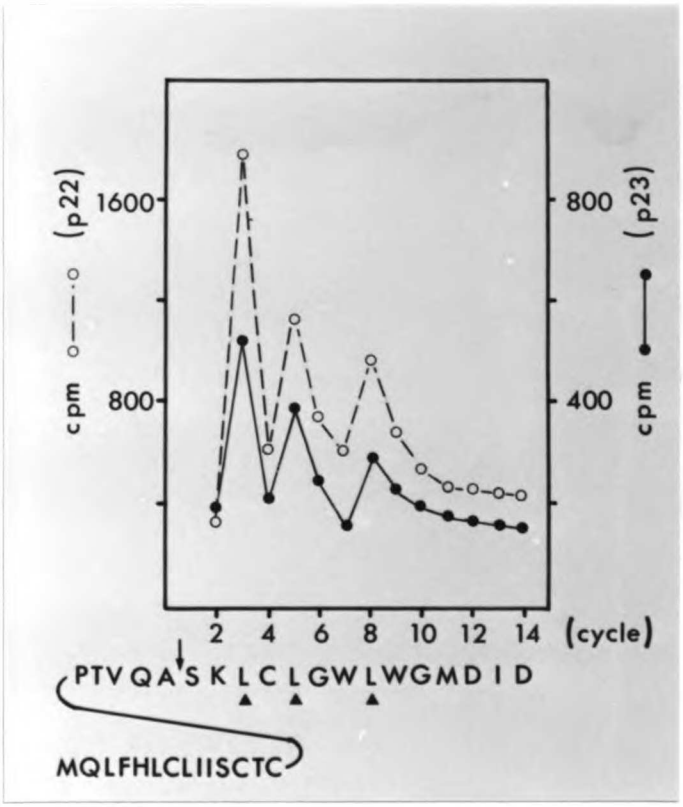

A.
1 2 3 4 5 6 7 8 9 10
p25
p21
globin
0 0 0 8.4 17 50 0 8.4 17 50 SRP (nM)
B.
% of Synthesis
100
80
60
40
20
0
0 10 20 30 40 50 60
SRP (nM)
p21
p25
pPL
pGH

peptide, is not affected in its synthesis by SRP (Walter et al, 1981). Figure 2B shows a quantitative comparison of the inhibition of p25 with that of two authentic mammalian secretory proteins, preprolactin (pPL) and pregrowth hormone (pGH). While preprolactin synthesis was inhibited by 80% at 10 nM SRP, the synthesis of pregrowth hormone and p25 requires about 50 nM SRP to be inhibited to the same level. This indicates that the affinity with which the signal peptide of p25 is recognized by SRP falls into the same range as those observed for signal peptides of at least one mammalian secretory protein.

To characterize further the processing of p25 by microsomal membranes, we determined the exact cleavage site by sequencing the amino terminus of both processed products, p22 and p23. Translation of p25 mRNA in the presence of microsomal membranes (RM; not depleted of SRP, see methods) was performed in the presence of ^{3}H-leucine. p22 and p23 were resolved by preparative SDS-PAGE and electroeluted. After the proteins were tested for their purity in an analytical SDS-PAGE (data not shown), the samples were subjected to sequential Edman degradation. The products released in each degradation cycle were analyzed for their content of radioactivity (Fig.3). Both processed bands gave an identical sequence pattern with peaks of radioactivity in cycles 3, 5 and 8. This distribution pattern of leucine residues is found only once in the p25 coding sequence with leucines in positions 22, 24 and 27 after the initiating methionine. These results show that both p22 and p23 have the same amino terminus and localize the cleavage site between amino acids 19 and 20 of p25. This processing site is consistent with the empirical consensus rules for signal peptidase cleavage (vonHeijne, 1984), with respect to both the distance from the hydrophobic core of the signal peptide and the amino acids found at

FIGURE 3: Amino terminal sequence analysis of p22 and p23.

Translation products after one hour of labeling with ^{3}H-leucine in the presence of microsomal membranes (RM; containing SRP, see methods) were resolved by preparative SDS-PAGE. The p22 and p23 protein bands were electroeluted as described (Hunkapiller et al, 1981) and their purity was determined by analytical SDS-PAGE (data not shown). Each labeled protein (about 70,000 cpm) was subjected to sequential Edman degradation in a gas phase sequenator. The radioactive content of the products of each cycle was determined. Each point in the figure corresponds to the total radioactivity released per cycle. In the third cycle, 69 % and 21 % of the radioactivity incorporated per leucine residue was recovered for p22 and p23, respectively. The sequence of the first 33 amino acids of p25 is indicated in the single letter code at the bottom of the figure. The triangles and the arrow indicate the leucine residues (aligned with the radioactive peaks) and the signal peptidase cleavage site, respectively.


cpm ○—·○ (p22)
1600
800
800
400
cpm ●—● (p23)
2 4 6 8 10 12 14 (cycle)
PTV Q A S K L C L G W L W G M D I D
MQLFHLCLIISCTC

