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Aspects of protein translocation across the membrane of the endoplasmic reticulum

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ASPECTS OF PROTEIN TRANSLOCATION ACROSS THE MEMBRANE OF THE ENDOPLASMIC RETICULUM

by

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ASPECTS OF PROTEIN TRANSLOCATION ACROSS THE MEMBRANE OF THE ENDOPLASMIC RETICULUM

Deborah L. Zimmerman

Abstract

Understanding the mechanism of protein translocation across the membrane of the endoplasmic reticulum (ER) will require the identification of the membrane proteins involved in the process as well as assays to evaluate the roles of these proteins in translocation. In this thesis I describe three approaches which I have taken toward understanding how protein translocation occurs across the membrane of the ER in higher eukaryotic cells.

Using canine microsomal membranes I developed a system whereby membrane proteins could be partially solubilized from the membrane with detergent and then be reconstituted into functional vesicles. Thus, it was demonstrated that microsomal vesicles could be partially solubilized using the detergent n-octyl-β-glucopyranoside (OG) and then reconstituted into functional vesicles once the detergent was removed. Purified proteins could potentially be incorporated into such reconstituted vesicles by addition to the partially solubilized extract before reconstitution to assess the role of individual membrane proteins in translocation.

As a step toward identifying novel membrane proteins involved in translocation and to address the question of whether nucleotide hydrolysis is required at the level of the membrane to facilitated translocation, we utilized an analog of ATP (8-N_3ATP) that can be covalently coupled to ATP-binding proteins. Membranes treated with 8-N_3ATP became inactivated for translocation activity indicating that an ATP-binding protein is required for translocation. Moreover, two of the major ATP-binding proteins in the ER membrane were found to be proteins with putative roles in translocation, signal sequence receptor (SSR) and a putative ribosome receptor which we term ERp180.

Lastly, I describe the identification of a ribosome binding protein in the ER termed ERp180. We have addressed the role of this protein in translocation, and found that it is not strictly required for this process. Biochemical analysis reveals that although ERp180 is tightly associated with microsomal vesicles, it is probably not a classical integral membrane protein. Further analysis reveals that the protein has an elongated structure, is purified from the membrane as a dimer and has some

homology to myosin in the tail region. All of these findings are consistent with the proposed role of this protein as a structural component of the ER membrane.

Peter Walter, June 12, 1992

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BACKGROUND

Biological membranes define the topological domains within the cell. In higher eukaryotes these include the major organelles and a multitude of other vesicular structures. This type of compartmentalization requires that individual proteins be sorted to the correct cellular location in order to function. Since protein synthesis begins in the cytoplasm, noncytoplasmic proteins must be targeted either during or after their synthesis to the correct organelle. Once targeted, the proteins are transferred or translocated across the biological membrane which delineates that organelle. Protein targeting and translocation is being studied for protein segregation into mitochondria, chloroplasts, peroxisomes, the nucleus and the endoplasmic reticulum (Schatz, 1986).

Proteins destined for secretion and the resident proteins of the ER, golgi, and plasma membrane are synthesized by membrane bound ribosomes on the ER (Palade, 1975). Ribosomes synthesizing these classes of proteins must be selectively targeted to the ER membrane shortly after protein synthesis begins (Walter and Lingappa, 1986). Concomitant with their synthesis the nascent polypeptide chains pass through the ER membrane such that the completed polypeptide resides either within the lumen of the organelle or in the ER membrane (Palade, 1975). Once inside the lumen, nascent chains must fold into the correct conformation, and when appropriate, must assemble into oligomeric protein complexes before they exit from the ER (Gething and Sambrook, 1991; Rothman, 1991).

PROTEIN TRANSLOCATION ACROSS THE ER

The signal hypothesis and in vitro protein translocation

In the past two decades much has been learned about how ribosomes synthesizing secretory proteins are specifically targeted to the ER membrane in higher eukaryotic cells (Rapoport, 1990; Walter and Lingappa, 1986). Most of the evidence supports the basic tenents of the "signal hypothesis" which was first proposed by Blobel and Dobberstein in 1975 (Blobel and Dobberstein, 1975). The signal hypothesis was based on the observation that secretory proteins are distinguished from cytoplasmic proteins by an amino terminal extension of 20-30 amino acids which is removed upon translocation into the ER (Blobel and Dobberstein, 1975). The hypothesis proposed that the amino terminal extension acts as a signal which specifies that ribosomes synthesizing secretory proteins be targeted to the ER membrane. An extension of the hypothesis is that once a ribosome is targeted, the signal sequence is recognized by specific receptors in the membrane that promote the formation of a protein tunnel which translocates the nascent chain across the membrane (Blobel and Dobberstein, 1975).

Most of our current knowledge of how secretory proteins are specifically targeted to and translocated across the ER membrane has been obtained through the use of in vitro systems which faithfully reproduce the events (Blobel and Dobberstein, 1975). Two heterologous components can reconstitute the translocation event, a cell free translation system and microsomal vesicles derived from the ER of pancreatic cells (Blobel and Dobberstein, 1975). Thus, ribosomes from a translation extract programmed with mRNA encoding a secretory protein are efficiently targeted to the microsomal membranes, and the nascent chain is efficiently sequestered within the lumen of the vesicles (Blobel and Dobberstein, 1975).

The ability to reconstitute translocation allowed the purification and characterization of some of the components involved in the process. Using this system, two components which coordinately target ribosomes to the membrane were identified biochemically, signal recognition particle (SRP) (Walter and Blobel, 1980) and the SRP receptor (SR) (Gilmore *et al.*, 1982a; Gilmore *et al.*, 1982b; Meyer and Dobberstein, 1980; Meyer, 1980). The study of these two components has resulted in a considerable amount of insight about how secretory proteins are targeted to the ER membrane.

Targeting to the ER

As mentioned above, secretory proteins are distinguished from cytoplasmic proteins by a signal sequence of 20-30 amino acids (Blobel and Dobberstein, 1975; von Heijne, 1983). Comparison of all known signal sequences reveals no primary sequence conservation between them. However, two structural features are required for a protein sequence to function as an ER targeting signal (1) a hydrophobic core of 8 - 15 amino acids (Blobel and Dobberstein, 1975; von Heijne, 1983) and (2) a polar region of 5 to 6 amino acids following the hydrophobic core including small side chain amino acids at positions 1 and 3 prior to the cleavage site (von Heijne, 1983). In most cases the signal sequence is located at the amino terminus of the protein, and thus, is the first part of the nascent polypeptide chain to emerge from the ribosome. Moreover, a ribosome which has synthesized only the signal sequence contains all the information required to target the ribosome-nascent chain complex to the membrane (Garcia and Walter, 1988; Perara *et al.*, 1986).

When the signal sequence emerges from the ribosome the small (11 S), cytoplasmic ribonucleoprotein, SRP, recognizes and binds to the ribosome and

nascent chain to form a ternary complex (Walter, 1981; Walter and Blobel, 1981a; Walter and Blobel, 1981b). Further elongation of the nascent chain is then slowed or completely arrested until the ribosome is targeted to the membrane through a high affinity interaction between SRP and the SRP receptor (SR), an integral membrane protein complex composed of two subunits, SRα and SRβ (Gilmore *et al.*, 1982b; Tajima *et al.*, 1986; Walter and Blobel, 1981b; Wolin and Walter, 1989). Once the ribosome and nascent chain are targeted, they become disengaged from SRP and SR, and the ribosome/nascent chain complex is handed over to other membrane components that facilitate the transmembrane movement of the nascent chain. Further elongation of the nascent chain occurs concomitant with its translocation across the microsomal membrane (Blobel and Dobberstein, 1975). Thus, SRP and SR function in concert to initiate the formation of a ribosome membrane junction; however, they do not facilitate the actual process of protein translocation across the membrane.

The role of GTP in protein targeting

Connolly and Gilmore first demonstrated that GTP is required to target nascent chains to the ER membrane and to establish a functional ribosome-membrane junction (Connolly and Gilmore, 1986; Connolly *et al.*, 1991). Subsequently, it has been demonstrated that both SRP and SRP receptor are GTP binding proteins (Connolly and Gilmore, 1989; Miller, J. and Walter, P. unpublished). Accordingly, the 54 kD subunit of SRP and both subunits of SRP receptor contain short segments of amino acids that are highly conserved among members of the GTPase superfamily (Bernstein *et al.*, 1989; Bourne *et al.*, 1991; Connolly and Gilmore, 1989; Römisch *et al.*, 1989; Miller, J. and Walter, P.,

unpublished). Moreover, all three proteins have been shown by photocrosslinking to bind to GTP (Miller, J. and Walter, P., unpublished), and it is likely that GTP binding by all of these polypeptides is required to facilitate nascent chain targeting. Thus, multiple rounds of GTP binding and hydrolysis may insure the proper vectorial delivery of the nascent chain to the site of translocation.

By making site directed mutations in SRα, Rapiejko and Gilmore have demonstrated that protein targeting resulting in the subsequent translocation of the nascent chain requires that this protein have a functional GTP binding site (Rapiejko and Gilmore, 1992). The mutations, which appear to block a guanine nucleotide exchange reaction in the GTP binding site, inhibit the initial contact between SRP and SRP receptor (Rapiejko and Gilmore, 1992). The requirement for an exchange reaction at this step is consistent with the earlier finding that following nascent chain targeting, the hydrolysis of GTP is required to recycle SRP and SRP receptor (Connolly *et al.*, 1991). Currently, there is no evidence that GTP hydrolysis by either SRP or SR contributes to the vectorial movement of the remainder of the nascent chain across the membrane (Connolly *et al.*, 1991). The possible requirement of nucleotides during protein translocation is discussed below.

The binding of SRP to the ribosome/nascent chain complex, and its subsequent interaction with a receptor in the ER membrane accounts for the targeting of secretory proteins to the ER, but unfortunately, it leaves us with a still unsatisfactory view of the events which occur during the translocation process itself. That is, how does the ribosome become tightly bound to the membrane, and how does the vectorial transfer of the nascent chain across the membrane occur? In this thesis I describe some experiments which begin to address both of these questions.

MEMBRANE PROTEINS INVOLVED IN TRANSLOCATION

Evidence for a dynamic protein translocating machinery

Translocation is likely to be mediated by a dynamic, multisubunit complex which is integrated within the ER membrane. Thus, some proteins would be permanent members of a "core" assembly associated with the ribosome binding site, while others like the SRP receptor might interact only transiently with the core proteins. Evidence for such a dynamic assembly or "translocon" has recently been provided by Simon and Blobel (Simon and Blobel, 1991).

Using electrophysiological techniques Simon and Blobel demonstrated the existence of large channels on microsomal vesicles. When nascent chains were released from membrane bound ribosomes under physiological salt conditions, the number of channels measured on the ER membrane increased dramatically, suggesting that the channels are effectively plugged by the nascent chains. However, the channels closed when the ribosomes were dislodged from the membrane subsequent to nascent chain release, indicating that the presence of the ribosome is required to maintain an open channel (Simon and Blobel, 1991). These results are the first direct evidence for the existence of a dynamic protein conducting channel in the ER. Moreover, they underscore the role of the ribosome as an important ligand for the translocation machinery.

SRP receptor and signal peptidase

Prior to the onset of the studies described in this thesis, two membrane components of the translocation machinery had been purified using activity assays

after detergent solubilization of microsomes: signal peptidase, the protein which cleaves the signal sequence off the nascent polypeptide chain (Jackson and Blobel, 1977), and the SRP receptor (Gilmore *et al.*, 1982a; Gilmore *et al.*, 1982b; Meyer and Dobberstein, 1980; Meyer *et al.*, 1982a). Signal peptidase is an integral membrane protein complex composed of six polypeptides, two of which are glysoslyated (Evans *et al.*, 1986). However, it has been demonstrated for the bacterial signal peptidase isolated from E. coli that only one polypeptide chain is required to carry out peptide cleavage (Wolfe *et al.*, 1983). The individual polypeptides of mammalian signal peptidase are present in the membrane in quantities equimolar with membrane bound ribosomes (Evans *et al.*, 1986). Thus, it has been proposed that the complex has a noncatalytic role in translocation in addition to signal sequence cleavage, but no other function has yet been ascribed to it. Moreover, cloning and sequence analysis of two of the polypeptide chains revealed no homology with other known proteins except signal peptidase from yeast (Bohni *et al.*, 1988; Greenburg *et al.*, 1989).

SR as discussed briefly above, is a heterodimeric protein complex composed of an α and a β subunit (Tajima *et al.*, 1986). SRα is a 72 kD polypeptide with a large hydrophilic domain which is cytoplasmically localized (Lauffer *et al.*, 1985). The amino acid sequence of the protein predicts one potential transmembrane domain; however, it is still uncertain whether SRα is a bona fide integral membrane protein (Lauffer *et al.*, 1985; Lauffer, unpublished). In contrast SRβ, a 30 kD polypeptide, which also contains one transmembrane domain is clearly a classical integral membrane protein (Miller, J. and Walter, P., unpublished). The complex is present in the membrane in quantities which are substochiometric (approximately 1:6) with the number of membrane bound ribosomes; thus, it is believed that the protein acts catalytically to target the SRP ternary complex to the membrane (Tajima *et al.*, 1986).

As discussed above both subunits of SR are GTP binding proteins. It was recently shown that GTP binding by SRα is required to facilitate the interaction

between SRP and SR, and GTP hydrolysis is required to recycle SRP from the membrane (Connolly *et al.*, 1991; Rapiejko and Gilmore, 1992). It is likely that GTP binding is also required to assure the proper transfer of the ribosome and nascent chain to the appropriate components of the translocation machinery. However, this function has not yet been directly demonstrated for SR, and the putative "translocon" components have not yet been identified.

Other potential translocon components

Identification of SSR, mp39 and TRAM

Together, signal peptidase and SRP receptor account for eight polypeptides involved in protein translocation, and the known activities which they carry out are not sufficient to account for vectorial protein translocation. In recent years several approaches have been taken to identify additional membrane components required for translocation. One approach has been to crosslink translocating nascent chains to membrane proteins which they contact as they traverse the membrane and then attempt to identify the crosslinked proteins based on their inferred molecular weight and other biochemical properties.

By translating a message in the presence of a modified lyslyl-tRNA a lysine residue containing a photoactivatable crosslinker can be introduced into the nascent chain (Krieg *et al.*, 1986; Wiedmann *et al.*, 1987). In this way the crosslinking reagent can be activated while the nascent chain is engaged with the translocation machinery, and the nascent chain will become covalently linked to proteins in close proximity. Moreover, the crosslinker can be incorporated specifically into either the signal sequence or the mature part of the nascent chain (Krieg *et al.*, 1989; Wiedmann *et al.*, 1989).

By incorporating modified lyslyl-tRNA into the signal sequence of preprolactin and allowing the signal sequence to insert into the membrane, Weiland and colleagues identified a putative signal sequence receptor in the ER (Wiedmann *et al.*, 1987). The protein identified by crosslinking was a glycoprotein with a molecular weight of approximately 35 kD. Rapoport's group subsequently identified a major ER protein with these properties and termed the protein SSR for "signal sequence receptor" (Prehn *et al.*, 1990). Further purification revealed that SSR is a heterotetrameric protein composed of two 25 kD subunits (SSRβ) as well as two 35 kD subunits (SSRα) (Görlich *et al.*, 1990). Originally, it was proposed that this protein is a major component of the translocating pore; however, this has never been shown experimentally (Görlich *et al.*, 1990). Moreover, it has recently been found that another glycoprotein of similar molecular weight, termed TRAM, is responsible for the majority of crosslinks seen during signal sequence insertion (Gorlich *et al.*, 1992).

Krieg and colleagues used a similar crosslinking approach to identify a 39 kD integral membrane glycoprotein (mp39) which crosslinks to the signal sequence as well as to mature parts of the nascent chain (Krieg *et al.*, 1989). In addition, Thrift et al. have demonstrated that an mp39-like glycoprotein crosslinks to the transmembrane regions of nascent membrane proteins as they are being integrated (Thrift *et al.*, 1991). They further demonstrated that an association between the nascent membrane protein and the mp39-like protein continues until translation has terminated. Mp39 has not yet been purified, thus the relationship between this protein, TRAM and SSR has yet to be determined. Moreover, the possible role of SSR in translocation remains to be determined.

Two lines of evidence indicate that SSR might still play a role in translocation. First, Fab fragments derived from antibodies raised against SSR block translocation activity of microsomal membranes (Hartmann *et al.*, 1989), and

second, SSR is found to be in close proximity to mature parts of the nascent chain as they traverse the membrane (Thrift *et al.*, 1991). However, two groups have now demonstrated that reconstituted microsomal vesicles depleted of SSR are still capable of translocation (Gorlich *et al.*, 1992; Migliaccio *et al.*, 1992). Thus, if SSR is involved in this process, its function might be either redundant or auxiliary.