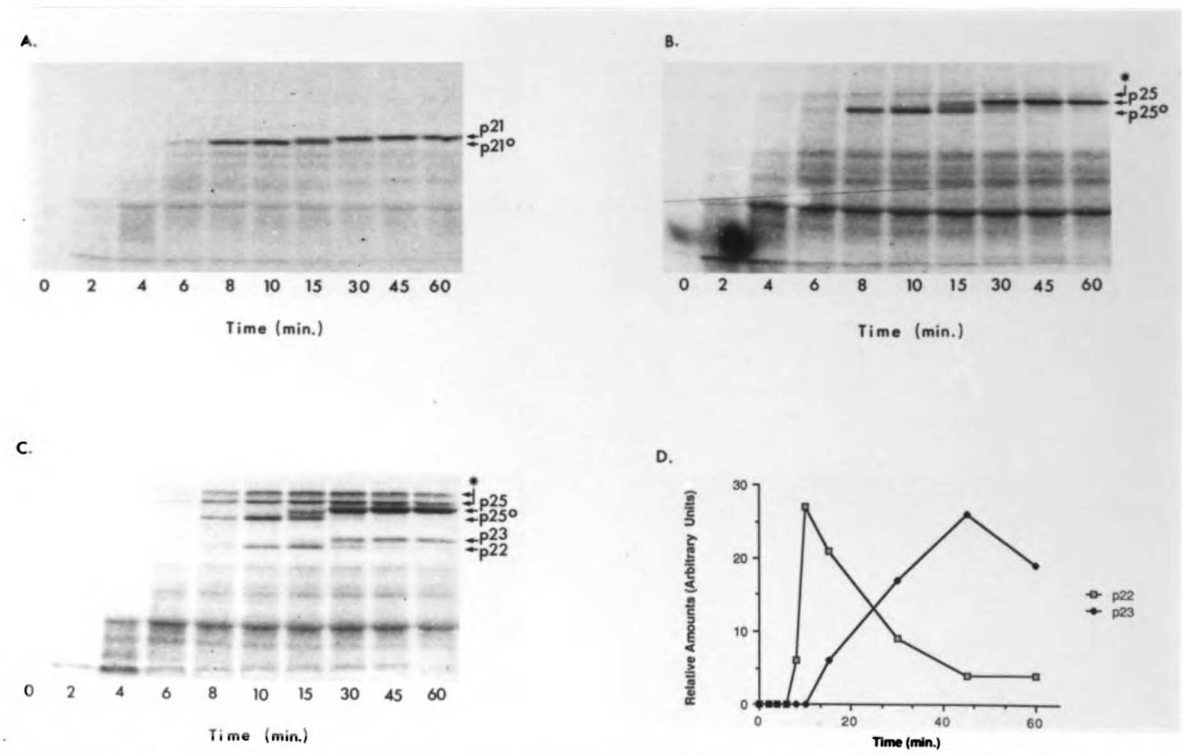
positions -1 and -3 of the cleavage site. Thus, we conclude that the observed processing of p25 protein is performed by signal peptidase and that at least the amino terminal portion of the protein has reached the interior of the microsomal vesicles.

p22 is post-translationally modified to p23.

Since both p22 and p23 are products of signal peptidase cleavage (i.e. contain the same amino terminus; see Figure 3), and are derived from a unique precursor (p25), we reasoned that one could be the product of a secondary modification of the other. To establish a precursor/product relationship between these two proteins, we analyzed the appearance of p22 and p23 at different time points during translation, instead of after 60 min as in the previous experiments. For this purpose, translation reactions were synchronized by the addition of initiation inhibitors after two min (see Methods). Samples were taken at different times and the products were resolved by SDS-PAGE. The results of experiments for both p21 and p25 mRNAs are shown in Figure 4. For p25, the experiment was carried out both in the absence (Fig. 4B) or presence (Fig. 4C) of microsomal vesicles. In the absence of RMs (Fig. 4A and 4B), the appearance of the primary translation products for both p21 and p25 were first observed after 8 min of incubation. We observed that both the p21 and p25 translation products at the 8 min time point migrated slightly faster on the gel than the products obtained after a 60 min incubation. A shift to the slower migrating species was observed around 15 min of incubation. This shift was not due to a discrete pause in elongation. It occurred independently of ongoing protein synthesis since in control experiments where 100 μM cycloheximide was added after 8 min of incubation identical results were obtained (data not shown). Thus we conclude that the shift in

FIGURE 4: Time course in synchronized translations of p21 and p25.

Samples of synchronized translations (see methods) were taken at 0, 2, 4, 6, 8, 10, 15, 30, 45 and 60 min. of incubation and the products were analyzed by SDS-PAGE. Panel A, shows the results obtained for the pHBVc/p21 mRNA. $p21^{o}$ indicates the unmodified p21 protein. Translation of the pHBVc/p25 mRNA was performed in the absence (Panel B) or the presence (Panel C) of microsomal membranes (RM; containing SRP, see methods). As for p21, $p25^{o}$ indicates the unmodified p25 protein. The bands observed above p25 (panels B and C asterisk), correspond to the products of minor mRNA contaminants in our extracts. They are not immunoprecipitated with anti-HBV core antibodies (as in figure 1). Panel D shows a quantitative analysis of the results obtained for p22 and p23. To obtain this data, a longer exposure than the one shown in panel C, was scanned as for figure 2.


A.
p21
p21°
0 2 4 6 8 10 15 30 45 60
Time (min.)
B.
*
p25
p25°
0 2 4 6 8 10 15 30 45 60
Time (min.)
C.
*
p25
p25°
p23
p22
0 2 4 6 8 10 15 30 45 60
Time (min.)
D.
Relative Amounts (Arbitrary Units)
30
20
10
0
0 20 40 60
Time (min.)
p22
p23

electrophoretic mobility was produced by a post-translational modification of both p21 and p25. This modification was independent of the presence of microsomal membranes in the reactions (Figure 4A and 4B) and therefore must have been catalyzed by a cytoplasmic enzyme(s).