Identification of an NEM sensitive factor and a putative ribosome receptor

Another approach to identify novel proteins required for translocation has been to treat membranes with proteases or other disruptive reagents and try to correlate loss of function with the biochemical modification of a particular protein. For example, by using a combination of proteolysis and treatment with N-ethylmaleimide (NEM), a potent alkylating reagent, the role of an NEM sensitive protein has been demonstrated by Nicchitta and Blobel (Nicchitta and Blobel, 1989). The NEM sensitive component was shown to be required for protein translocation but not for nascent chain targeting or signal sequence insertion (Nicchitta and Blobel, 1989). However, the protein itself has not yet been identified. Using a similar type of biochemical "knockout" approach, we have demonstrated the requirement for an ATP-binding membrane protein in translocation (Zimmerman and Walter, 1991; chapter 3), and similar experiments have recently been reported by Klappa et al (Klappa *et al.*, 1991).

Savitz and Meyer identified a putative ribosome receptor in the ER by combining proteolytic treatment of the membrane with a functional assay for ribosome binding to the membrane (Savitz and Meyer, 1990). Thus, the authors generated large proteolytic fragments from the ER membrane and found that this fraction could inhibit ribosome binding (Savitz and Meyer, 1990) They purified a fragment of 160 kD which was responsible for the inhibition and then showed that the fragment was derived from a 180 kD parent molecule (Savitz and Meyer, 1990).

Biochemical analysis of the protein indicated that it is an integral membrane protein which is localized in the ER. Moreover, when the purified protein was reconstituted into liposomes, it did display some ribosome binding activity (Savitz and Meyer, 1990). However, experiments in our lab have brought into question the importance of this protein as a receptor which plays a key role in ribosome binding during protein translocation (Collins and Gilmore, 1991; Nunnari *et al.*, 1991; Zimmerman and Walter, 1991). For example, we have shown that the protein fractionates away from the ribosome binding sites which can be assayed for on the ER membrane and that at least half of the protein can be proteolyzed from microsomal membranes, and the membranes are still active for translocation (Nunnari *et al.*, 1991)(Zimmerman and Walter, 1991; Chapter 3). Experiments addressing alternative functions of this protein will be described later in this thesis.

All the approaches described above have aided in identifying potential components of the translocon. However, given the constraints of working within a complex membrane system, it has been difficult to study the individual proteins in the process of translocation. To characterize translocation unambiguously, identified membrane proteins must be solubilized from microsomal membranes, purified and reconstituted into liposomes of controlled compostion.

RECONSTITUTION OF PROTEIN TRANSLOCATION

Two characteristics of the translocation process make it difficult to reconstitute into liposomes. First, translocation is a vectorial process; it is defined as the passage of the polypeptide chain from one side of the membrane to the other. Thus, any analysis of this process requires that the lipid bilayer remain intact. Second, the purification and analysis of membrane components requires that they, at least temporarily, be taken out of the context of the membrane. Thus, to assay

translocation the integrity of the lipid bilayer must be maintained, but to purify and study individual components involved, the integrity of the lipid bilayer must be disturbed. At the time that these studies began no reconstitution system had been developed whereby membrane proteins could be solubilized from the membrane with detergent and then reconstituted into translocation competent proteoliposomes once the detergent was removed. We and other groups have attempted to develop reconstitution systems which faithfully reproduce all the events required for protein translocation across a lipid bilayer. As a result, several reconstitution systems have been developed over the past 5 years which accomplish this goal to varying extents (Nicchitta and Blobel, 1990; Yu *et al.*, 1989; Zimmerman and Walter, 1990).

We describe in chapter 2 a reconstitution system that allows faithful reconstitution of translocation competent vesicles from partially solubilized microsomal membranes (Zimmerman and Walter, 1990).This system provides the advantage that protease protection of newly translocated chains, the most stringent criteria for translocation, can be reproducibly obtained. However, it has the disadvantage that the membrane proteins are not completely solubilized, and thus, cannot be easily fractionated into individual components. The microsomes are solubilized using the detergent octyl glucoside in physiological salt, thus, we expect that salt sensitive interactions between proteins are not disrupted by the treatment. Moreover, this procedure has the additional advantage that reconstitution is fast and simple; thus, it might be possible to use this system to complement the activity of biochemically inactivated proteins such as the 8-N_3ATP sensitive protein described in chapter 3 (Zimmerman and Walter, 1991). A similar system was simultaneously reported by Yu et al. (Yu *et al.*, 1989).

Nicchitta and Blobel were able to assemble translocation-competent proteoliposomes from microsomes completely solubilized by the detergent cholate

under high salt conditions (Nicchitta and Blobel, 1990). Translocation competence in this system was determined primarily by the dependence of the reaction on SRP. The amount of translocated substrate which was protected from degradation by exogenously added protease varied considerably from experiment to experiment (Nicchitta and Blobel, 1990). Thus, protease protection is not yet a reliable indicator of translocation for this system.

With this caveat notwithstanding, Nicchitta et al. have used this system to demonstrate that the translocation machinery can be fractionated prior to reconstitution such that the processes of precursor binding and translocation can be biochemically uncoupled (Nicchitta *et al.*, 1991). Moreover, they have recently demonstrated that the role of specific proteins in translocation can be assessed by immunodepletion from the detergent extract prior to reconstitution (Migliaccio *et al.*, 1992). Thus, immunodepletion of SR from the solubilized extract leads to a complete inhibition of translocation after reconstitution, but immunodepletion of SSR apparently has no affect. Eventually, cholate solubilization the complete fractionation and purification of the minimum components required for targeting and translocation. At the very least, it should allow the assessment of the role of individual proteins in translocation.

RIBOSOME BINDING TO THE ER MEMBRANE

The Ribophorins

The endoplasmic reticulum consists of many interconnected cisternae located in the perinuclear region of the cell (Palade, 1975). Two forms of ER, the rough and the smooth ER, can be characterized morphologically. The rough endoplasmic

reticulum (RER) is distinguished from the smooth endoplasmic reticulum (SER) by the presence of attached ribosomes which synthesize secretory proteins (Palade, 1975). Thus, the RER is the site of protein translocation into the ER, while the SER is the site of synthesis for many of the lipid components required for cell membranes. Since distinct roles can be attributed to these two different forms of ER, it is likely that to some extent these two forms of ER have distinct protein components associated with these activities. On the basis of this assumption, early studies by Kreibich et al. identified two putative ribosome receptors from the RER, ribophorins I and II (Kreibich *et al.*, 1978; Kreibich *et al.*, 1978).

The ribophorins had biochemical properties expected for ribosome receptors; they fractionated exclusively with the RER; they could be chemically crosslinked to ribosomes in the membrane, and after solubilization of microsomal membranes, they pelleted with ribosomes (Kreibich *et al.*, 1978; Kreibich *et al.*, 1978). Subsequently, it has been demonstrated that these proteins pellet after solubilization whether or not ribosomes are present (Hortsch *et al.*, 1986). Moreover, the protease sensitivity of the ribophorins differs from the protease sensitivity of the ribosome binding sites which can be assayed on the ER membrane (Hortsch *et al.*, 1986). There is currently no evidence that these proteins contribute to ribosome binding on the RER membrane. A possible role for the ribophorins in protein translocation has been demonstrated by antibody blocking experiments (Yu *et al.*, 1990); however, the ribophorins have recently been identified as part of a complex of three proteins which comprise oligosaccharyltransferase, the protein which catalyzes the N-linked glycosylation of asparagine residues on nascent polypeptide chains as they traverse the membrane of the ER (Kelleher *et al.*, 1992).

Two putative ribosome receptors in the ER

The electrophysiological experiments by Simon and Blobel described above indicate that the ribosome is an important player in protein translocation and probably must become specifically engaged with at least some of the components which form a protein conducting channel through the ER membrane. This finding gives greater significance to an older observation by Borgese et al. (Borgese, 1974). Using microsomal membranes stripped of all bound ribosomes Borgese et al. demonstrated that there are a saturable number of ribosome binding sites on microsomal membranes (Borgese, 1974). The ribosome binding sites are proteinaceous in nature and can be solubilized from microsomal membranes and reconstituted into liposomes in active form (Borgese, 1974)(Yamaguchi *et al.*, 1981). This reconstitution assay provided a means for the identification and purification of a ribosome receptor from the ER.

Using the ribosome binding assay two approaches have been used to identify and purify the ribosome binding activity from the ER membrane. As described above, Savitz and Meyer generated proteolytic fragments from ER membrane proteins with cytoplasmically exposed domains. They used the proteolytic fragments to compete with intact membranes for ribosome binding. Thus, they looked for inhibition of ribosome binding to the membrane and used the inhibition which they observed to purify a 160 kD protein fragment. Using antibodies raised against the 160 kD protein, they purified the parent molecule, a 180 kD ER specific membrane protein (Savitz and Meyer, 1990).

Independently, we identified the 180 kD protein as a potential ribosome binding protein in the ER based on its selective solubility in the presence or absence of membrane bound ribosomes (chapter 4), and Collins et al. demonstrated that this protein can be crosslinked to membrane bound ribosomes on the ER (Collins and

Gilmore, 1991). However, Nunnari et al. used the ribosome binding assay developed by Yamaguchi and colleagues in conjunction with reconstitution to attempt to purify directly the ribosome binding activity (Nunnari *et al.*, 1991; Yamaguchi *et al.*, 1981). They confirmed that the ribosome binding sites could be quantitatively reconstituted into lipid vesicles. Moreover, by fractionating the solubilized membrane proteins prior to reconstitution and carefully quantifying the ribosome binding sites, they demonstrated that the ribosome binding sites do not fractionate with the 180 kD protein. The binding sites do fractionate with a number of smaller basic proteins; however, the specific protein(s) responsible for ribosome binding has not yet been identified (Nunnari *et al.*, 1991). Interestingly Tazawa et al. used a similar approach to identify a 34 kD protein which can be crosslinked to 60 S ribosomal subunits bound to reconstituted liposomes (Tazawa *et al.*, 1991). Definitive confirmation of this protein as a ribosome receptor will require its purification and a direct demonstration that this protein has the ribosome binding properties which have been characterized in microsomal membranes.

THE ROLE OF NUCLEOTIDES IN TRANSLOCATION

In the past ten years several attempts have been made to elucidate the nucleotide requirements for protein translocation across the ER membrane. As mentioned above, the role of GTP in nascent chain targeting has been clearly demonstrated; however, it is still not known what the nucleotide requirements are to translocate the nascent chain across the membrane. Since the transfer of large hydrophilic proteins across the lipid bilayer is a thermodynamically unfavorable process, it is likely that there is a further requirement for nucleotide hydrolysis to provide the energy for polypeptide chain transfer across the membrane. However, it has been difficult to demonstrate a role experimentally.

ATP hydrolysis is required for protein transport across many cellular membranes. For example, ATP is required for protein import into chloroplasts (Flugge and Hinz, 1986; Grossman *et al.*, 1980) and mitochondria (Eilers *et al.*, 1987; Pfanner and Neupert, 1986) and for translocation across bacterial membranes and the ER membrane (Chen and Tai, 1987; Hansen *et al.*, 1986; Lill *et al.*, 1989; Rothblatt and Meyer, 1986; Waters and Blobel, 1986). However, for some of these systems at least part of the requirement for ATP can be attributed to the need to keep substrate proteins in a "translocation competent" or "unfolded" state, as has been demonstrated for mitochondrial import (Pfanner and Neupert, 1986) and post-translational translocation across the ER membrane in the yeast S. cerevisiae (Chirico *et al.*, 1988; Deshaies *et al.*, 1988).

The question of whether ATP is required for translocation across mammalian microsomal membranes has been difficult to address experimentally, because in this system translocation is tightly coupled to translation. Since polypeptide chain elongation requires both ATP and GTP, these nucleotides are required to assay co-translational translocation. However, studies by Garcia and Walter, demonstrated that the tight coupling of translation and translocation did not require ongoing elongation but required only that termination had not occured (Garcia and Walter, 1988). Thus, it was possible to determine the nucleotide requirements to translocate pre-elongated nascent chains.

Studies using pre-elongated nascent chains as substrates have shown that ATP is required for protein translocation across the membrane of mammalian ER (Garcia and Walter, 1988; Mueckler and Lodish, 1986; Perara *et al.*, 1986). However, these studies do not distinguish whether the ATP requirement involves a membrane protein directly involved in translocation or a cytosolic component required to maintain the unfolded state of the substrate. Thus, the question of whether ATP hydrolysis is required for the vectorial movement of the nascent

chain across the membrane has not yet been answered. Moreover, with the direct demonstration of the translocon as a large and dynamic complex of proteins, it is likely that ATP will provide some of the energy required for the assembly and/or disassembly of the translocon. In this thesis I describe experiments which indicate that ATP is required for translocation and the independent identification of two ATP-binding proteins with putative roles in translocation, SSRα and the 180 kD putative ribosome receptor.

THE OBJECTIVE OF THIS STUDY

At the time that this thesis was started very little was known about the process of protein translocation at the level of the membrane. Only two membrane proteins involved in the process had been identified, SRP receptor and signal peptidase (Evans *et al.*, 1986; Gilmore *et al.*, 1982a; Gilmore *et al.*, 1982b; Meyer *et al.*, 1982a). The identification of the SRP receptor has aided greatly in the understanding of how protein targeting to the membrane occurs but has added little to our knowledge of how translocation across the membrane occurs. The study of this process has been difficult for primarily two reasons. First, it is likely to be carried out by a complex and highly dynamic assembly of membrane proteins, and second, these proteins must function in the context of an intact lipid bilayer. The goal of this thesis was to overcome these barriers and gain a greater understanding of how protein translocation across the ER membrane occurs. I describe three ways in which this has been accomplished.

First, I describe an assay whereby membrane components can be partially solubilized from the lipid bilayer and then reconstituted into lipid vesicles in a functionally active form. Such an assay can potentially be used to identify further

proteins which mediate translocation across the ER; for example, by using detergent extracts from active membranes to complement microsomes which are biochemically inactivated by the modification of specific proteins.

Second, I demonstrate that ATP is required for protein translocation and describe a method to inactivate specifically microsomes for protein translocation using an analog of ATP, 8-N_3ATP. Thus, by combining reconstitution with translocation inactivation by 8-N_3ATP, it should be possible to purify the ATP binding protein(s) involved.

Finally, I describe the identification of a novel ribosome binding protein simultaneously described by Savitz and Meyer as a ribosome receptor on the ER membrane. However, we found that this protein does not fractionate with the majority of ribosome binding sites which can be assayed for in the membrane. Moreover, at least half of the molecule can be proteolyzed and stripped from the membrane, and yet microsomes are still active for translocation (Nunnari *et al.*, 1991). Thus, I describe a series of experiments which aid in the understanding of what the role of this protein might be.

CHAPTER 2:

Reconstitution of Protein Translocation Activity From Partially Solubilized Microsomal Vesicles

ABSTRACT

We have used a reconstitution assay to demonstrate that protein translocation activity can be recovered after microsomal vesicles derived from the rough endoplasmic reticulum have been partially solubilized with n-octyl-β-glucopyranoside. Two independent approaches were used to establish conditions for partially solubilizing microsomal membranes. When the lipid bilayer was disrupted by detergents to the extent that the integrity of the lipid bilayer had been perturbed, membranes were inactive for translocation. However, detergent treated membranes could be reconstituted in good yield into a translocation competent form once the detergent was removed.

INTRODUCTION

In higher eukaryotes, ribosomes synthesizing secretory and some integral membrane proteins are specifically targeted to the membrane of the rough endoplasmic reticulum. These ribosomes become bound to the membrane, and the nascent protein chains they synthesize are translocated across the RER membrane (Blobel and Dobberstein, 1975). The events and components that facilitate ribosome targeting have been well characterized (Walter and Lingappa, 1986). However, very little is known about how nascent chains are translocated into the lumen of the RER and which membrane proteins facilitate this process.

The translocation of nascent chains is likely to involve the concerted action of a complex assembly of RER membrane proteins, termed translocon (Walter and Lingappa, 1986). Some of these proteins may play an active role in facilitating the movement of the nascent chain across the membrane by serving as a protein motor and/or a proteinaceous tunnel (Blobel and Dobberstein, 1975). Other proteins may help to target and anchor ribosomes to the membrane, or may enzymatically modify the nascent chain but may not themselves contribute to its vectorial movement. To date, only two RER membrane proteins with known roles in this translocation process have been purified, the SRP receptor (Gibbs *et al.*, 1984; Gilmore *et al.*, 1982a; Gilmore *et al.*, 1982b; Meyer *et al.*, 1982a; Meyer *et al.*, 1982b) and signal peptidase (Evans *et al.*, 1986). Other membrane proteins have recently been shown by photoaffinity labelling to be in close proximity to the nascent chain as it is translocated across the membrane (Krieg *et al.*, 1989; Wiedmann *et al.*, 1987). Since these proteins are integral membrane proteins that are in intimate contact with the nascent chain as it spans the membrane, they are thought to play a more direct role in the translocation of the nascent chain.

Despite the progress which has been made in recent years in identifying membrane proteins that participate in translocation, further analysis of the role that these and other proteins play in this process remains a formidable task. Such analysis would be greatly facilitated if the translocation assembly could be reconstituted from detergent extracts of microsomal vesicles. As a first step toward developing a strategy for such a reconstitution, we wanted to determine whether translocation activity can be recovered after the integrity of the RER membrane has been highly disrupted by detergent. In the current work we have treated microsomal vesicles with sufficient detergent to partially solubilize the membranes and have developed a method for recovering sealed vesicles from the detergent disrupted microsomes. We have shown that although partially solubilized microsomes are incompetent for translocation, translocation competence can be restored to the membranes once the detergent is removed.

EXPERIMENTAL PROCEDURES

Materials

[^{35}S] Methionine (800 Ci/mmol) was purchased from Amersham Corp., Arlington Heights, Ill; Nikkol BL-8SY (octa-ethyleneglycol mono-n-dodecyl ether) from Nikko Chemicals Co., Ltd., Tokyo, Japan; hydroxylapatite (Bio-gel HTP) from Bio-Rad Laboratories, Richmond, Ca.; ConA Sepharose from Pharmacia, Uppsala, Sweden, and methyl α-D-mannopyranoside, n-octyl-β-glucopyranoside (OG) and L-α-phosphatidylcholine (PtdCho) were from Sigma, St. Louis, Mo.

Preparation of Salt Extracted and EDTA Stripped Microsomal Membranes

Canine rough microsomes that were EDTA stripped and/or salt extracted were prepared as previously described (Walter and Blobel, 1983).

Purification of Signal Peptidase

Salt extracted rough microsomes were incubated at a final concentration of 0.5 equivalents / μl in a buffer containing 150 mM KOAc / 50 mM triethanolamine-HOAc, pH7.5 (TEA) / 1 mM dithiothreitol (DTT) / 1 mM Nikkol BL-8SY on ice for 30 minutes. One equivalent is defined as the material derived from 1 μl of rough microsomal membranes which are at a concentration of 50 A_{280} units / ml (Walter and Blobel, 1983). The detergent extract (16 ml) was underlayered with 8 ml of cushion (50 mM TEA / 500 mM sucrose / 150 mM KOAc / 1 mM DTT) and centrifuged for 30 min at 45,000 rpm (184,000 x g_{av}) in a Beckman Ti50.2 rotor. The pellet fraction was resuspended in 8 ml of a buffer containing 526 mM KOAc / 53 mM TEA / 1 mM DTT / 21 mM sodium phosphate, pH 6.8 / 10% glycerol with a

Dounce homogenizer, and 400 µl of 20% Nikkol BL-8SY (1% final) was slowly added to the suspension under constant agitation. After a 30 min incubation on ice the detergent suspension was centrifuged at 40,000 rpm (100,000 x g_{av}) for 2 hr in a Ti50.2 rotor. The supernatant was collected and applied to a hydroxylapatite column (1 ml of resin for each 10 ml of supernatant) equilibrated with a buffer containing 50 mM TEA / 500 mM KOAc / 1 mM DTT / 20 mM sodium phosphate, pH 6.8 / 0.1% Nikkol BL-8SY / 10% glycerol.

The flow-through fraction from the hydroxylapatite column was loaded (8 ml / hour) onto a ConA Sepharose column (1 ml resin for each 8 ml of sample) equilibrated with the same buffer. The column was washed with 2 column volumes of a buffer containing 50 mM TEA / 100 mM KOAc / 1 mM DTT / 0.4% Nikkol BL-8SY and eluted (2 ml / hr) with 2 column volumes of a buffer containing 50 mM TEA / 100 mM KOAc / 1 mM DTT / 250 mM sucrose / 750 mM methyl α-D-mannopyranoside / 0.4 mg/ml PtdCho. The eluent was brought to 15 mM sodium phosphate, diluted 3-fold with a buffer containing 50 mM TEA / 100 mM KOAc / 1 mM DTT / 100 mM sucrose / 0.4% Nikkol BL-8SY / 0.4 mg/ml PtdCho, adjusted to pH6.8 and loaded onto an hydroxylapatite column (150 µl resin for each 10 ml of sample) equilibrated with the same buffer. Only about half of the signal peptidase bound to this column. The flow-through fraction was reloaded onto a second hydroxylapatite column (150 µl resin for each 10 ml of sample) equilibrated with the same buffer. The second column was eluted with one column volume of a buffer containing 50 mM TEA / 500 mM KOAc / 250 mM sucrose / 10 mM sodium phosphate, pH6.8 / 0.4% Nikkol BL-8SY / 0.4 mg/ml PtdCho and contained essentially homogeneous signal peptidase. From 1000 equivalents of rough microsomes, 40 ng of homogeneous signal peptidase was obtained. This is comparable with the yield from the previously reported purification (Evans *et al.*,

1986). Each μg of the purified protein complex contained approximately 25 units of activity (Evans *et al.*, 1986).

Con A Blots

In vitro ^{14}C labelling of ConA was done by reductive methylation as reported (Fisher *et al.*, 1982). After transfer of the protein to nitrocellulose (Fisher *et al.*, 1982), the blots were blocked for 30 minutes with ConA buffer (50 mM Tris-HCl, pH7.5 / 140 mM NaCl / 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$ / 0.02% NaN_3) containing 1% (w/v) bovine hemoglobin. The blots were then incubated overnight with the same buffer containing 15 μg of [^{14}C] ConA (2000 cpm/μg) per lane of proteins on the nitrocellulose filter. After the incubation, blots were washed 3 times, 10 min each wash, with 140 mM NaCl, dried under a lamp and exposed directly to Kodak X-Omatic AR film.

Turbidity measurements

Salt washed and EDTA stripped microsomes were incubated at a final concentration of 1 equivalent / ml in a buffer containing 125 mM sucrose / 50 mM TEA / 150 mM KOAc / 1 mM $Mg(OAc)_2$ / 1 mM DTT containing the appropriate concentration of OG. After a 30 min incubation on ice, 50 μl of sample was diluted into 550 μl of the same buffer without detergent, and the absorbance was measured at 500 nm with a spectrophotometer.

Detergent Treatment and Reconstitution of Stripped Microsomal Membranes

Salt washed and EDTA stripped microsomes were incubated at a final concentration of 1 equivalent / μl in 125 mM sucrose / 50 mM TEA / 150 mM KOAc / 1 mM $Mg(OAc)_2$ / 1 mM DTT containing the appropriate concentration of OG. After a 30 min incubation on ice the extracts were diluted ten-fold with cold

buffer. The diluted samples were centrifuged for 5 min at 80,000 rpm (228,000 x g_{av}) in a Beckman TL 100.2 rotor at 4° C. Supernatant fractions were saved for analysis by SDS-PAGE. Pellet fractions were resuspended in two times the original volume of cold 50 mM TEA / 250 mM sucrose / 1 mM DTT and were again centrifuged at 80,000 rpm for 5 min. The pellet fractions were resuspended to a final concentration of 2 equivalents / µl in 50 mM TEA / 250 mM sucrose / 1 mM DTT. Control membranes containing no OG underwent the same treatment as detergent treated membranes. For the assays described here we found some slight variability between membrane preparations (not shown), and the exact OG concentrations required to get the desired degree of solubilization or disruption was determined empirically for each batch of microsomes.

The samples shown in Figure 2 were detergent treated as described above, and after a 30 min incubation on ice, 75 µl of extract was centrifuged at 25 psi for 3 minutes in an A-110 rotor in a Beckman Airfuge.

Translocation Assays

Wheat germ translation extracts and SRP were prepared as previously described (Erickson and Blobel, 1983; Walter and Blobel, 1980). Translations were programmed with preprolactin mRNA as described (Walter and Blobel, 1980), except that RNA transcripts obtained from 2.5 ng of plasmid (contained in 1µl) were translated in each 10 µl of reaction containing 25 µCi of [^{35}S] methionine. In the reactions shown in Figure 5, 4 equivalents of membranes were included per 20 µl of reaction, and where indicated 5 mM OG was included in the reactions. The ionic conditions were kept constant in all the reactions. Aliquots of each reaction were used for protease protection assays or were prepared for SDS-PAGE as described (Garcia and Walter, 1988). Protease protection assays were done as previously reported (Garcia, 1988)

RESULTS

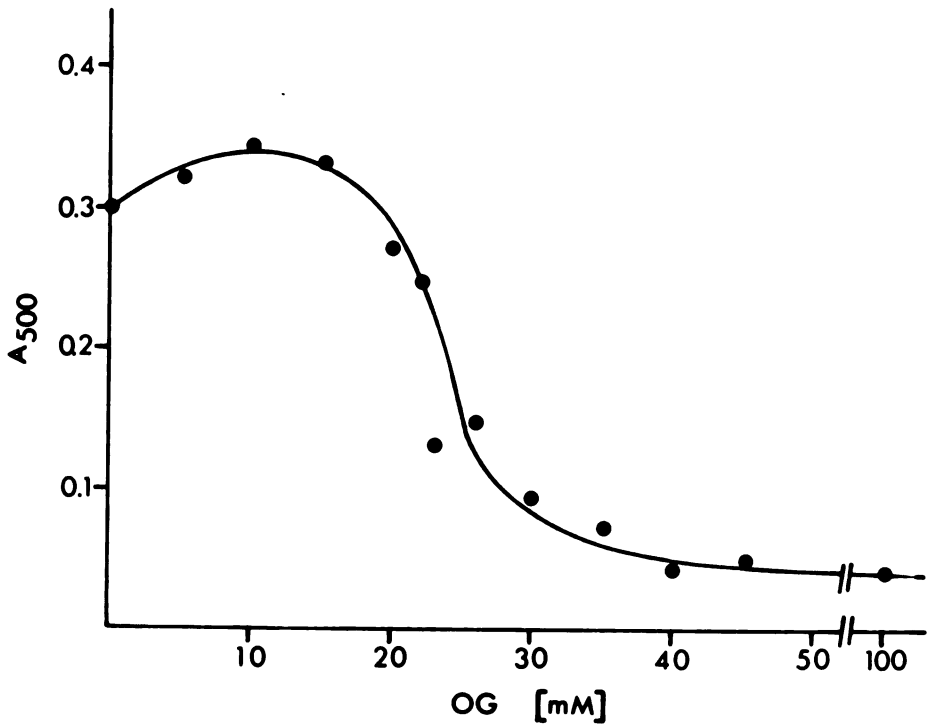
Before attempting to reconstitute protein translocation activity from detergent extracts of microsomal membranes, we established conditions whereby all the microsomes in a suspension would be disrupted by detergent. To analyze the extent of microsome disruption, a turbidity assay was used to follow the solubilization of the lipid bilayer by detergent, and a blotting assay was used to measure the concentration of detergent required to release the lumenal contents from the microsomes or to solubilize an ER membrane protein known to be involved in translocation.

Measuring the turbidity, or optical density, of a membrane-detergent suspension is a commonly used method for determining the extent of solubilization of membrane vesicles (Goni *et al.*, 1986). A three stage model has been proposed to describe the solubilization of lamellar structures into mixed micelles (Lichenberg, 1985), and these stages can be monitored by measuring the turbidity of treated membranes (Paternostre *et al.*, 1988). Thus, by measuring turbidity as optical density at 500 nm, we were able to plot the stages of solubilization of microsomal vesicles as a function of detergent concentration (Fig. 1). The rise in turbidity that occurs between 0 mM and 13 mM OG in Figure 1 represents the first stage of the solubilization process. Free detergent molecules partition between the aqueous medium and the lipid bilayer during this stage. The presence of detergent in the vesicles makes them larger, and this is thought to account for the increased turbidity (Goni *et al.*, 1986; Lichenberg, 1985). Between 13 mM and 40 mM OG there is a rapid decrease in turbidity that represents stage II of the solubilization process. The bilayers are saturated with detergent, and lipid-detergent micelles begin to form as more lipids are extracted from the bilayer (Lichenberg, 1985; Paternostre *et al.*, 1988). Note that the midpoint of this change occurs at around the critical micelle

concentration of the detergent, i.e. the concentration of detergent above which micelles are formed (25 mM OG in aqueous solution). By stage III (around 50 mM OG in Fig. 1) the bilayers are completely solubilized; all the lipids are present in mixed lipid-detergent micelles, and the suspension is no longer turbid (Goni *et al.*, 1986; Lichenberg, 1985; Paternostre *et al.*, 1988).

Figure 1. The effect of OG on the turbidity of a microsomal membrane suspension.

The turbidity (A_{500}) of salt-extracted and EDTA-stripped microsomes was measured after a 30 minute incubation with various concentrations of OG (see materials and methods).

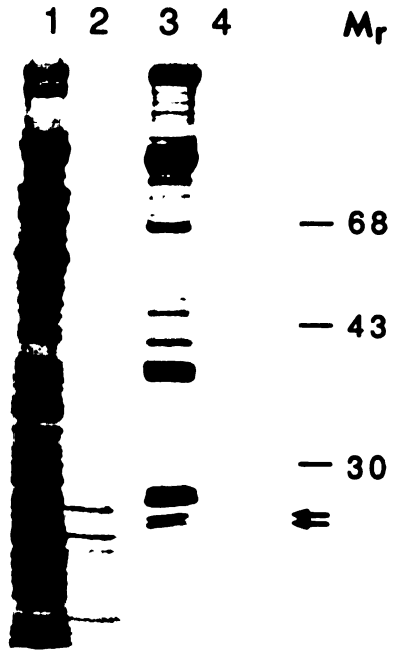

0.4
0.3
0.2
0.1
A500
10
20
30
40
50
100
OG [mM]

According to the data presented in Figure 1, microsomal vesicles are at stage II of solubilization after treatment with 13 - 40 mM OG. Thus, their lipid bilayers are fully saturated with detergent and partially solubilized. Microsomes treated with OG concentrations in this range should still pellet after centrifugation carrying integral membrane proteins with them. Thus, as an independent measure of solubilization, we have followed the sedimentation behavior of a known membrane protein, the glycosylated subunit of signal peptidase.

Signal peptidase is an integral membrane protein complex of six polypeptides, which includes two glycoproteins (Evans *et al.*, 1986). The glycoproteins migrate differently on SDS-PAGE but have identical amino acid sequences which contain a hydrophobic transmembrane region (Shellness *et al.*, 1988). These bands can be visualized by using [^{14}C] ConA to probe protein blots of either the purified signal peptidase complex (Fig. 2, lane 4) or the microsomal membranes (Fig. 2, lane 3) with [^{14}C] ConA (Evans *et al.*, 1986). By following the signal peptidase polypeptides during our purification protocol, we determined that they are the only glycoproteins in microsomal membranes that migrate at 22 and 23 kD on SDS-PAGE (data not shown). Thus, [^{14}C] ConA blots can be used to probe microsomal membrane fractions for the presence of the signal peptidase glycoproteins (indicated by a double arrow, compare Fig. 2, lanes 3 and 4). Since soluble glycoproteins can also be identified on [^{14}C] ConA blots (Kreibich and Sabatini, 1974), we also used this procedure to follow the behavior of the glycoproteins in the ER lumen after detergent treatment and fractionation of the microsomal membrane.

Figure 2. Purified signal peptidase compared with salt-extracted microsomal membranes.

Signal peptidase (lanes 2 and 4) was purified from salt-extracted microsomal membranes (lanes 1 and 3) as described (see materials and methods). Twenty equivalents of microsomes (lanes 1 and 3) or 1 mg of purified signal peptidase (lanes 2 and 4) were resolved by SDS-PAGE on 10-15% gradient gels. After electrophoresis samples were visualized by Commassie blue staining (lanes 1 and 2) or were transferred to nitrocellulose, probed with [^{14}C] ConA and visualized by exposure to X-Omat AR Kodak film (lanes 3 and 4) (see materials and methods). The signal peptidase glycoproteins are indicated by a double arrow. Molecular weights (Mr) are indicated.