Presently we do not know the chemical nature of this post-translational modification. However, since it is carried out by a cytoplasmic enzyme(s) it provided us with a convenient marker for the topographical disposition of the products of signal peptidase cleavage with respect to the microsomal membrane (see also below). Figure 4C shows that p22 was formed first as the primary cleavage product. The bulk of p22 was subsequently modified and converted to p23. Again, the conversion was insensitive to the addition of cycloheximide at 8 min of incubation (data not shown) and, therefore, corresponds to a post-translational modification of the signal peptidase cleaved form of p25. However, in marked contrast with what we observed for p21 and p25, not all p22 is converted to p23 during the time extent of our analysis. A quantitative analysis of the time course of appearance of these two proteins is shown in Figure 4D. Note that after 45 minutes no more of the remaining p22 (about 20 %) was converted to p23. These results suggest that initially two distinct populations of p22 were formed: about 80 % of p22 was not completely translocated across the membrane and therefore was accessible to the cytoplasmic enzyme(s) that converted it to p23, whereas about 20 % was completely translocated and therefore not modified.

Topography of p22 and p23.

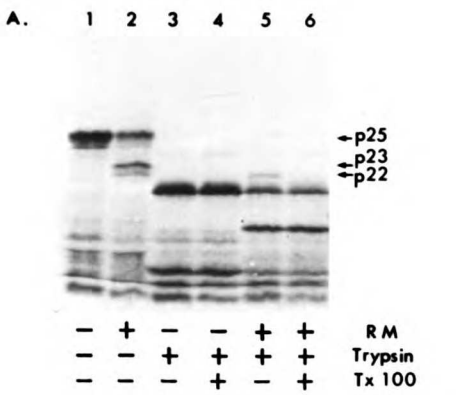
In order to establish directly the topography of p22 and p23 with respect to the microsomal membrane, we used two additional experimental approaches: protease protection by the lipid bilayer (Figure 5) and

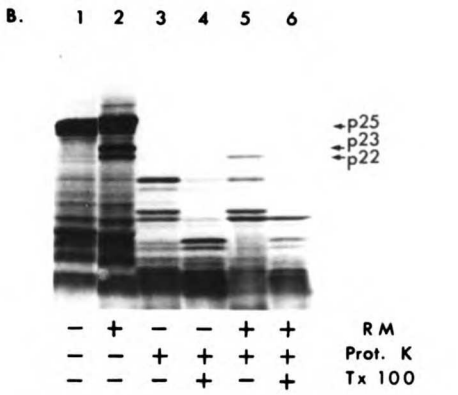
Figure 5: Protease protection analysis.

The pHBVc/p25 mRNA was translated for one hour at 26 °C, in the absence (lanes 1, 3 and 4) or presence (lanes 2, 5 and 6) of microsomal membranes (RM; containing SRP, see methods). The products of a 10 µl translation were treated (lanes 3-6) with trypsin (panel A) or protease K (panel B) at 0 °C for 30 min. Triton X-100 (0.4 % final) was added to the reactions in lanes 4 and 6, prior to the protease addition. After the proteases were inhibited (see methods), the products were resolved by SDS-PAGE. The positions at which p22, p23 and p25 migrate are indicated. These results are representative of five experiments carried out independently. Note that the fraction of p22 that is resistant to proteases depends on the degree to which cytoplasmically disposed p22 has been modified to p23 (see figures 4 and 7). In the unsynchronized translations shown here, the modification has not reached completion and its extent is different for the experiments of panel A and B; thus p22 is not equally protected.

These results also allowed us to map the protease sensitive site(s). Digestion of p25 with trypsin and protease K yields a 20 kD and 18 kD

fragments respectively, which are very resistant to further digestion. Trypsin digestion of a mix of p25, p22 and p23 (panel A, lanes 5-6) results in an additional fragment of about 17-18 kD. Since p25 and p23 differ on their amino terminus due to the removal of the signal peptide, it follows that the protease sensitive site(s) lies about 5-6 kDa from the carboxy terminus of both proteins. We have tested other proteases (elastase, protease V8, thermolysin and subtilysin; each at 1 mg/ml) and found that they also cleaved the protein in approximately the same location. This indicates that the core protein has at its amino terminus a domain that is extremely resistant proteases (see also Takahashi et al, 1983), but that the carboxy terminal portion can easily be digested.