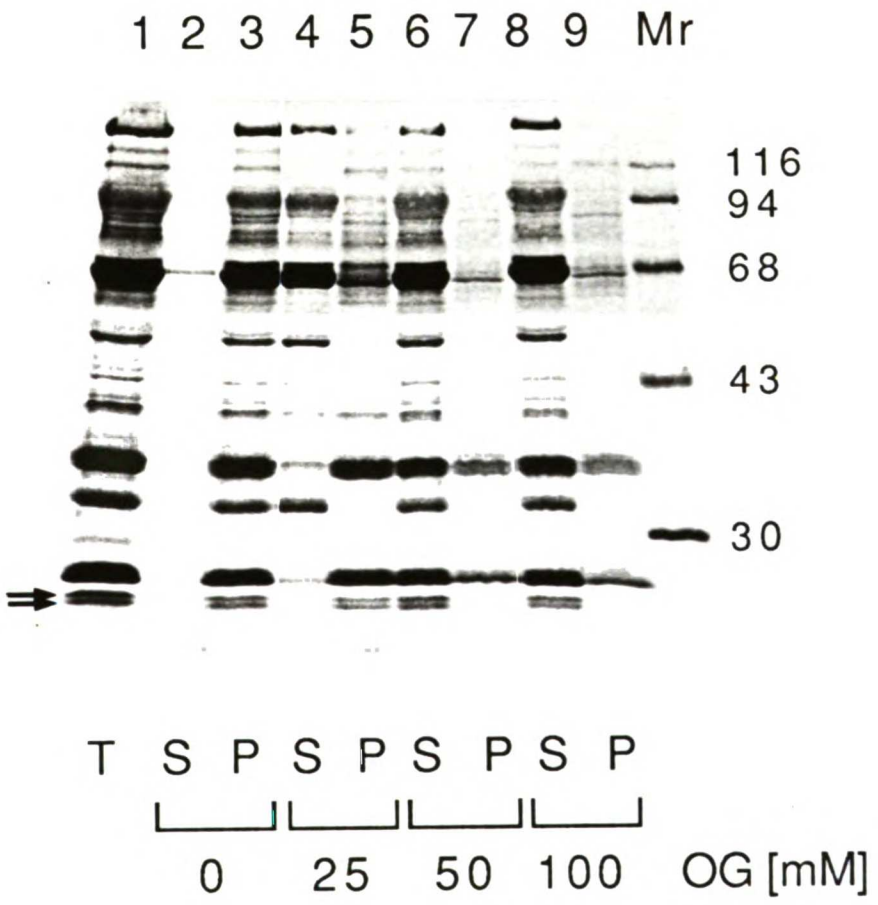

1 2 3 4
M_r
68
43
30

Microsomes incubated with increasing concentrations of OG were separated into supernatant and pellet fractions by centrifugation in an Airfuge (see materials and methods). We then probed protein blots of each fraction with [^{14}C] ConA. We found that treatment of microsomes with 25 mM OG resulted in the release of lumenal glycoproteins (compare Fig. 3, lane 4 to lane 5), without solubilizing integral membrane proteins such as signal peptidase (double arrow) and SRP receptor (localized by probing protein blots with monoclonal antibodies to both subunits (not shown)). In contrast, when membranes were treated with 50 mM OG, signal peptidase was recovered in the supernatant fraction (Fig. 3, lane 6), indicating that the lipid bilayer had been solubilized. We have shown by independent means that OG does not dissociate the signal peptidase complex (not shown). Thus, the presence of the signal peptidase glycoproteins in these fractions indicates that the whole complex has been solubilized.

Note that even after treatment with 50 mM OG some glycoproteins remained in the pellet fraction (Fig. 3, lane 7). By Western blotting we determined that both subunits of the SRP receptor were in the pellet fraction (data not shown). The amount of material in this fraction did not significantly decrease when a higher detergent concentration was used (Fig. 3, lane 9), and turbidity measurements confirmed that the vesicles were completely solubilized under these conditions (Fig. 1, see discussion below). Since under low ionic strength conditions the SRP receptor proteins pellet even in the absence of an intact lipid bilayer, they may comprise a network held together by protein-protein interactions.

Figure 3. The release of lumenal proteins and the solubilization of membrane proteins from microsomal vesicles occur at different and discrete OG concentrations.

One hundred equivalents of salt-extracted microsomes were incubated with the indicated amount of OG, and were centrifuged into supernatant (S) and pellet (P) fractions as described (see materials and methods). Twenty equivalents of salt-extracted microsomes (T) (lane 1) or of each fraction (lanes 2-9) were resolved by electrophoresis on a 10-15% gradient gel in SDS. Proteins were blotted onto nitrocellulose, and the filter was probed with [^{14}C] ConA and exposed to X-Omat AR Kodak film. Note that the samples in lanes 2 and 3 were subjected to the same treatment as the samples in lanes 4 - 9 except that no OG was present in the suspension. Molecular weight standards (Mr) are indicated.


1 2 3 4 5 6 7 8 9 Mr
116
94
68
43
30
T S P S P S P S P
0 25 50 100 OG [mM]

Microsomes treated with OG concentrations near the CMC of OG (25 mM) are saturated with detergent and disrupted, yet they still contain both signal peptidase and SRP receptor. We developed a simple procedure for recovering sealed membrane vesicles from this "extract". Microsomes were incubated with OG at or below 25 mM. The detergent concentration was then reduced ten-fold by diluting out the suspension with buffer containing no detergent, and the samples were separated into supernatant and pellet fractions by centrifugation. The pellet fractions were resuspended in detergent-free buffer and were recovered after a second centrifugation. The glycoproteins in each fraction were visualized by [^{14}C] ConA blotting (Fig. 4A). The amount of lumenal proteins released into the supernatant fraction increased with increasing detergent concentration (Fig. 4A, lanes 2, 4, 6 and 8). The microsomes treated with 25 mM OG released most of their lumenal contents into the supernatant fraction (Fig. 4A, lane 8), indicating that the integrity of the vesicles was highly disrupted. By probing protein blots with antibodies against immunoglobulin heavy chain binding protein, or BiP, a soluble protein which resides in ER lumen (Bole *et al.*, 1986) we determined that the detergent treated membranes contain less that 2% of the amount of BiP normally present in rough microsomal vesicles (data not shown). However, as noted above, all the signal peptidase (indicated by a double arrow) remained in the pellet fraction (Fig. 4A, lane 9), indicating that the vesicles were not completely solubilized. By electron microscopy we have shown that reconstituted vesicles are unilamellar and have a similar morphology to the starting membranes (not shown).

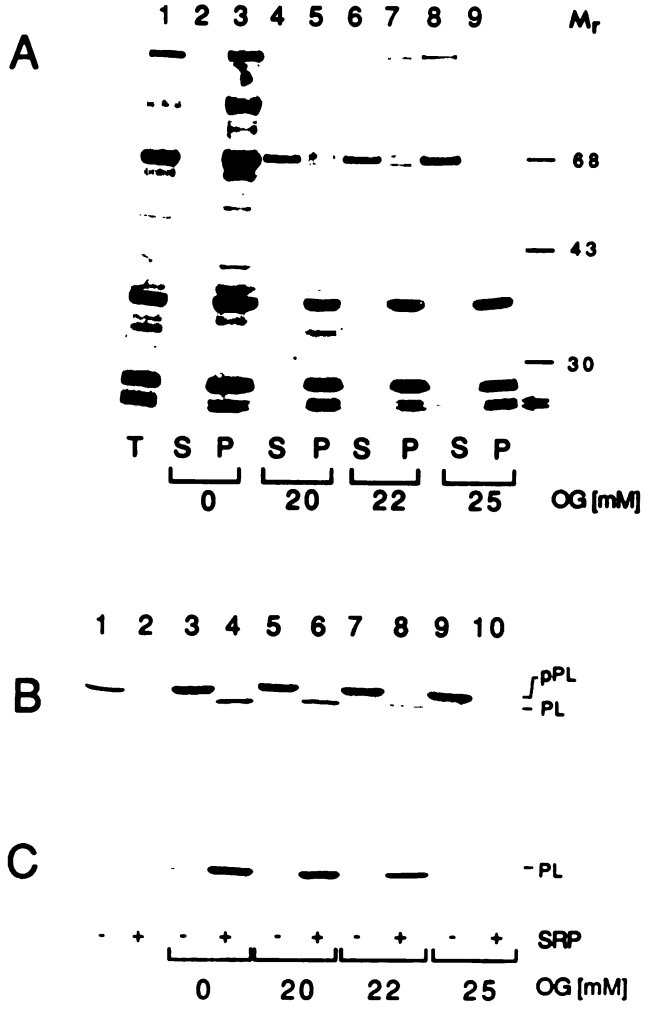
We tested the pellet fractions for activity in a co-translational translocation assay. Preprolactin was synthesized in a wheat germ translation extract (Fig. 4B, lane 1), and its synthesis was arrested by the addition of 10 nM SRP. SRP is required to target the nascent chain and ribosome to the RER membrane, but in the absence of added membranes it forms a ternary complex with the ribosome and nascent chain and arrests or slows further synthesis of the nascent chain (Fig. 4B, lane 2). When mock-treated microsomes were added to the translation extract, preprolactin was processed to prolactin in the presence of SRP (Fig. 4B, lane 4), revealing that the nascent protein chain had been transferred to the interior of the microsome and thereby became susceptible to cleavage by signal peptidase (Palade, 1975). Note that in the absence of added SRP some processing was detected (Fig. 4B, lanes 3, 5, 7 and 9) due to a small amount of residual SRP present on the microsomes. Most importantly, when detergent-treated microsomes reconstituted by the procedure described above were included in the translation mixture, SRP-dependent processing of preprolactin to prolactin was also detected (Fig. 4B, lanes 5-10). The amount of processed prolactin decreased with increasing detergent concentration (Fig. 4B, lanes 5-10), so that for microsomes treated with 25 mM OG, the amount of protein translocation activity recovered appeared to be only slightly above background (Fig. 4B, lanes 9 and 10).

Signal peptidase normally cleaves proteins after they reach the interior of the microsome (Blobel and Dobberstein, 1975). However, detergent extracted signal peptidase can cleave full length substrate proteins independently of ongoing translation or translocation (Jackson and Blobel, 1977). Although signal peptidase has not been solubilized in the assayed samples, the lumenal content proteins have been released, and hence, it is possible that the active site of signal peptidase has become exposed to proteins on the outside of the microsome. Thus, processing of preprolactin to prolactin may not be an adequate criteria for translocation. We

therefore subjected aliquots of the translation extracts shown in Figure 4B to digestion with protease K before preparing the samples for SDS-PAGE (Fig. 4C). Only prolactin which has been translocated into the lumen of a sealed vesicle should be protected from digestion by the protease (Walter and Blobel, 1983). Translocation competent vesicles, as determined by protease protection of mature prolactin, were recovered from membranes treated with each concentration of detergent used (Fig. 4C, lanes 3 - 10). In this assay the microsomal membranes recovered after treatment with 25 mM OG are more clearly dependent on the presence of SRP for protein translocation (Fig. 4C, compare lanes 9 and 10). It seems that by subjecting the translation products to digestion with protease, translocated prolactin is distinguished from prolactin produced by a fraction of signal peptidase that loses its latency during the detergent treatment. At detergent concentrations above 25 mM OG, no translocation competent membranes are recovered (data not shown).

Figure 4. Translocation competent vesicles can be recovered from detergent-treated microsomes.

(*A*) Glycoprotein profile of fractions recovered after detergent treatment of microsomes. Supernatant (S) and pellet (P) fractions were processed as described (see materials and methods) and analyzed by [^{14}C] ConA blotting. Twenty equivalents of each fraction or total salt-extracted and EDTA stripped microsomes (T) were loaded in each lane. The bands corresponding to signal peptidase are indicated with a double arrow. (*B*) Translocation reactions (see materials and methods) were carried out in the absence (lanes 1 and 2) or in the presence (lanes 3 to 10) of microsomes recovered after detergent treatment (see materials and methods). OG [mM] indicates the concentration of OG which the membranes had been incubated with prior to their recovery after the detergent was diluted and removed. Membranes treated with no OG were processed in a similar manner to the detergent treated membranes. SRP was included at 10 nM where indicated. The precursor protein, preprolactin (pPL), and processed prolactin (PL) are indicated. (C) One half of each reaction in (B) was treated with protease K before being prepared for SDS-PAGE.


A
1 2 3 4 5 6 7 8 9
M_r
68
43
30
T S P S P S P S P
0 20 22 25
OG [mM]
B
1 2 3 4 5 6 7 8 9 10
pPL
PL
C
PL
- + - + - + - + - +
SRP
0 20 22 25
OG [mM]

Taken together, the data in Figures 1 and 3 strongly suggest that after treatment with 23 - 25 mM OG all the microsomal vesicles in the population are saturated with detergent and partially solubilized. We wanted to rule out the possibility that the recovered activity is derived from a small subset of vesicles in the extract that actually contain little or no detergent. If there were any vesicles in the extract which were not affected by the detergent, they should be active for translocation whether or not the detergent is subsequently removed. We therefore assayed aliquots of detergent treated vesicles before the reconstitution procedure for translocation activity (Fig. 5).

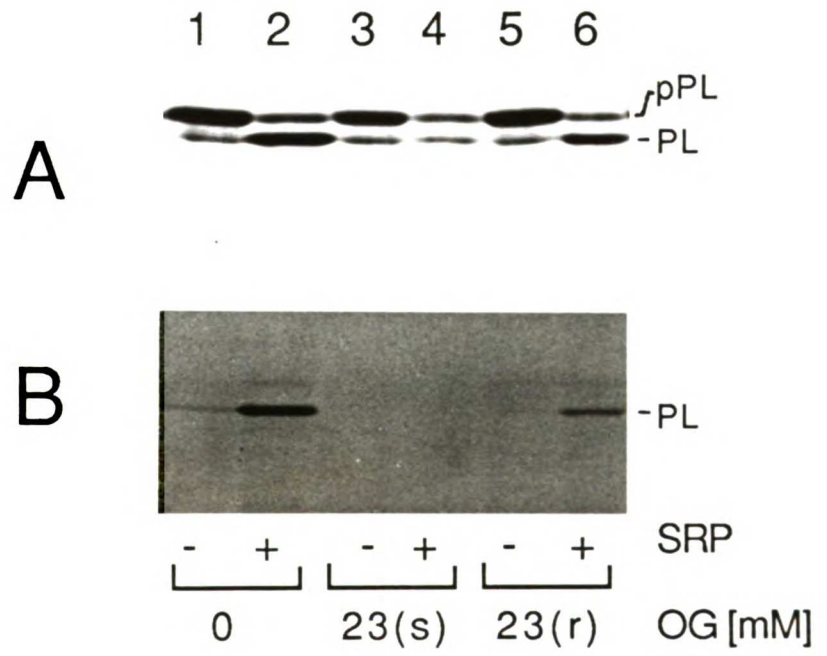
Microsomes were treated with 23 mM OG, and aliquots were either assayed directly for translocation activity (Fig. 5, lanes 3 and 4) or were diluted and washed prior to being assayed (Fig. 5, lanes 5 and 6). Note that the detergent-depleted (i.e. reconstituted) membranes displayed enhanced processing of preprolactin to prolactin in the presence of SRP (Fig. 5A, compare lanes 5 and 6). The membrane fraction assayed before detergent removal displayed no enhanced processing in the presence of SRP (Fig. 5A, compare lanes 3 and 4). This indicates either that SRP targeting does not occur, or that targeting occurs but does not result in a productive interaction between the nascent chain and the membrane components involved in translocation.

When the translations were subjected to digestion with protease K, the processed prolactin produced when reconstituted membranes and SRP were included in the reaction was protected from digestion (Fig. 5B, lanes 5 and 6). However, as expected, when detergent-saturated membranes were used, no such protease protection was observed (Fig. 5B, lanes 3 and 4). Similar results were obtained when membranes were treated with 24 mM or 25 mM OG (data not shown). In order to rule out the possibility that the small amount of OG present in the reactions containing detergent-saturated membranes inhibited translocation, we

performed the translocation assays with mock treated membranes in the presence of the same concentration of detergent (5 mM OG final). This concentration of detergent had no effect on translation or on translocation (Fig. 5A and B, lanes 1 and 2). Taken together, these results suggest that translocation competent vesicles were not present in the detergent extract, and that translocation competent vesicles form from detergent-disrupted membranes that reconstitute after detergent removal.

Figure 5. The recovery of translocation competent vesicles from detergent treated microsomes requires the removal of OG.

(A) Translocation reactions (see materials and methods) were carried out in the presence of mock treated microsomes (lanes 1 and 2), microsomes treated with 23 mM OG, 23(s), (lanes 3 and 4) or microsomes reconstituted after treatment with 23 mM OG, 23(r), (lanes 5 and 6) (see materials and methods). OG was included at 5 mM in the reactions shown in lanes 1 and 2. SRP was included at 10 nM where indicated. The precursor protein, preprolactin (pPL), and processed prolactin (PL) are indicated. (B) One half of each reaction shown in (B) was treated with protease K before being prepared for SDS-PAGE.


1 2 3 4 5 6
pPL
PL
A
B
PL
- + - + - +
SRP
0 23(s) 23(r)
OG [mM]

DISCUSSION

We have developed a method for reconstituting sealed vesicles from partially solubilized microsomal membranes. We have demonstrated that although partially solubilized microsomes are incompetent for translocation, reconstituted vesicles are translocation competent. Translocation by the reconstituted vesicles is SRP-dependent, indicating that nascent preprolactin chains are cotranslationally targeted to the membranes via an interaction between SRP and its receptor. Furthermore, no translocation is observed if preprolactin is added to reconstituted membranes post-translationally (not shown). Thus, we believe that in addition to signal peptidase (Evans *et al.*, 1986) and SRP receptor (Gill *et al.*, 1986; Gilmore *et al.*, 1982a; Gilmore *et al.*, 1982b; Meyer *et al.*, 1982a; Meyer *et al.*, 1982b), all other components required for translocation are active after partial solubilization and reconstitution.

In addition to preprolactin, prepro-α-factor, the precursor for the yeast pheromone a-factor, was used as a substrate for translocation by microsomes reconstituted after treatment with 23 mM OG. Prepro-α-factor contains three asparagine residues which become glycosylated upon translocation (Julius *et al.*, 1983). However, although prepro-α-factor was translocated across reconstituted membranes, as determined by cleavage of its signal sequence, no glycosylation of the translocated protein was detected (data not shown). Thus, disruption of the membrane by OG renders the microsomal vesicles inactive for glycosylation. This result is in agreement with the findings of Rothman et al (Rothman *et al.*, 1978) who showed that the ability of microsomal vesicles to glycosylate the glycoprotein of vesicular stomatitis virus in an in vitro translocation reaction decreased when vesicles were reconstituted after treatment with increasing amounts of Triton X-100 relative to vesicle concentration.

We were able to reconstitute vesicles from microsomes treated with up to 25 mM OG. At this detergent concentration most of the lumenal proteins are released from the microsomes. By Western blotting we detect <2% of immunoglobulin heavy chain binding protein (Bole *et al.*, 1986) in reconstituted membranes after extraction with 25 mM OG (data not shown). Thus, it seems unlikely that any lumenal proteins play an active role in translocation, although formally this possibility cannot be ruled out by our experiments. Our findings are in agreement, however, with Bulleid and Freedman (Bulleid and Freedman, 1988) who reported that translocation activity can be recovered from microsomal membranes alkali-treated at pH 9. This treatment does not perturb the integrity of the lipid bilayer per se, but leads to the release of lumenal contents from the microsomes, presumably by causing the microsomal vesicles to open transiently (Fujiki *et al.*, 1982).

When microsomes were treated with 23 mM OG, about 30% of the translocation activity of control microsomes was reconstituted. The percent of activity reconstituted decreased with increasing OG concentration, and so far we have been unable to recover activity when microsomes were treated with detergent concentrations in excess of 25 mM OG. Perhaps at higher detergent concentrations some of the lipid that is solubilized forms into lipid micelles and is excluded from protein-containing bilayers when the detergent is removed. Thus, upon detergent removal protein containing bilayers might not reform into sealed vesicles. Alternatively, the higher detergent concentration may affect the translocon itself. Our finding that detergent-treated membranes display no SRP-dependent cleavage supports this second view. We would expect that if all the components of the translocon were still in contact with one another, then SRP-dependent targeting followed by efficient processing by signal peptidase would occur even in the absence of sealed vesicles.

Our ability to partially solubilize and then reconstitute microsomal vesicles, provides a first step toward achieving reconstitution from completely solubilized vesicles. In addition, it may provide a way to incorporate membrane proteins which have been completely solubilized into partially solubilized microsomal membranes. Thus, it may now be possible to complement biochemically inactivated membrane proteins with solubilized active components.

CHAPTER 3:

An ATP-binding Membrane Protein is Required for Protein Translocation Across the Endoplasmic Reticulum Membrane

ABSTRACT

The role of nucleotides in providing energy for polypeptide transfer across the endoplasmic reticulum membrane is still unknown. To address this question we treated ER derived mammalian microsomal vesicles with a photoactivatable analog of ATP, 8-N_3ATP. This treatment resulted in a progressive inhibition of translocation activity. Approximately twenty microsomal membrane proteins were labeled by [α^{32}P] 8-N_3ATP. Two of these were identified as proteins with putative roles in translocation, αSSR, the 35 kD subunit of the signal sequence receptor complex, and ERp180, a putative ribosome receptor. We found that there was a positive correlation between inactivation of translocation activity and photolabeling of αSSR. In contrast, our data demonstrate that the ATP-binding domain of ERp180 is dispensable for translocation activity and does not contribute to the observed 8-N_3ATP sensitivity of the microsomal vesicles.

INTRODUCTION

Protein transport across cellular membranes is fundamental for organelle biogenesis and cell growth. The transfer of large hydrophilic proteins across the lipid bilayer is thermodynamically unfavorable, and therefore, energy must be expended in the process. In many cases, part of the energy appears to be provided by the hydrolysis of ATP. For example, ATP is required for protein import into chloroplasts (Flugge and Hinz, 1986; Grossman *et al.*, 1980) and mitochondria (Eilers *et al.*, 1987; Pfanner and Neupert, 1986) and for translocation across bacterial membranes and the ER membrane (Chen and Tai, 1987; Hansen *et al.*, 1986; Lill *et al.*, 1989; Rothblatt and Meyer, 1986; Waters and Blobel, 1986).

Part of the requirement for ATP can be attributed to the need to keep substrate proteins in a "translocation competent" or "unfolded" state, as has been demonstrated for mitochondrial import [Pfanner, 1987 #617] and post-translational translocation across the ER membrane in the yeast S. cerevisiae (Chirico *et al.*, 1988; Deshaies *et al.*, 1988). It is likely that there is a further requirement for nucleotide hydrolysis to provide the energy for polypeptide chain transfer across the membrane. However, it has been difficult to address this problem experimentally.

Translocation across mammalian ER occurs in at least four discrete steps: signal sequence recognition by SRP, targeting to the ER via the SRP receptor, nascent chain insertion into the membrane and subsequent translocation of the polypeptide chain (Rapoport, 1990). Both SRP and SRP receptor bind GTP (Connolly and Gilmore, 1986) Miller and Walter, unpublished), and GTP binding is required to complete the first three steps of translocation (Connolly and Gilmore, 1986;

Connolly *et al.*, 1991). Thus, multiple rounds of GTP binding and hydrolysis may insure the proper vectorial delivery of the nascent chain to the site of translocation. However, GTP hydrolysis by SRP and SRP receptor probably does not contribute to the vectorial movement of the remainder of the nascent chain across the membrane (Connolly *et al.*, 1991).

In studies which further elucidate the nucleotide requirements for protein translocation, Garcia and Walter found that there is a requirement for ATP to translocate pre-elongated nascent chains across the ER membrane (Garcia and Walter, 1988). Similarly, Mueckler and Lodish found that ATP hydrolysis is required to translocate and insert an integral membrane protein (Mueckler and Lodish, 1986). These studies do not distinguish whether ATP is required by a cytosolic protein which unfolds the pre-elongated nascent chains, or whether ATP is used by an ER membrane protein which acts during translocation. If the second case is true, then there should be at least one ATP binding protein in the ER membrane which is required for translocation. We have tested this directly by using a photoactivatable analog of ATP, 8-N_3ATP, to crosslink the ATP binding proteins in the membrane and assess their role in translocation.

EXPERIMENTAL PROCEDURES

Materials

8-N_3ATP, [α^{32}P] was purchased from ICN Biomedicals; 8-N_3ATP and puromycin were from Sigma; the ECL Western blotting detection system was from Amersham.

Preparation of microsomal membranes

Salt washed and EDTA stripped microsomes (EKRMs) were prepared as previously described (Walter and Blobel, 1983) except that stocks of microsomes were stored in a buffer containing 10 mM TEA, 250 mM sucrose, 100 μM $Mg(OAc)_2$ (Buffer A) at a concentration of 3 equivalents / μl. One equivalent is defined as the material derived from 1 μl of rough microsomal membranes that are at a concentration of 50 280 A units / ml (Walter and Blobel, 1983).

Preparation of pKRMs was adapted from a procedure by Adelman, et al. (Adelman *et al.*, 1973). Rough microsomes were brought to a final volume of 0.5 equivalents / μl in a buffer containing 50 mM TEA, 250 mM sucrose, 10 mM $Mg(OAc)_2$, 500 mM KOAc, pH 7.5, 1 mM DTT, 1 mM puromycin and incubated on ice for one hour, followed by successive incubation for 10 min at 37°C and room temperature. The membranes were loaded on top of a 2 ml cushion (1.8 M sucrose, 50 mM TEA, 1 mM DTT, 100 mM KOAc, pH 7.5, 5 mM $Mg(OAc)_2$) and centrifuged at 4°C for 20 hours at 40,000 rpm in a Beckman SW-40 rotor. The membranes sedimenting at the interface were collected and resuspended in twice their original volume in a buffer containing 50 mM TEA, 250 mM sucrose, 1 mM DTT (buffer B). The membranes were pelleted to remove excess sucrose and were resuspended to their original volume in buffer B. RMs were extracted twice with this procedure.

Photolabeling with 8-N_3ATP

Reaction volumes ranged from 50 µl - 200 µl in a buffer containing 10 mM TEA, 250 mM sucrose. $Mg(OAc)_2$ was equimolar with the final nucleotide concentration and 0.5 mM GTP was included in all reactions. Microsomes were included in the reaction at a final concentration of 1.5 equivalents / µl. For each reaction all components except nucleotides and / or 8-N_3ATP were mixed together and kept on ice. GTP and / or ATPγS were added to the reaction mix just prior to addition of 8-N_3ATP. The samples were transferred to siliconized wells of a 1/16" S/P serological ring slide placed on ice and irradiated with UV light of 366 nm by a hand held lamp (Mineralight model UVGL-25 from UVP) at a distance of 3 cm for five minutes. After UV irradiation the reactions were quenched by addition of an equal volume of buffer containing 10 mM TEA, 250 mM sucrose, 60 mM DTT and were transferred to centrifuge tubes fitting a TLA 100.2 rotor. The ring slide plate was rinsed with an equal volume of buffer and this was added to the corresponding sample. The membranes were pelleted by centrifugation at 70,000 rpm for 10 min (trypsinized / mock trypsinized microsomes were centrifuged for 15 min). Pelleted microsomes were resuspended in 3 times their original volume in buffer A and pelleted again under the same conditions. The microsomes were resuspended to 3 equivalents / µl in buffer A and subjected to two more rounds of 8-N_3ATP treatment as described. Aliquots of microsomes were saved at each step for analysis.

Photolabeling with [α^{32}P] 8-N_3ATP

Photocrosslinking with [α^{32}P] 8-N_3ATP was carried out as described above with the following differences. The final reaction volumes ranged from 5 µl - 20 µl, and microsomes were included in the reactions at a final concentration of 1.5 - 2 equivalents / µl. Prior to addition, an aliquot of anhydrous [α^{32}P] 8-N_3ATP was

dried under a gentle stream of nitrogen, resuspended at 4°C in buffer A to a final concentration of 100 - 200 µM and immediately diluted into the reaction mixture to the appropriate final concentration. Where included, nucleotides were added to the reaction mix just before addition of [α^{32}P] 8-N_3ATP. Samples were UV irradiated, and the reactions were quenched as described above. Samples were prepared for SDS-PAGE (Garcia and Walter, 1988) or immunoprecipitation as described (Krieg *et al.*, 1986).

Translation / translocation assays

Rabbit reticulocyte translation extracts were prepared as previously described (Jackson and Hunt, 1983). Translations were programmed with synthetic preprolactin RNA or SRα RNA as described (Andrews *et al.*, 1989). Translocation assays were as described (Andrews *et al.*, 1989). Reconstitution of trypsin treated microsomes with SRα was as previously described (Andrews *et al.*, 1989).

Protease treatment of microsomes

pKRMs at a concentration of 2 equivalents / µl in buffer B were adjusted to 2 µg / ml of trypsin or 100 µg / ml proteinase K and incubated on ice for one hour. The protease was inactivated by addition of phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 2 mM and incubation was continued for an additional 15 minutes. Trypsinized membranes were pelleted by centrifugation at 4° C in a TLA 100.2 rotor at 75,000 rpm for 10 minutes and resuspended to 1 equivalent / µl in a buffer containing 50 mM TEA, pH 7.5, 100 mM sucrose, 1 mM PMSF. The membrane suspension was diluted with an equal volume of buffer containing 1 M KOAc pH 7.5, 50 mM TEA pH 7.5, 1 mM PMSF, underlayered with a cushion of 50 mM TEA, pH 7.5, 500 mM sucrose and centrifuged for one hour at 70,000 rpm. Membrane pellets were resuspended in buffer B to 0.5 equivalents / µl and pelleted

again for 60,000 rpm for 1 hour. The trypsinized pKRMs (TpKRMs) were finally resuspended in buffer B at a concentration of 3 equivalents / µl.

Immunoprecipitations and Western blotting

Immunoprecipitations (Krieg *et al.*, 1986) and Western blotting were performed as described (Fisher *et al.*, 1982) with the following exceptions. The primary antibodies were detected using the enhanced chemiluminescent Western blotting detection system (Amersham). Blots were incubated with horseradish peroxidase-labeled secondary antibodies at a dilution of 1:10,000 and were detected as described in the Amersham manual.

RESULTS

Microsomes photolabeled with 8-N_3ATP are inhibited for translocation activity

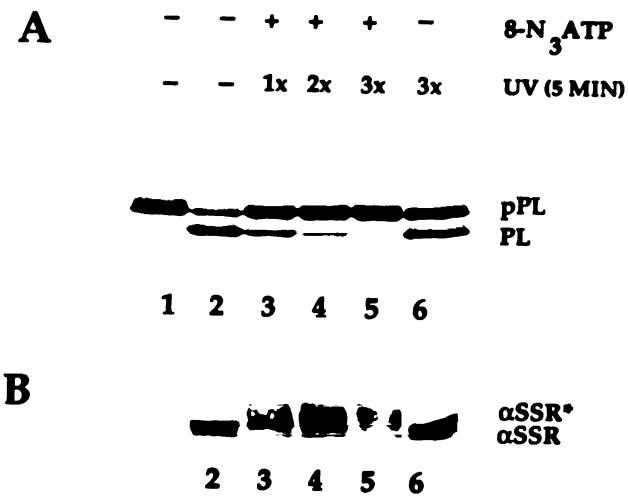
8-N_3ATP is an ATP analog which can be used to photocrosslink ATP-binding proteins. Upon exposure to UV light, the azide group on the probe becomes activated to a nitrene, and the nucleotide analog becomes covalently attached to the protein to which it is bound (Potter and Haley, 1983). Thus, ATP-binding proteins which require nucleotide hydrolysis for activity might be inactivated by this procedure. To determine if an ATP binding protein in the ER membrane is required for protein translocation, we asked whether microsomes photocrosslinked with 8-N_3ATP are impaired for translocation activity (Fig. 1A).

As shown in Figure 1A, full length preprolactin synthesized in a reticulocyte lysate translation extract was efficiently processed to prolactin when untreated microsomes were added to the extract (Fig. 1A, compare lanes 1 and 2). However, after photocrosslinking with 8-N_3ATP microsomes had a reduced capacity for

translocation; thus, they were 68% active compared with untreated membranes (Fig. 1A, compare lanes 2 and 3). After continued photocrosslinking their activity compared with untreated membranes was reduced to approximately 25% and finally 3%, as assessed by a decrease in processed prolactin and an increase in full length preprolactin (Fig. 1A, lanes 4 and 5). These results suggest that there are microsomal components involved in translocation which are sensitive to photocrosslinking with 8-N_3ATP. Mock treated microsomes exposed to UV in the absence of 8-N_3ATP were still fully active for translocation (Fig. 1A, lane 6), indicating that neither UV irradiation alone nor subsequent handling of the microsomes resulted in a significant reduction in translocation activity.

Figure 1. Inhibition of protein translocation activity by 8-N_3ATP correlates with photolabeling of αSSR.