A.
1 2 3 4 5 6
p25
p23
p22
− + − − + + RM
− − + + + + Trypsin
− − − + − + Tx 100


B.
1 2 3 4 5 6
p25
p23
p22
− + − − + + RM
− − + + + + Prot. K
− − − + − + Tx 100

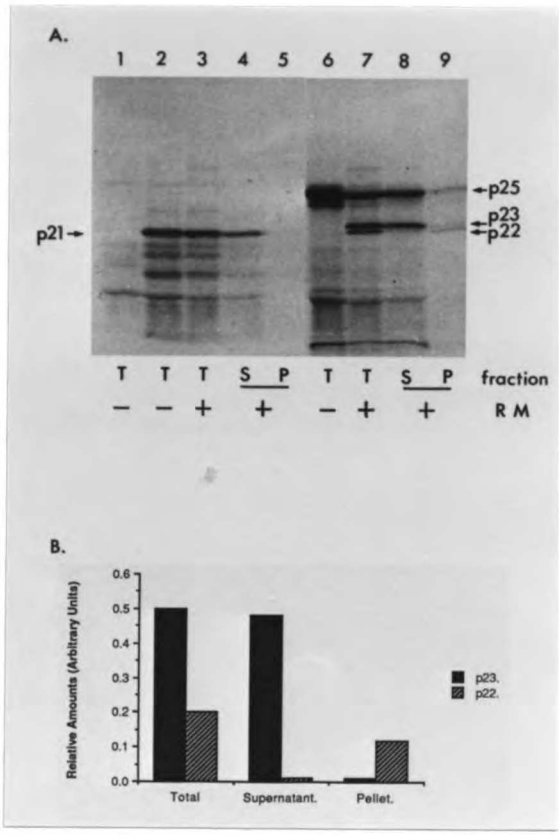
cosedimentation with the microsomal vesicles (Figure 6). Proteins which are not translocated or, as is the case for some integral membrane proteins, are only partially translocated across the lipid bilayer will remain sensitive to externally added proteases while those that are completely in the lumen of the microsomal vesicles will be protected from proteolytic digestion. Figure 5 shows the results for translation products obtained after 60 minute incubations with two different proteases, trypsin (Panel A) and protease K (Panel B). Although p25 is completely digested in both cases, the resulting pattern of bands is complex because both proteases leave discrete fragments which are resistant to further digestion even in the absence of microsomal membranes (Fig. 5A and 5B, lanes 3, see also Takahashi et al, 1983). Furthermore, the addition of the nonionic detergent Triton X-100 allows further digestion of these fragments by protease K (Fig. 5B, lane 4). When translation of p25 was carried out in the presence of microsomal membranes (Figure 5A and 5B, lanes 2, 5 and 6), we found that p22 was protected from proteases (Fig. 5A and 5B, lanes 5) unless the membranes were disrupted with detergent (Fig. 5A and 5B, lanes 6). These results indicate that p22 after one hour of incubation was completely translocated. In contrast p23 was entirely sensitive to both proteases, indicating that it was exposed on the cytoplasmic side of the microsomal membranes. Thus, these results are consistent with the observed modification pattern of the signal peptidase cleaved products of p25 (see above).

The cosedimentation assays shown in figure 6 further confirm this conclusion. Translation products obtained after one hour of incubation were layered on top of a sucrose cushion (see methods). Membrane (pellet) and cytoplasmic (supernatant) fractions were separated by centrifugation

FIGURE 6: Sedimentation analysis.

PANEL A: Translations of pHBVc/p21 (lanes 2-5) or pHBVc/p25 (lanes 6-9) mRNAs were carried out for one hour at 26 ^{o}C, in the absence (lanes 2 and 6) or presence (lanes 2-5 and 7-9) of microsomal membranes (RM; containing SRP). Translation products made in the presence of RM were fractionated into a supernatant (lanes 4 and 8; that correspond to the cytoplasmic fraction) and a pellet (lanes 5 and 9; that correspond to the microsomal membrane fraction) fraction by ultracentrifugation in a Beckman airfuge (see methods).

PANEL B: Quantitative representation of the distribution of p22 and p23 in the supernatant and pellet fractions (lanes 7, 8 and 9 from panel A). The total amounts of p22 and p23 indicated, correspond to the products obtained without fractionation. To obtain these data, a longer exposure that the one shown in panel A was scanned as indicated in figure 2. These results are representative of five independent experiments. Some loss of p22 (about 10-20 %) was observed and is likely due to incomplete recovery of the pellet fraction. Qualitatively very similar results were obtained when the ionic strength was reduced (by a 1/10 dilution with distilled water), or increased (by addition of potassium acetate to 1 M), or when the translation products were treated with 2 M urea prior to the fractionation procedure.


A.
1 2 3 4 5 6 7 8 9
p21
p25
p23
p22
T T T S P T T S P fraction
− − + + − + + R M
B.
Relative Amounts (Arbitrary Units)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
p23.
p22.
Total
Supernatant.
Pellet.

(see Methods). In this assay, p21 always remained in the supernatant fraction (Fig. 6A, compare lanes 4 and 5). This result is expected since p21 does not contain a signal peptide and therefore will not be targeted to the microsomal membrane during translation. The same result was obtained for the bulk of residual p25 that has not been targeted and cleaved by signal peptidase (Fig. 6A, compare lanes 8 and 9). In contrast p22 behaved as expected for a protein that is translocated into the microsomal vesicles; the bulk of p22 cosedimented with the membrane vesicles and was recovered in the pellet fraction (Fig. 6A, compare lanes 8 and 9; also see Fig. 6B for quantification). Under identical conditions, in vitro synthesized and processed preprolactin is quantitatively recovered in the pellet fraction (data not shown). p23, on the other hand, was quantitatively recovered in the supernatant fraction (Fig. 6A, lanes 8 and 9 and Fig. 6B). Thus, in spite of the fact that both p22 and p23 are the result of proteolytic processing of p25 by signal peptidase and, therefore, must have been, at least partially, translocated across the membrane to contact the active site of the enzyme, only p22 is found associated with the vesicles. Surprisingly, p23 appears to be localized free in the cytoplasm as indicated by its sedimentation behavior, its modification by cytoplasmic enzyme(s) and its susceptibility to proteases.

In summary, we can conclude that only a fraction of p22 (about 20 to 30 %) was completely translocated across the microsomal membranes and located in the lumen of the vesicles, whereas translocation of the bulk of p22 (70 to 80 %) appears to be aborted after being processed by signal peptidase and the protein is released into the cytoplasm. The fraction of p22 that was sequestered inside of the microsomal vesicles corresponds to the fraction of p22 in figure 4C and 4D that was not modified to p23 even