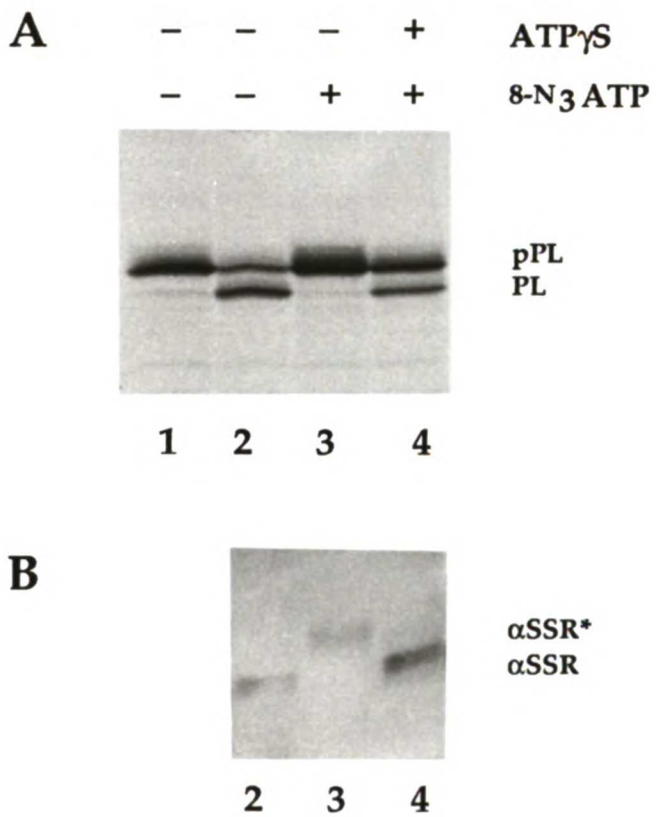
(A) Translation / translocation reactions were carried out in the absence of EKRMs (lane 1) or presence of EKRMs that were either untreated (lane 2), treated with 5 mM 8-N_3ATP (lanes 3, 4 and 5) or mock treated by UV irradiation (lane 6). EKRMs were UV irradiated for 1x, 2x and 3 x five min as indicated. Samples were analyzed by SDS-PAGE. The precursor pPL and processed form of preprolactin (PL) are indicated. (B) The microsomal membranes used in panel A were analyzed by Western blotting with antiserum against αSSR. αSSR and photolabeled αSSR (αSSR*) are indicated. UV treatment of membranes in the presence of 5 mM ATP instead of 8-N_3ATP had no affect on translocation activity (data not shown); thus, the inactivation caused by treatment with 8-N_3ATP requires the presence of the photoactivatable azido group. When activated and quenched 8-N_3ATP was added separately to a translocation assay no effect on translation or translocation was observed (data not shown). Thus, the effect observed is not a primary effect on translation or a nonspecific inhibition due to the presence of the activated 8-N_3ATP.


A
- - + + + - 8-N3ATP
- - 1x 2x 3x 3x UV (5 MIN)
pPL
PL
1 2 3 4 5 6
B
αSSR*
αSSR
2 3 4 5 6

If 8-N_3ATP is binding to bona fide ATP binding sites, then the binding should be competed for by ATP or other ATP analogs. Using a three-fold excess ATPγS, we found that microsomes photolabeled with 8-N_3ATP were >50% protected from inhibition compared with microsomes treated in the absence of ATPγS (Fig. 2A, compare lanes 3 and 4). In this experiment some degree of inhibition of translocation activity is expected because binding of 8-N_3ATP is irreversible during the time of irradiation, whereas binding of ATPγS is reversible. When microsomes are irradiated with UV in the presence of 5 mM ATP alone, no affect on translocation activity was observed (data not shown), thus the inactivation caused by treatment with 8-N_3ATP requires the presence of the photoactivatable azido group.

Figure 2. ATPγS inhibits 8-N_3ATP crosslinking to membrane proteins.

(A) Translation / translocation assays were carried out in the absence (lane 1) or presence of EKRMs. EKRMs were mock-treated by UV irradiation for 15 min (lane 2), or photolabeled with 5 mM 8-N_3ATP in the absence (lane 3) or presence of 10 mM ATPγS (lane 4). The precursor pPL, and processed PL are indicated. (B) The microsomal membranes indicated in panel A were analyzed by Western blotting with antiserum against αSSR (lanes 2 - 4). αSSR and photolabeled αSSR (αSSR*) are indicated.


A
– – – + ATPγS
– – + + 8-N3 ATP
pPL
PL
1 2 3 4
B
αSSR*
αSSR
2 3 4

α signal sequence receptor (SSR) and ERp180 cross-link to 8-N_3ATP

Our results indicate that at least one ATP-binding protein in the membrane causes an inhibition of translocation activity when it is crosslinked by 8-N_3ATP. In order to identify the 8-N_3ATP-binding proteins in the membrane which are the potential targets for the inhibition, microsomal membranes were photolabeled with [α^{32}P] 8-N_3ATP, and the profile of labeled proteins was examined by SDS-PAGE. Approximately 20 membrane proteins were crosslinked with the ATP analog (Fig. 3, lane 1). All the photolabeling observed can be competed for by excess unlabeled 8-N_3ATP (Fig. 3, lane 2), indicating that the binding sites for [α^{32}P] 8-N_3ATP are saturable. Moreover, ATPγS competed out nearly all photolabeling by [α^{32}P] 8-N_3ATP (Fig. 3, lane 3), indicating that the binding of 8-N_3ATP to these proteins was specific.

Two of the major [α^{32}P] 8-N_3ATP labeled proteins approximately comigrate with proteins that are thought to be involved in protein translocation, αSSR, a 35 kD subunit of the signal sequence receptor complex [Wiedmann, 1987 #204] and the 180 kD protein, which we term ERp180, identified as a putative ribosome receptor by Savitz and Meyer (Savitz and Meyer, 1990). We tested the identity of the [α^{32}P] 8-N_3ATP labeled products by immunoprecipitation with antibodies raised against these proteins.

SSR is an integral membrane glycoprotein comprised of a 35 kD α subunit (αSSR) and a 22 kD β subunit (βSSR) (Görlich *et al.*, 1990; Wiedmann *et al.*, 1989). αSSR was identified by photoaffinity labeling to be in close proximity to the nascent chain as it is being translocated across the membrane (Krieg *et al.*, 1989; Wiedmann *et al.*, 1987). Although its function is still unknown, it is thought that SSR is actively

involved in translocation and may comprise part of a protein translocation channel (Simon and Blobel, 1991). Antibodies raised against αSSR (Görlich *et al.*, 1990) immunoprecipitate the 35 kD [α^{32}P] 8-N_3ATP labeled product, suggesting that αSSR itself is an ATP-binding protein (Fig. 3, lane 6). αSSR is predicted to have a single transmembrane spanning domain and a carboxy-terminal cytoplasmic tail of approximately 5 kD which is sensitive to degradation by proteolysis (Prehn *et al.*, 1990). When [α^{32}P] 8-N_3ATP labeled microsomes are treated with proteinase K prior to immunoprecipitation, a photolabeled product of αSSR is no longer detected (Fig. 3, lane 5), indicating that 8-N_3ATP crosslinks to αSSR in the cytoplasmic domain.

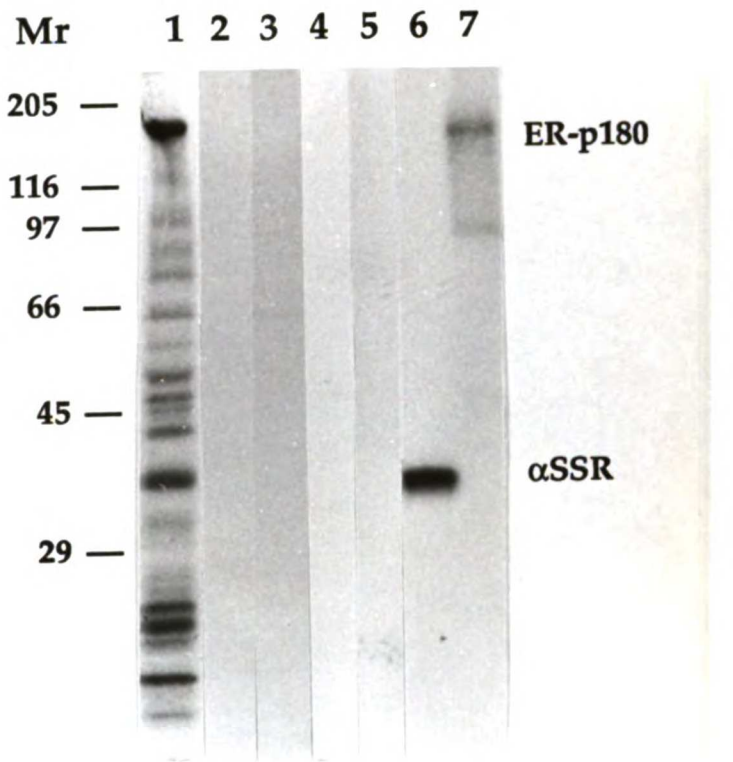
In a similar manner we confirmed the identity of the 180 kD crosslinked product as ERp180 (Fig. 3, lane 7). ERp180 was originally identified as a ribosome receptor, because a soluble proteolytic fragment derived from this protein inhibits ribosome binding to microsomal membranes (Savitz and Meyer, 1990). However, experiments done in our lab show that this protein does not fractionate with the majority of ribosome binding sites which can be assayed for in microsomal membranes (Nunnari *et al.*, 1991). Thus, the role for ERp180 in translocation, if any, remains to be determined. However, it is intriguing that two of the major [α^{32}P] 8-N_3ATP labeled proteins in the ER membrane are implied to function during translocation and thus are potential targets for the inhibition of translocation activity observed.

No photolabeled products were immunoprecipitated by antibodies which recognize βSSR, the α subunit of SRP receptor (SRα), signal peptidase or immunoglobulin heavy chain binding protein (BIP) (data not shown). BIP is a soluble protein residing in the ER lumen which is known to bind to ATP (Kassenbrock and Kelly, 1989), and thus might be expected to crosslink 8-N_3ATP. However, all the crosslinked sites are sensitive to degradation by exogenously added protease (Fig. 2, lane 4), indicating that they are all cytoplasmically exposed. Thus,

under the conditions used [α^{32}P] 8-N_3ATP labels only ATP-binding sites exposed to the cytoplasm.

Figure 3. Analysis of [α^{32}P] 8-N_3ATP labeled microsomal membrane proteins.

EKRMs were photolabeled for 5 min with 25 μM [α^{32}P] 8-N_3ATP in the absence (lane 1), or presence of either 15 mM unlabeled 8-N_3ATP (lane 2), or 1 mM ATPγS (lane 3). EKRMs photolabeled with 25 μM [α^{32}P] 8-N_3ATP were treated with proteinase K and were analyzed by SDS-PAGE either directly (lane 4) or after immunoprecipitation with antiserum raised against αSSR (lane 5). EKRMs were photolabeled with 25 μM [α^{32}P] 8-N_3ATP and prepared for immunoprecipition with antibodies against αSSR (lane 6) or ERp180 (lane 7). αSSR, ERp180 and protein standards (Mr) x 10^{-3} are indicated. No proteins were labeled when the samples were incubated with [α^{32}P] 8-N_3ATP but not exposed to UV light or when the [α^{32}P] 8-N_3ATP was activated and quenched before being incubated with the membranes (data not shown). Most proteins became crosslinked when the exposure time to activating light was between 1 and 5 min (data not shown), the time scale which is indicative of specific binding (Potter and Haley, 1983). No additional proteins were labeled when the time of UV exposure was increased to 15 min (data not shown). Thus, none of the labeling seen is due to the presence of a long-lived reactive group or a secondary reactive group created by extended exposure to UV light.


Mr
1 2 3 4 5 6 7
205
116
97
66
45
29
ER-p180
αSSR

Inactivation of translocation activity by 8-N_3ATP correlates with photolabeling of αSSR

We observed that when crosslinked to 8-N_3ATP, αSSR undergoes a mobility shift when analyzed by SDS-PAGE (Fig. 1B). We took advantage of this mobility shift to assess the extent to which αSSR is modified in membranes crosslinked by 8-N_3ATP and to compare this with the amount of inhibition of translocation activity observed. Thus, when microsomes photolabeled with 8-N_3ATP were analyzed, we found that the extent of αSSR crosslinked correlates with the amount of inhibition of translocation activity observed (Fig. 1B). Thus, after one round of 8-N_3ATP labeling, approximately 30% - 40% of αSSR was crosslinked (Fig. 1B, compare lanes 2 and 3). Moreover, the percentage of αSSR crosslinked increases to >90% after three rounds of 8-N_3ATP labeling (Fig. 1B, lane 5). As expected, when microsomes were mock treated, no mobility shift was detected, indicating that the altered migration is indeed due to crosslinking by 8-N_3ATP (Fig. 1B, lane 6).

We have already demonstrated that the presence of ATPγS during photocrosslinking protects the membranes from the inhibition of translocation activity caused by 8-N_3ATP crosslinking. Thus, we compared the extent of aSSR crosslinked in membranes photolabeled in the presence and absence of 8-N_3ATP and ATPγS (Fig. 2B). Accordingly, when microsomes were photolyzed in the presence of 8-N_3ATP alone, all αSSR was shifted compared with mock treated membranes (Fig. 2B, compare lanes 2 and 3). Moreover, when microsomes were photolabeled in the presence of both 8-N_3ATP and 10 mM ATPγS the amount of αSSR crosslinked was greatly reduced (Fig. 2B, compare lanes 3 and 4). Thus, the protection conferred by ATPγS on translocation activity directly correlates with the

reduced amount of αSSR crosslinked. With respect to the role of αSSR in translocation, these findings are only a correlation and do not demonstrate that αSSR is the 8-N_3ATP sensitive target required for translocation.

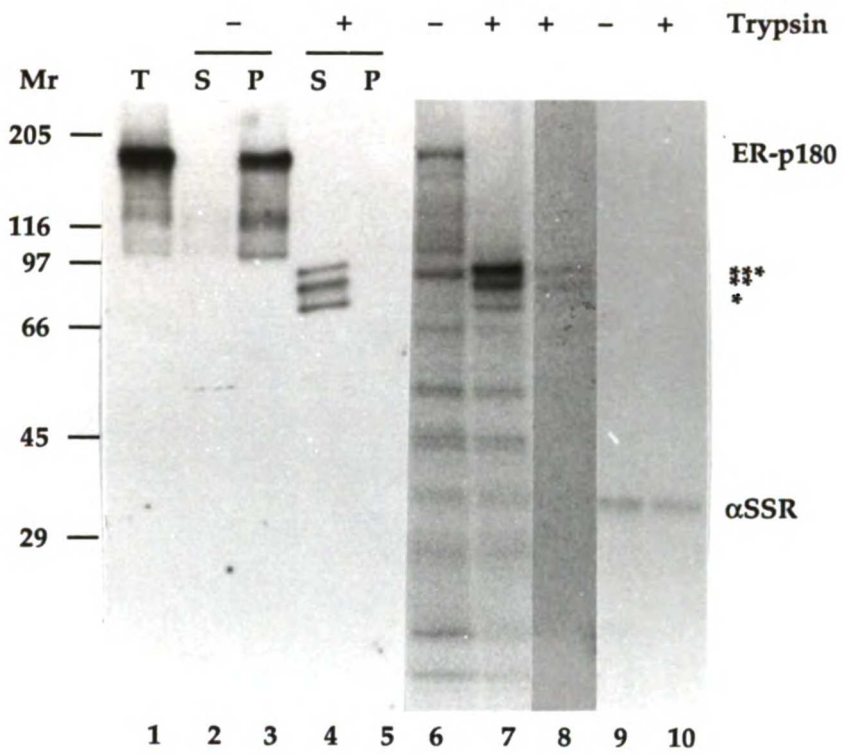
ERp180 is proteolyzed from trypsinized microsomes

ERp180, has a large cytoplasmic domain which is extremely sensitive to proteolysis (Nunnari *et al.*, 1991; Savitz and Meyer, 1990). In order to further characterize this protein with respect to 8-N_3ATP labeling, we used mild proteolysis conditions to cleave this domain from the membrane. Puromycin stripped, salt washed microsomes (pKRMs) were treated with a low concentration of trypsin, and the membranes were fractionated away from soluble proteolytic fragments by centrifugation. The protein composition of both fractions was analyzed with respect to ERp180 by Western blotting with antibodies raised against this protein (Nunnari *et al.*, 1991) (Fig. 4). All of ERp180 was recovered in the membrane pellet after mock treatment of the microsomes (Fig. 4, compare lanes 2 and 3); whereas, three proteolytic fragments were recovered in the supernatant fraction after trypsin treatment (Fig. 4, lane 4). Moreover, neither intact ERp180 nor any detectable degradation products pelleted with the microsomes after trypsinization (Fig. 4, lane 5).

In order to map the site of [α^{32}P] 8-N_3ATP crosslinking to ERp180, microsomes were photolabeled with [α^{32}P] 8-N_3ATP and then treated with trypsin as described. The [α^{32}P] 8-N_3ATP label was found to be crosslinked to the trypsin derived fragments (Fig. 4, lane 8). Thus, the site of [α^{32}P] 8-N_3ATP binding to ERp180 is in the protease sensitive cytoplasmic domain. In contrast to ERp180, many other sites crosslinked by 8-N_3ATP are unaffected by mild trypsinization (Fig. 4, compare lanes 6 and 7). For example, αSSR, which is less sensitive to proteolysis than ERp180, is photolabeled in trypsinized membranes (TpKRMs) (Fig. 4, compare

Figure 4. ERp180 is sensitive to mild proteolysis.