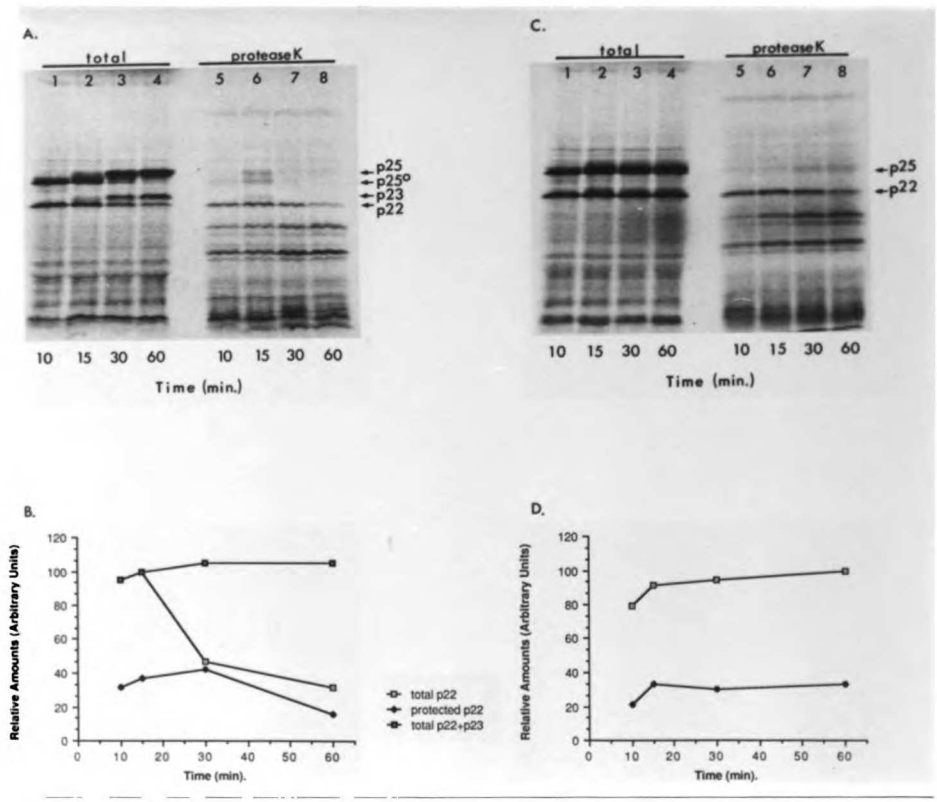
upon prolonged incubations. Conversely, the fraction released into the cytoplasmic compartment became accessible to the same cytoplasmic enzymes that modify p21 and p25 in the absence of microsomal membranes (see Fig. 4A and 4B) and, therefore, was converted to p23 (see model in figure 9).

To confirm this notion, we analyzed the protease sensitivity of the products obtained at different time points during translation. Reactions were programmed with p25 mRNA and incubated in the presence of microsomal membranes. Samples of the reaction were removed at 10, 15, 30 and 60 min and transferred to an ice-water bath. Half of each sample was analyzed directly by SDS-PAGE and the other half was treated with protease K before electrophoresis (Fig. 7A). Both unmodified ($p25^{o}$) and modified p25 were completely sensitive to protease K. Only p22 was resistant to the protease at all time points examined (Fig. 7A, lanes 5 to 8), whereas p23 was completely degraded. A quantitative analysis of these results demonstrates (Fig. 7B), however, that only a fraction of p22 (about 30 %) at the early time points was protected by the lipid bilayer. In similar experiments using preprolactin as control, we found that processed preprolactin was quantitatively protected at all the time points examined (data not shown). Thus, the lack of protection of the bulk of p22 at early time point was not due to an intrinsic leakiness of the microsomal vesicles but rather was specific for p22.

During the course of our experiments, we observed that the modification reaction of the HBV core proteins was ATP-dependent. Thus, the modification reaction could be blocked by depletion of ATP at an early time point after the appearance of p22 and p25. Depletion of ATP was performed by the addition of an excess of glycerol kinase and glycerol at 10 min after translation (see legend of figure 7). The results of such an

FIGURE 7: Protease protection analysis during time course of translations.

Samples (20 µl) of synchronized translations were taken at 10, 15, 30 and 60 min. of incubation. Each sample was divided in two: one half was directly subjected to SDS-PAGE (lanes 1-4), and the other half was treated with protease K before electrophoresis (lanes 5-8). In the experiment shown in panel C, E.coli glycerol kinase was added to a final concentration of 250 µg/ml (from a stock of 10 mg/ml in 50 % glycerol) at 10 min of incubation. Panels B and D show the quantification of the the experiments in panels A and C, respectively. The relative amounts of p22, p23 and protected p22 were determined as for figure 2.


A.
total
protease K
1 2 3 4 5 6 7 8
p25
p25°
p23
p22
10 15 30 60 10 15 30 60
Time (min.)
C.
total
protease K
1 2 3 4 5 6 7 8
p25
p22
10 15 30 60 10 15 30 60
Time (min.)
B.
Relative Amounts (Arbitrary Units)
0 20 40 60 80 100 120
0 10 20 30 40 50 60
Time (min).
total p22
protected p22
total p22+p23
D.
Relative Amounts (Arbitrary Units)
0 20 40 60 80 100 120
0 10 20 30 40 50 60
Time (min).

experiment are shown in figure 7C. In this case, as in figure 7A, only p22 was protected from protease at all the time points examined, whereas, the unmodified form of p25 was protease sensitive. However, a quantitative analysis (Fig. 7D) shows that only about 30 % of the p22 protein was protected from protease, i.e. was translocated into the microsomal vesicles. This result demonstrates that the modification reaction is a secondary process that has no effect on the localization of the processed forms of p25.

A processed form of p25 lacking the nucleic acid binding domain is also partitioned between the lumen of the microsomal vesicles and the cytoplasm.