Material derived from 10 equivalents of pKRMs (lane 1) or of supernatant (S) and pellet (P) fractions of mock treated (lanes 2 and 3) or trypsin treated pKRMs (lanes 4 and 5) was analyzed by Western blot with antibodies against ERp180. pKRMs were photolabeled with [α^{32}P] 8-N_3ATP and were prepared for SDS-PAGE (lane 6) or immunoprecipitation with antibodies against αSSR (lane 9). [α^{32}P] 8-N_3ATP labeled pKRMS were treated with trypsin and were prepared for SDS-PAGE (lane 7) or immunoprecipitation with antibodies raised against ERp180 (lane 8) or αSSR (lane 10). αSSR and ERp180 are indicated. Trypsin derived fragments of ERp180 are indicated by asterisks. Protein standards are indicated (Mr) x 10^{-3}.


– + – + + – + Trypsin
Mr T S P S P
205
116
97
66
45
29
ER-p180
‡‡*
*
αSSR
1 2 3 4 5 6 7 8 9 10

Trypsinized microsomes are sensitive to 8-N_3ATP

We showed above that TpKRMs no longer have the 8-N_3ATP binding domain of ERp180, and thus, this protein should no longer be a target for 8-N_3ATP in TpKRMs. Therefore, if photolabeling of ERp180 leads to the inhibition of translocation activity that we observe, then TpKRMs should not be inhibited for translocation activity by 8-N_3ATP. To test this TpKRMs and mock treated pKRMs were photolabeled as described and were assayed for translocation activity (Fig. 5). Since the α-subunit of the SRP receptor is required for translocation (Walter *et al.*, 1979), but is itself very protease sensitive, we used an assay that allows activity to be restored to membranes depleted of SRα by mild trypsinization (Andrews *et al.*, 1989; Walter *et al.*, 1979). Thus, TpKRMs were inactive for translocation compared with pKRMs, as assessed by protection of prolactin by exogenously added protease (Fig. 5, compare lanes 2 and 7), but when TpKRMS were supplemented with SRα translated from synthetic RNA, translocation activity was restored (Fig. 5, lane 9).

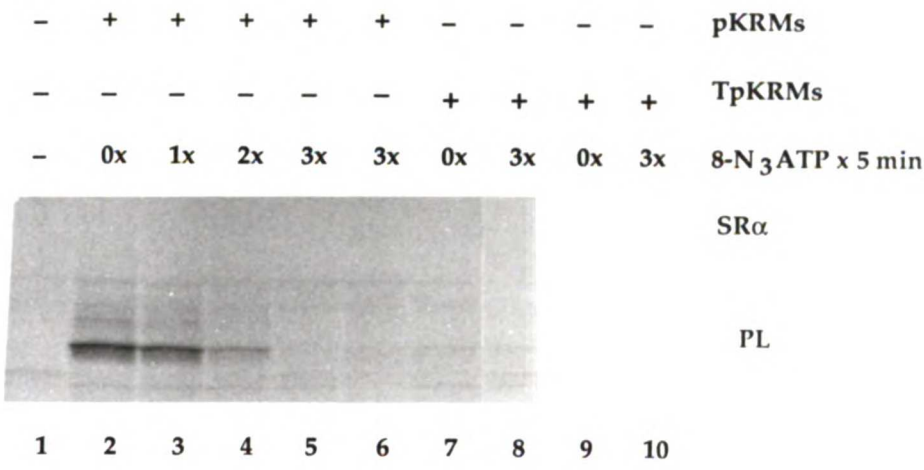
As expected, unproteolyzed membranes were inhibited for translocation by 8-N_3ATP treatment (Fig. 5, lanes 3 - 5); whereas, TpKRMS were inactive for translocation activity both before and after treatment with 8-N_3ATP (Fig. 5, lanes 7 and 8). In contrast to uncrosslinked TpKRMs, translocation competence was not restored to 8-N_3ATP treated TpKRMs when SRα was added back to them (Fig. 5, compare lanes 8 and 10). Thus, TpKRMs which no longer have the 8-N_3ATP binding site of ERp180 are still sensitive to 8-N_3ATP treatment and, it is unlikely that photolabeling of this protein is responsible for the inhibition of translocation activity that we observe.

We showed above that TpKRMs are dependent on newly added SRα for translocation activity (Fig. 5, compare lanes 7 and 9). Supplying new SRα does not

restore translocation competence to either TpKRMs (Fig. 5, lane 10) or pKRMs (Fig. 5, lane 6) after treatment with 8-N_3ATP. Since SRα binds GTP in its trypsin sensitive cytoplasmic domain, and this domain is restored to TpKRMs after 8-N_3ATP treatment, then these data further demonstrate that photolabeling of SRα does not cause the inhibition of translocation activity that we observe.

Figure 5. Trypsinized microsomal membranes are inhibited for protein translocation activity by 8-N_3ATP.

Translocation reactions were carried out in the absence (lane 1) or in the presence of pKRMs (lane 2), pKRMs treated with 5 mM 8-N_3ATP for five minutes one (lane 3), two (lane 4) and three times (lanes 5 and 6), trypsinized pKRMs (lanes 7 and 9), or trypsinized pKRMs treated for a total of 15 min with 8-N_3ATP (lanes 8 and 10). Translation reactions were supplemented with SRα where indicated. All reactions were treated with proteinase K before being prepared for SDS-PAGE. The processed form of prolactin (PL) is indicated.


– + + + + + – – – – pKRMs
– – – – – – + + + + TpKRMs
– 0x 1x 2x 3x 3x 0x 3x 0x 3x 8-N3ATP x 5 min
SRα
PL
1 2 3 4 5 6 7 8 9 10

DISCUSSION

We have shown that microsomes photolabeled with 8-N_3ATP are inactive for translocation. The requirements for photolabeling are those expected if inhibition is due to 8-N_3ATP crosslinking to one or more ATP-binding proteins which function during translocation. Our results further demonstrate that the target protein(s) is a resident membrane protein of the ER, since microsomes stripped of all ribosomes and loosely bound cytosolic factors are sensitive to 8-N_3ATP treatment. We find that there are upward of twenty substrates for 8-N_3ATP, any of which could be responsible for the inactivation observed. However, it is intriguing that two of the major targets for 8-N_3ATP are proteins previously proposed to have roles in translocation, αSSR and ERp180.

αSSR is an integral membrane glycoprotein which forms a complex with another 25 kD glycoprotein (Görlich *et al.*, 1990). Two types of experiments have implicated this protein complex in translocation. Photocrosslinking studies have demonstrated that αSSR is in close proximity to the nascent chain during translocation (Krieg *et al.*, 1989; Wiedmann *et al.*, 1989; Wiedmann *et al.*, 1987), and F_{ab} fragments which recognize αSSR block protein translocation in vitro (Hartmann *et al.*, 1989). We have found that αSSR is quantitatively crosslinked in membranes inactivated for translocation. Moreover, when microsomes are partially inactivated for translocation activity, the amount of αSSR crosslinked correlates with the inhibition of translocation activity observed. Although this result is intriguing, more experiments will need to be done to determine whether photolabeling of αSSR causes the inhibition of translocation activity which we observe. Moreover, it will be important to determine whether the purified SSR complex has an intrinsic ATPase activity, or whether another closely associated

protein actually binds to 8-N_3ATP in the membrane putting the photoactivatable azido group in close proximity to αSSR.

The second 8-N_3ATP binding protein which we have identified, ERp180, has a putative role as a ribosome receptor (Savitz and Meyer, 1990). However, we have demonstrated that microsomes which have been mildly trypsinized no longer have the ATP binding domain of ERp180, yet they remain sensitive to 8-N_3ATP treatment. Thus, it is unlikely that photolabeling of ERp180 causes the inhibition that we observe after photolabeling. Moreover, these results raise doubts about whether ERp180 plays an essential role in protein translocation in general. As shown above, translocation competence is restored to TpKRMs, when they are supplemented with SRα. Thus, under the conditions used, the only trypsin sensitive protein required for translocation is SRα. Since the proteolytic products derived from ERp180 that we can detect range in size from 70 kD - 100 kD (Fig.4 , lane 4), it can be concluded that proteolysis of at least half of this protein does not impair microsomes for translocation activity.

Previous studies have shown that ATP is required for protein translocation across the membrane of mammalian ER when pre-elongated nascent chains are used as a substrate (Garcia and Walter, 1988; Mueckler and Lodish, 1986; Perara *et al.*, 1986). However, these studies could not distinguish whether the ATP requirement involved a cytosolic component or a membrane protein. Thus, our results are the first demonstration that a putative ATP-binding protein in the ER membrane is required for translocation.

Connolly and Gilmore found that, in contrast to the longer chains used in the other studies, an 86 amino acid truncated form of preprolactin requires GTP, but not GTP hydrolysis, for translocation (Connolly and Gilmore, 1986). Since GTP is needed for nascent chain targeting and signal sequence insertion (Connolly *et al.*, 1991), then proper delivery of these chains to the membrane may be sufficient to

ensure their subsequent translocation into the lumen. Thus, it might be expected that 8-N_3ATP treatment of membranes would not affect translocation of short nascent chains. In contrast, we found that 8-N_3ATP treated membranes are blocked for translocation at the level of signal sequence insertion (data not shown). Thus, it is possible that the ATP-binding protein(s) which is crosslinked is required for translocation of both long and short nascent chains. However, more steps might be required at the level of the membrane to translocate the longer chains, and ATP binding and hydrolysis may not be required until a later step.

Our results demonstrate that crosslinking of an ER protein by 8-N_3ATP renders microsomal membranes inactive for translocation activity. Thus, it might be possible to restore translocation competence to 8-N_3ATP treated membranes by adding back uncrosslinked protein, thereby providing an assay to purify the protein involved. We are currently using affinity chromatography to purify the ATP-binding proteins from the ER membrane and will use the reconstitution assays currently available to try and complement 8-N_3ATP inactivated microsomes with the purified proteins and identify the required component (Nicchitta and Blobel, 1990; Yu *et al.*, 1989; Zimmerman and Walter, 1990) .

Chapter 4:

A ribosome binding protein in the endoplasmic reticulum membrane

ABSTRACT

Ribosomes synthesizing proteins destined for the secretory pathway become specifically engaged with the ER membrane. Part of the interaction between the ribosome and the membrane is mediated by the nascent chain; however, there is now compelling evidence for the presence of specific ribosome binding proteins in the membrane. We have identified an ER protein, termed ERp180, which binds with high affinity to both 60 S and 40 S ribosomal subunits. ERp180 is tightly associated with the ER membrane but does not fractionate as a classical integral membrane protein. Crosslinking studies indicate that purified ERp180 is a dimer, and partial protein sequencing reveals that part of the protein is homologous to myosin in the tail region and thus might have regions with a coiled-coil structural motif.

INTRODUCTION

Ribosomes synthesizing secretory proteins are selectively targeted to the ER membrane by SRP and SRP receptor (Walter and Lingappa, 1986). Once targeted, the ribosomes become attached to the membrane by their large subunits, and the nascent chains are translocated into the ER lumen (Walter and Lingappa, 1986). Thus, ribosome binding to the ER is a crucial step in the events which lead to the translocation of proteins across the membrane. Early studies indicated that this event was mediated only in part by the nascent chain (Sabatini and Blobel, 1970). It was therefore thought that there are specific membrane-bound receptors for ribosomes in the ER membrane. Recent results by Simon and Blobel demonstrating the existence of a large protein translocating channel in the ER membrane underscore the role of these putative receptors in maintaining the open state of the channel (Simon and Blobel, 1991).

Despite the acknowledged importance of ribosome receptors as active players in the translocation process, the direct identification of these proteins has remained elusive. Many attempts have been made to identify proteins responsible for the direct binding of ribosomes to the ER. For example, the ribophorins I and II which are localized exclusively to the rough ER were identified by Kreibich et al. as potential ribosome receptors for the ER (Kreibich *et al.*, 1978; Kreibich *et al.*, 1978) However, it has subsequently been shown that these proteins do not contribute to ribosome binding on the ER membrane and are instead components of oligosaccharyl transferase (Kelleher *et al.*, 1992; Yoshida *et al.*, 1987).

Early studies by Borgese and colleagues demonstrated that there are a saturable number of ribosome binding sites on the membrane and that the binding sites are proteinaceous in nature (Borgese, 1974). Moreover, it was demonstrated

that a protein fraction with high affinity for ribosomes could be isolated from microsomal membranes and could be reconstituted into liposomes in functional form (Yamaguchi *et al.*, 1981). This later finding provided the first direct assay for an ER ribosome receptor, and in recent years a number or groups have attempted to use this assay to identify the protein(s) responsible for this activity (Nunnari *et al.*, 1991; Savitz and Meyer, 1990).

Savitz and Meyer succeeded in purifying a 160 kD proteolytic fragment from microsomal membranes which inhibits ribosome binding to the ER membrane (Savitz and Meyer, 1990). Reasoning that this fragment must be derived from the ribosome receptor in the membrane which mediates this binding activity, they purified the parent molecule from which the 160 kD fragment was derived. The parent molecule is a 180 kD protein which the authors describe as being an integral membrane protein localized in the ER, and they call this protein "a ribosome receptor in the RER membrane". However, they do not demonstrate that this protein is responsible for the saturable binding of ribosomes which can be measured on microsomal membranes (Savitz and Meyer, 1990). Moreover, Nunnari et al. demonstrated that the 180 kD protein fractionates away from the ribosome binding activity, and that all the binding activity can be accounted for by a subset of smaller basic proteins (Nunnari *et al.*, 1991).

Using biochemical criteria we independently identified a 180 kD protein as a ribosome binding protein in the ER membrane. Based on the molecular weight of the intact protein and of proteolytic products generated with the protease thermolysin, we believe that this protein is identical to the 180 kD protein identified by Savitz and Meyer as a ribosome receptor for the RER (Nunnari *et al.*, 1991). We show by cell fractionation that this protein is localized to the ER and is tightly associated with the membrane. The 180 kD protein binds with high affinity to both

the large and small subunits of ribosomes. Thus, the nature and specificity of the binding interaction is uncertain.

Protein sequence data revealed that the protein has some homology to myosin in the tail region, and thus, might be a member of the family of proteins which display the coiled-coil motif. Sucrose density gradient analysis in conjunction with preliminary EM studies revealed that the protein has an elongated shape, and crosslinking studies revealed that the purified protein is a homodimer. These findings are consistent with the proposed role for this ER membrane protein as a structural component which has the capacity to become engaged with ribosomes.

EXPERIMENTAL PROCEDURES

Materials

Nikkol BL-8SY (octaethylene glycol mono-*n* -dodecly ether) and Nikkol BD-8SY (octa-ethylene glycol mono-n-decyl ether) were from Nikko Chemicals Co., Ltd., Tokyo, Japan.

Preparation of microsomal membranes

Canine rough microsomal membranes (RM) and salt washed microsomal membranes (KRM) were prepared as previously described (Walter and Blobel, 1983). Mock puromycin treated (mKRMs) and puromycin treated microsomes (pKRMs) were prepared as previously described (Zimmerman and Walter, 1991).

Preparation of antibodies

ERp180 was gel purified on a 5 - 15% acrylamide gel by SDS-PAGE. Polyclonal antiserum was raised in rabbits as previously described [Kellog, 1989 #751]. Antibodies which recognize specifically ERp180 were immunoselected from the polyclonal serum by absorption to ERp180 which was blotted onto nitrocellulose. A preparative SDS-PAGE gel containing only ERp180 in the molecular weight range of 180 kD was blotted onto nitrocellulose and stained with ponceau S. The band corresponding to ERp180 was cut out, and the nitrocellulose strip was incubated in blocking buffer containing 10% (weight / volume) powdered milk in phosphate buffered saline (PBS) solution. The strip was then incubated overnight at 4^{o} C with a buffer containing 10% (weight / volume) of powdered milk, 0.2% tween 20, and 50% (volume / volume) rabbit serum in PBS. The strip was washed three times for 20 min each wash in the same buffer without the serum. The antibody was then eluted

from the strip by the addition of 200 µl of HCl, pH 2.1 for 60 seconds. The acid eluate was removed and added to 40 µl of 1 M Tris.

Solubility assay

pKRMs or mKRMs were diluted to a concentration of 1 eq/µl in a buffer containing 50 mM TEA, 100 mM sucrose, 150 mM KOAc, 1 mM $Mg(OAc)_2$, 1 µM EGTA, 1 mM DTT and 30 mM Nikkol BD-8SY. The extracts were incubated on ice for 15 min and were centrifuged for 3 min at 25 psi in a Beckman A-110 Airfuge rotor. The supernatant and pellet fractions were saved and prepared for analysis by SDS-PAGE and Western blotting.