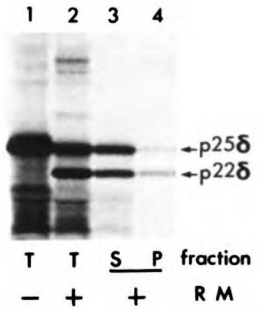
Because of the very unusual behavior of p22, we were intrigued by the possibility that the extremely charged carboxy terminal domain could adversely affect the translocation process. Thus, this domain could be the responsible for the aborted translocation. To investigate this possibility, a deletion mutant was constructed (pHBVc/p25δ, see methods for details) containing a termination codon precisely at the amino terminal boundary of the charged cluster. When the p25δ mRNA was translated in the absence of microsomal membranes, a major translation product of about 17 kD (p25δ) was observed (Fig. 8A, lane 1). If microsomal membranes were present during translation a corresponding processed form (p22δ) of about 15 kD was also observed (Fig. 8A, lane 2). To determine the topography of p22δ only the cosedimentation assay could be used, because p22δ and p25δ are resistant to both trypsin and protease K (the cleavage sites for both proteases map near the position where the termination codon was introduced, see legend of fig. 5). The cosedimentation assay was performed as in figure 6. As expected, residual p25δ remains in the supernatant (Fig. 8A, compare lanes 3 and 4). Interestingly, the bulk of p22δ also remained in the

FIGURE 8: Sedimentation analysis of p22δ and p25δ.

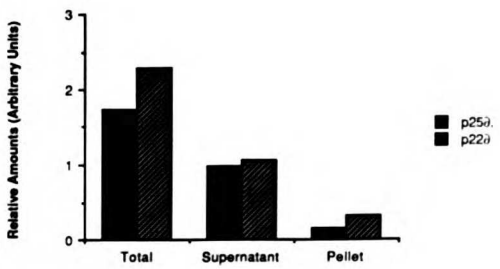
PANEL A: Translations of pHBVc/p25δ mRNA were performed in the absence (lane 1) or presence (lanes 2-4) of microsomal membranes (RM; containing SRP, see methods). Translation reactions done in the presence of RM were fractionated into a supernatant (lane 3) and a pellet fraction (lane 4) as indicated in the methods.

PANEL B shows the quantification of the amounts of p22δ and p25δ found in an unfractinated reaction, in the supernatant and in the pellet fractions. The intensity of each protein band was determined as in figure 2. However, to compare both proteins in this case the intensity of p22δ was multiplied by 1.5 to account for the loss of one of the three methionines due to removal of the signal peptide.

A.


1 2 3 4
p25δ
p22δ
T T S P fraction
– + + R M

B.


Relative Amounts (Arbitrary Units)
3
2
1
0
Total
Supernatant
Pellet
p25∂
p22∂

supernatant (Fig. 8A, compare lanes 3 and 4), indicating that, as was for p22, a large fraction of the protein was not translocated. Figure 8B shows a quantitative analysis which indicates that approximately 20 to 30 % of p22δ is found in the pellet fraction. Thus, we can conclude that removal of the nucleic acid binding domain does not affect the partitioning of the signal peptidase processed form between the lumen of the microsomal vesicles and the cytoplasmic compartment.

Interestingly, in this experiment no heterogeneity in the electrophoretic mobility of the p25δ and p22δ protein bands was observed. In a time course of translation neither protein showed any shift in the electrophoretic mobility during incubations similar to those of figure 4 (data not shown). Since both proteins are found in the cytoplasmic fraction (where the modifying enzyme(s) resides), this result indicates that, in contrast to the other HBV core proteins, p22δ and p25δ are not substrates for the modifying enzyme(s) described above. The result suggests that the site of modification reside in the nucleic acid binding domain of the HBV core proteins. This result also confirms that the abortion of translocation and the release of the protein into the cytoplasm occurs independent of its modification.

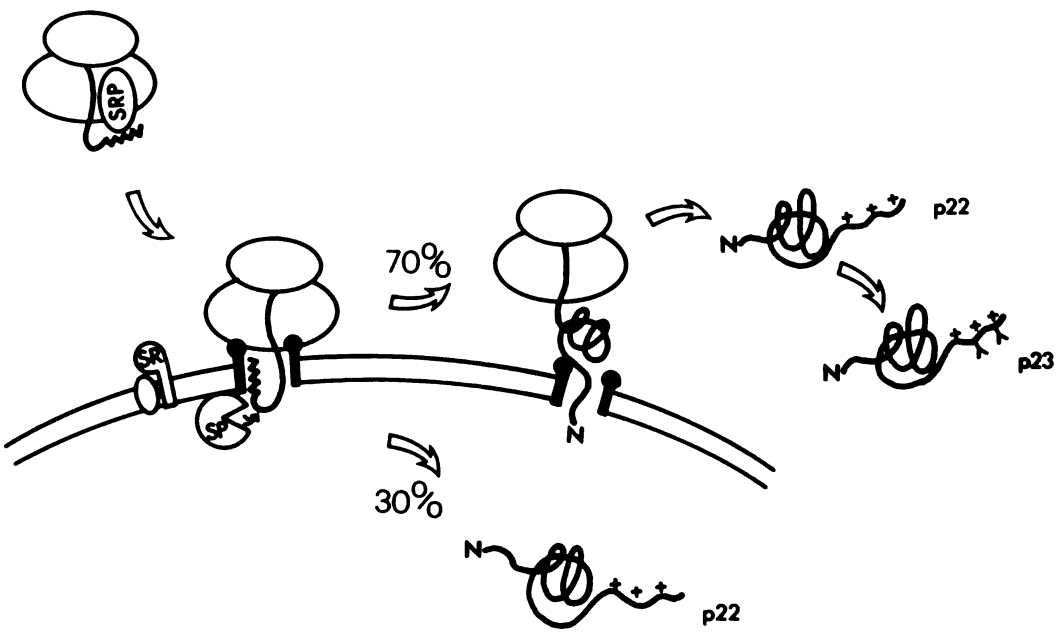
DISCUSSION

Implications for the viral life cycle

Using *in vitro* protein translation and translocation assays we have determined that the precore region of the HBV genome encodes a functional signal sequence that directs nascent p25 to the membrane of the endoplasmic reticulum. This targeting event is indistinguishable in its molecular requirements, i.e. its SRP and SRP receptor dependence, from that observed for other secretory and membrane proteins. Once targeted to the ER

FIGURE 9: Model for the targeting of p25 to the endoplasmic reticulum (ER) membrane.

SRP recognizes the signal peptide of p25 as the protein is being synthesized by free ribosomes. The binding of SRP results in the arrest of the synthesis of p25. This inhibition of translation is released when the arrested complex interacts (through SRP) with the SRP receptor (SR) in the membrane of the ER. A translocation competent junction of the ribosome to the membrane is established. During translocation of p25, the cleavage site becomes accessible to signal peptidase (SP). However, translocation of 70 % of the protein that has been cleaved by SP is aborted and the protein is released into the cytoplasm. Only 30 % of the processed p25 is correctly translocated and is found in the lumen of the microsomal vesicles.


SRP
70%
SR
SR
N
p22
N
p23
30%
N
p22

membrane, p25 is engaged in the translocation machinery and in the process becomes a substrate for signal peptidase after the cleavage site has been exposed on the lumenal side of the membrane. Removal of the signal peptide creates p22. This protein differs from the "major" core protein (p21) initiated at the second AUG codon of the open reading frame since it contains 10 additional amino acids at its amino terminus.

This finding is consistent with the observation that forms of core protein can indeed be secreted in vivo. Proteolytic fragments of the core protein (of about 15 kD) can be found in the serum of infected patients. These fragments have been immunologically defined as HBV "e" antigen which is distinguishable from core antigenicity. More direct evidence of secretion of core related products (similar to those found in the serum of infected patients) comes from expression of its complete coding sequence in mammalian cells (Ou et al, 1986; Roossinck et al, 1986; McLachlan et al, 1987) and Xenopus oocytes (Standring et al, 1987). Furthermore, these secreted fragments contain the same amino terminus as the signal peptidase cleaved p22 (Standring et al, 1987). It follows that the second proteolytic processing event (to generate 15 kD products) occurs at the carboxy terminus of the protein. Since we observe intact translocated p22 in the lumen of the microsomal vesicles, this secondary cleavage event is likely to occur in a later compartment of the secretory pathway. It is possible that this processing is performed by the same enzyme that processes pro-proteins in the Golgi or secretory vesicles (that cleaves at Lys-Arg or Arg-Arg residues), since the fragment removed is very rich in arginines. Taken together, these studies demonstrate that the signal peptide in the precore region is responsible for the targeting of p25 to the ER and, thus, the secretion of the HBV "e" antigen.

Much to our surprise, we found that a large fraction of p22 was released into the cytoplasm (where it became modified to p23) after being targeted to the ER and processed by signal peptidase. We have shown that this property is specific for p22, since in similar experiments preprolactin (a typical secretory protein) remains stable inside of the microsomal vesicles (data not shown). We speculate that our results may reflect a novel cellular mechanism utilized by HBV to obtain different forms of a protein with respect to its structure and/or intracellular localization. At present we do not know that aborted translocation of p25 does indeed occur in vivo. There is, however, indirect evidence, since forms of core protein with molecular weights around 23 kD can be observed in intact virions isolated from the serum of infected patients (Wolfram Gerlich, personal communication) and upon expression of p25 mRNA in Xenopus oocytes (David Standring, personal communication), respectively. Unfortunately, amino terminal sequence information of these proteins is not yet available and will be required to confirm their correspondence to p23. Most interestingly, the 23 kD form found in intact virions is not present in core particles isolated from infected cells (W. Gerlich, personal communication) hinting that this protein may be acquired by the virus during the assembly/budding process (see below).

What purpose could the virus accomplish by producing a signal peptidase processed form and localizing part of the protein in the cytoplasm? While we do not know the answer to this question, we can discuss a few possibilities. For example, the partial translocation of a portion of the core protein across the ER membrane may put this protein into proper configuration to interact with other viral components on the cytoplasmic side of the membrane and thereby affect its assembly. p22 thus

localized in the vicinity of the ER could interact with membrane integrated surface protein and subsequently become "co-polymerized" into already assembled or partially assembled core particles. Thus a minor amount of p22 could provide a physical link between the core particles and the viral envelope. Such an interaction may facilitate assembly and give the virus a growth advantage, but as discussed above is unlikely to be essential for the assembly process. Furthermore, but not necessarily exclusive, considering that p22 has ten more amino acids at its amino terminus than p21, p22 may have novel properties that could allow the virus to utilize the protein for a function(s) in the cytoplasm, secretory pathway and/or blood stream that still remains to be discovered.

In the process of the membrane translocation studies, we noticed that all cytoplasmic forms of HBV core protein (p21, p25, and p22 after release from the membrane vesicles) were covalently modified in the translation extract. The modification did not appear to be peculiar to the wheat germ extract since similar experiments performed in a rabbit reticulocyte lysate translation system showed similar modification patterns (data not shown). Therefore, we conclude that the HBV core proteins serve as a substrate for a modification enzyme(s) that is(are) present in cytoplasm of cells as divergent as plants and mammals. We suggest that core protein may be similarly modified by a corresponding enzyme(s) in the host cell cytoplasm prior to its assembly into viral particles. We have not determined the chemical nature of the modification, but we know that the reaction requires ATP (Fig. 7). It has been described that the major core protein is phosphorylated <u>in vivo</u> (Roossinck and Siddiqui, 1987). We tested if the modification observed in our assays was phosphorylation by treating the translation products with alkaline phosphatase. Although the

electrophoretic mobility of modified p25 was slightly increased by this treatment (indicating that it is indeed phosphorylated), it still migrated significantly slower than the primary translation product ($p25^{o}$, data not shown). Thus, we conclude that the HBV core proteins can carry at least two different modifications, phosphorylation and the unknown modification described here. This latter modification is likely to occur within the charged carboxy terminal domain, because p25δ showed no shift in mobility even upon prolonged incubation with the translation extracts. Alternatively, the carboxy terminal domain could be required for substrate recognition by the modifying enzymes.

Implications for the protein translocation process

Regardless of the implications for the HBV life cycle, the study of p25 as a translocation substrate allowed us to address a variety of questions concerning the mechanism of protein translocation *per se*. First it should be noted that a fraction of p22 is completely translocated across the microsomal membrane *in vitro* (see above), consistent with the observation that HBV core protein related polypeptides are secreted *in vivo*. We are confident that translocation occurred to completion, since the carboxy terminal tail of p25 (the most protease sensitive part of the protein) was not removed in the protease protection assays and was not a substrate for the cytoplasmic modification enzyme(s). It is unlikely that the observed protease resistance was due to an interaction of the arginine rich region with negatively charged phospholipid head groups, since no interaction of p25 with membranes was observed when microsomal vesicles where added post-translationally (data not shown). Detection of completely translocated p22 is of particular interest, since the carboxy terminus of the protein comprises the nucleic acid binding domain and has a very high

charge density (36 amino acids, 17 of which are arginines). It has been a long-standing debate whether translocation of a protein across the membrane occurs through a protein pore (i.e. a hydrophilic environment) or whether the chain passes directly through the hydrophobic interior of the lipid bilayer. Our findings clearly favor protein translocation in a nonlipid environment. Even if one assumes a completely stretched out conformation of p25 during translocation (i.e. only about 12 amino acids would be required to span the membrane), still temporarily up to eight arginine residues would need to reside simultaneously within the bilayer. Thus if translocation were to proceed directly through a lipid environment, this could only be achieved at a significant energy cost.

The most unusual aspect of the translocation of p25 is our observation that the translocation process can be aborted after the protein has been processed by signal peptidase. Existing evidence in eukaryotes suggests that the catalytic site of signal peptidase is localized on the lumenal side of the membrane, i.e. signal peptidase activity is latent unless either the vesicles are dissolved in detergent or the substrate protein is translocated across the bilayer. It follows that p25 must have been at least partially translocated across the membrane to become processed. At some stage between processing and completion of protein synthesis the translocation process is aborted for a large fraction of the nascent polypeptide chains. We must assume that abortion of translocation is caused by some unusual characteristic of p25, since all other substrates tested (with the exception of integral membrane proteins) are completely translocated. It is possible that this unusual behavior is determined by some unknown feature of the signal peptide. However, we consider this possibility unlikely since the signal peptide behaves normally in its

interactions with SRP or the translocation machinery prior to its cleavage. Therefore, if the signal peptide determines this behavior, we must assume that it has a yet undefined function in the translocation process after its cleavage by signal peptidase.

An alternative mechanism to explain the abortion of translocation of p22 would assume, in analogy with the process of the insertion of integral membrane proteins, that translocation is terminated in response to the recognition by the translocation machinery of "stop-transfer" sequences in p25 (Blobel, 1980; Yost et al, 1983). However, p25 does not contain sequence stretches hydrophobic enough to resemble "classical" stop-transfer sequences. Yet it is still possible that a sequence within nascent p25 could interact (albeit with poor affinity, thereby giving rise to only 80 % efficiency of stop-transfer) with a site in the translocation apparatus that normally interacts with stop-transfer sequences and in doing so triggers the nascent protein to disengage from the translocation machinery. Whereas a membrane protein at this stage would attain a stable integrated configuration, p25 lacking sufficiently hydrophobic sequences would slip back into the cytoplasm.

A conceptually different alternative to explain the mechanism that causes aborted translocation, is that certain regions of p25 are simply difficult to translocate. We ruled out that such an interference could be caused by the charge clusters in the carboxy terminal nucleic acid binding domain (see above). Although there are no other unusual features apparent in p25, the possibility remains that portions of the protein may fold prior to translocation into a very tight domain (such as, for example, the protease resistant portion of core protein) that is then translocation incompetent. In order for this mechanism to be plausible there would need

to be sufficient slack in the nascent polypeptide chain between ribosome and membrane to allow it to assume a folded structure. In other words, at some stage after signal peptidase cleavage elongation must be considerably faster than translocation, and the two respective processes may, in fact, not be as tightly coupled as generally assumed. Presently, we cannot distinguish between these various possibilities, but we hope that through the construction of appropriate fusion proteins we will be able to map the cause for the peculiar behavior of p25 to defined determinants within its primary sequence and thereby learn about the underlying molecular mechanism.

Signal peptidase cleavage in the absence of translocation was previously observed in other experimental situations involving post-translational assays. Thus it was demonstrated that the signal peptide of maltose binding protein can be removed by purified bacterial signal peptidase that has been reconstituted into liposomes (Ohno-Iwashita et al, 1984). The protein in this case was not translocated into the lumen of the vesicles and the processed protein was not found associated with the lipid bilayer. Similarly, prepromellitin can be processed, but not be translocated across mammalian microsomal membranes that have been trypsinized or alkylated (Zimmermann and Mollay, 1986). In both of these cases it is likely that parts of the translocation machinery required for the translocation event beyond signal peptidase cleavage were either absent or rendered non-functional. Thus both of these observations differ from the one described here performed with intact and unperturbed microsomal vesicles and that were shown to be co-translational. Yet they demonstrate that at least for some proteins the initial membrane insertion that leads

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