Cell fractionation

To prepare cell fractions the procedure for the preparation of RMs was followed (Walter and Blobel, 1983). Briefly, canine pancreas was minced and homogenized in a volume of buffer equal to four times the weight of the pancreas. The homogenized suspension was centrifuged for 10 min at 3,000 rpm in a Beckman JA-20 rotor. The supernatant fraction was saved as the "total" cell extract and was further processed into subcellular fractions as described (Walter and Blobel, 1983). Equivalent amounts based on volume were saved from each fraction and were prepared for analysis by SDS-PAGE and Western blotting.

KOAc, EDTA and puromycin extraction of microsomal membranes

350 µl of RMs at a concentration of 1 eq/µl in a buffer containing 50 mM TEA, 250 mM sucrose and 1 mM DTT were diluted with an equal volume of the same buffer containing either 1 M KOAc, 1 M KOAc and 50 mM EDTA or 1 M KOAc and 4 mM puromycin. The suspensions were underlayered with a 300 µl sucrose cushion (50 mM TEA, 1.8 M sucrose, 100 mM KOAc and 1 mM DTT) and were centrifuged

for 20 h at 200,000 x g in a Beckman TLA100.2 rotor. The samples were separated into supernatant and membrane fractions. The cushion and pellet fractions were pooled as the "cushion". Aliquots of each fraction were saved for analysis by SDS-PAGE and Western blotting.

Purification of ERp180

All the steps described were performed at 4°C. KRMs at a concentration of 1 eq/µl in a buffer containing 50 mM TEA, 250 mM sucrose and 1 mM DTT were diluted with an equal volume of a buffer containing 300 mM KOAc, 50 mM TEA, 1 mM DTT, 3 mM Nikkol BL-8SY and protease inhibitors (pI). 1x pI is comprised of 0.5 µg/ml leupeptin, 0.5 µg/ml pepstatin and 50 µg/ml antipain. The extract was incubated on ice for 15 min, underlayered with a sucrose cushion (50 mM TEA, 500 mM sucrose, 150 mM KOAc, 1 mM DTT and 1x pI; 4 ml cushion / 20 ml of extract) and centrifuged for 1 h at 40,000 rpm in a Beckman Ti50.2 rotor. The pellet fraction was resuspended in a buffer containing 50 mM TEA, 250 mM sucrose, 1 mM DTT and 1x pI to a final concentration of 2 eq/µl and was diluted with an equal volume of a buffer containing 50 mM TEA, 250 mM sucrose, 1 M KOAc, 10 mM $Mg(OAc)_2$, 1 mM DTT, 2% Nikkol BL-8SY and 1x pI. The extract was incubated for 15 min on ice and was then centrifuged for 1 h at 40,000 rpm in a Beckman Ti50.2 rotor.

The pellet fraction (P-2) was resuspended in a buffer containing 50 mM TEA and 10 mM $Mg(OAc)_2$ at a final concentration of 20 eq/µl. The supernatant fraction was diluted to a final concentration of buffer containing 50 mM TEA, 250 mM sucrose, 150 mM KOAc, 5 mM $Mg(OAc)_2$, 0.3% Nikkol BL-8SY, 1 mM DTT and 1x pI. P-2 was added to this extract, and the suspension was incubated on ice for 15 min, underlayered with a sucrose cushion (50 mM TEA, 500 mM sucrose, 150 mM KOAc, 5 mM $Mg(OAc)_2$, 0.3% Nikkol BL-8SY, 1 mM DTT and 1x pI; 4 ml cushion / 20 ml extract) and centrifuged for 1 h at 40,000 rpm in a Beckman Ti50.2 rotor.

The pellet fraction was resuspended in a buffer containing 50 mM TEA, 250 mM sucrose, 5 mM $Mg(OAc)_2$, 0.3% Nikkol BL-8SY and 1x pI at a final concentration of 1 eq/µl and was brought to a final concentration of 500 mM KOAc from a 4 M stock solution. The suspension was incubated on ice for 15 min and was then centrifuged for 90 min at 40,000 rpm in a Beckman Ti50.2 rotor.

The supernatant fraction was brought to a final concentration of 10 mM sodium phosphate, pH 6.5 from a 500 mM stock solution and was loaded onto a hydroxylapatite column (1 ml resin for each 5 ml of supernatant) equilibrated with a buffer containing 50 mM TEA, 500 mM KOAc, 5 mM $Mg(OAc)_2$, 0.3% Nikkol BL-8SY and 10 mM sodium phosphate, pH 6.5. The column was washed with 6 column volumes of a buffer containing 50 mM TEA, 250 mM sucrose, 250 mM KOAc, 2 mM $Mg(OAC)_2$, 10 mM sodium phosphate, pH 6.5, 1 mM DTT and 1x pI. and was eluted with 2 column volumes of the same buffer containing 60 mM sodium phosphate, pH 6.5.

The eluate fraction from the hydroxylapatite column was diluted to a final concentration of buffer containing 50 mM TEA, 100 mM KOAc, 100 mM Sucrose, 1.6 mM $Mg(OAc)_2$, 3 mM Nikkol BL-8SY, 1 mM DTT, 15 mM sodium phosphate pH, 6.5 and 1x pI by addition of 3 volumes of a buffer containing 50 mM TEA, 50 mM KOAc, 50 mM sucrose, 1.5 mM $Mg(OAc)_2$, 2.2 mM Nikkol BL-8SY, 1 mM DTT and 1x pI. The suspension was loaded at a rate of 10 ml/h onto a DEAE-sepharose CL6B column (1 ml of resin for each 4 ml of suspension) equilibrated with a buffer containing 50 mM TEA, 100 mM KOAc, 1.6 mM $Mg(OAc)_2$, 3 mM Nikkol BL-8SY and 15 mM sodium phosphate, pH 6.5. The column was washed with 6 column volumes of a buffer containing 50 mM TEA, 100 mM KOAc, 100 mM Sucrose, 3 mM Nikkol BL-8SY, 1 mM DTT and 1x Pi. and then eluted with 2 column volumes of a buffer containing 50 mM TEA, 250 mM KOAc, 100 mM Sucrose, 3 mM Nikkol BL-8SY, 1 mM DTT and 1x pI.

The eluate fraction from the DEAE-sepharose column was diluted to 50 mM TEA, 150 mM KOAc, 100 mM sucrose, 3 mM Nikkol BL-8SY, 1 mM DTT and 1x pI. with 0.67 volumes of a buffer containing 50 mM TEA, 100 mM sucrose, 3 mM Nikkol BL-8SY, 1 mM DTT and 1x pI. and was loaded onto a CM-sepharose column () equilibrated with a buffer containing 50 mM TEA, 150 mM KOAc and 3 mM Nikkol BL-8SY. The column was washed with 10 volumes of buffer containing 50 mM TEA, 150 mM KOAc, 3 mM Nikkol BL-8SY, 250 mM sucrose, 1 mM DTT and 1x pI and was eluted with 2 column volumes of a buffer containing 50 mM TEA, 250 mM KOAc, 3 mM Nikkol BL-8SY, 250 mM sucrose, 1 mM DTT and 1x pI.

Generation of peptide sequences

ERp180 was purified as described above through the hydroxylapatite column step. The protein was further purified from the hydroxylapatite eluate by preparative SDS-PAGE on a 7% polyacrylamide gel. The band corresponding to ERp180 was electroeluted using a Schleicher and Schuell elutrap or by the method of Hunkapillar et al. (Hunkapillar *et al.*, 1983b). All subsequent steps were performed by Christopher W. Turck in the Howard Hughes Medical Institute and Department of Medicine, University of California San Fransisco. The eluted protein was cleaved with CNBr or trypsin and was subjected to reverse phase HPLC. The resolved peptides were sequenced by automated Edman degradation using an Applied Biosystems gas phase sequencer (Hunkapillar *et al.*, 1983a).

Carbonate extraction

50 µl of mKRMs or pKRMs at a concentration of 3 eq/µl were diluted with 2.45 ml of 0.1 M sodium carbonate, pH 11.2 and incubated at 0 ^{o}C for 30 min. The suspension was underlayered with a sucrose cushion (500 mM sucrose and 0.1 M sodium carbonate, pH 11.2) and centrifuged at 100,000 rpm for 1.5 h in a Beckman

TLA 100.3 rotor. The pellet fractions were resuspended in 25 µl of a buffer containing 50 mM TEA, 500 mM KOAc and 1x pI. The supernatant and an aliquot of the pellet fraction was saved for analysis by SDS-PAGE and Western blotting.

The remainder of the pellet fraction from the mKRMs was diluted with 175 µl of a buffer containing 50 mM TEA, 500 mM KOAc, 2 M sucrose and 1x pI and was transferred to a tube for the Beckman TLA 100.2 rotor. The suspension was overlayered first with 500 µl of a buffer containing 50 mM TEA, 500 mM KOAc, 1.5 M sucrose and 1x pI and then with 100 µl of the same buffer containing 100 mM sucrose. The samples were centrifuged at 70,000 rpm for 1 h in a Beckman TLA 100.2 rotor. The supernatant and membrane fractions were collected, and the membrane fraction was pelleted by centrifugation at 70,000 rpm for 30 min in a TLA 100.2 rotor and was then resuspended in a buffer containing 50 mM TEA, 250 mM sucrose and 1 mM DTT. The membrane and supernatant fractions were saved for analysis by SDS-PAGE and Western blotting.

Crosslinking with Disuccinimidyl suberate (DSS)

Purified ERp180 was at a concentration of 40 nM in a buffer containing 30 mM TEA, 150 mM KOAc, 150 mM sucrose, 1.8 mM Nikkol BL-8SY, 0.6 mM DTT and 0.6x pI. DSS was added from a 17.2 mM stock solution to a final concentration of 0.5 mM. The reactions were incubated for 0 min or 3 min at room temperature and were quenched with 1/2 volume of 1 M NH_4OAc, pH 7.5. The samples were TCA precipitated and prepared for analysis by SDS-PAGE on a 5% polyacrylamide gel and Western blotting.

Microsomal membranes were in a buffer containing 50 mM TEA, 125 mM sucrose, 0.5 mM DTT and 150 mM KOAc at a concentration of 0.1 eq/µl. DSS was

added to a final concentration of 1 mM. The reactions were incubated at RT for 0 min or 3 min and were quenched and processed as described above.

Preparation of ribosomal subunits

Ribosomal subunits from canine pancreas were gradient purified as previously described (Blobel and Sabatini, 1971). Gradient purified subunits were diluted with an equal volume of a buffer containing 20 mM Tris-HCl, pH 7.5, 500 mM KCl and 1.5 mM $MgCl_2$ and were pelleted by centrifugation in a Beckman TLA100.3 rotor for 3.5 h at 60,000 rpm. The pelleted subunits were resuspended in H_20 as concentrated stocks.

ERp180 binding to ribosomal subunits

ERp180 at a final concentration of 25 nM was incubated alone or with 60 S or 40 S ribosomal subunits in a buffer containing 50 mM TEA, 100 mM KOAc, 100 mM sucrose, 5 mM $Mg(OAc)_2$, 1.2 mM Nikkol BL-8SY and 1 mM DTT. The ribosomal subunits were at a concentration of 40 nM. The binding reactions proceeded for 10 min at 0 ^oC, and the samples were centrifuged for 20 min at 4 ^oC in a Beckman TLA100 rotor. The supernatant and pellet fractions were prepared for analysis by SDS-PAGE.

Western blot analysis

Western blotting was performed as described (Fisher *et al.*, 1982) except that where noted the primary antibodies were detected using the enhanced chemiluminescent Western blotting detection system (Amersham) as described (Zimmerman and Walter, 1991). Immunoselected antibodies raised against ERp180 were used at a dilution of 1:200.

ConA blots

[^{14}C]ConA blots were done as previously described (Zimmerman and Walter, 1990).

RESULTS

Identification of a potential ribosome binding protein from the ER

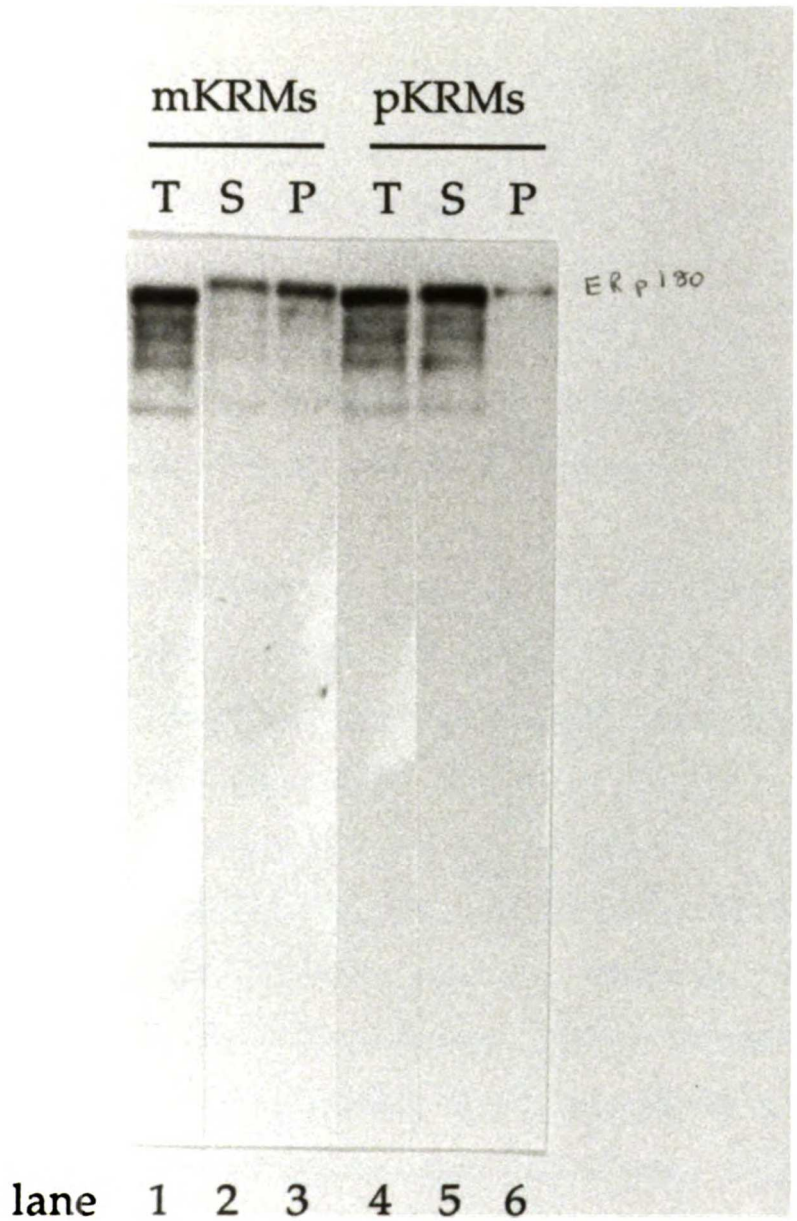
Many ER membrane proteins remain insoluble when microsomal membranes are treated with high concentrations of nonionic detergent under physiological salt conditions (Zimmerman and Walter, 1990). Insolubility is conditionally defined as pelleting after centrifugation at 100,000 x g for 1 hour, conditions under which ribosomes will pellet. Thus, it is possible that some ER proteins remain insoluble because they are tightly associated with ribosomes. Such proteins should then be soluble in the absence of ribosomes. In order to identify potential ribosome binding proteins based on such selective solubility, we compared the profile of membrane proteins which are insoluble after detergent treatment of rough microsomal membranes with that of ribosome stripped microsomes (data not shown).

By Comassie staining we identified one prominent 180 kD protein that was selectively solubilized in the absence of ribosomes (data not shown). Western blotting with antibodies raised against this protein confirmed that a significant amount of the 180 kD band was found in the pellet fraction after solubilization and fractionation of membranes which still had ribosomes specifically bound to them (Fig 1, compare lanes 2 and 3 with lane 1). However, when the membranes were first

treated with puromycin to remove the ribosomes, ERp180 fractionated mostly with the supernatant after solubilization and centrifugation (Fig. 1, compare lanes 5 and 6 with lane 4). Thus, ERp180 demonstrates selective solubility depending on the presence of ribosomes and is a potential candidate for a ribosome binding protein in the ER membrane.

Figure 1. ERp180 is selectively soluble.

mKRMs (lane 1) or pKRMs (lane 4) were solubilized as described (see Experimental Procedures) and were separated into supernatant (lanes 2 and 5) and pellet (lanes 3 and 6) fractions. Equivalent aliquots of total membranes (T) or supernatant (S) and pellet (P) fractions were prepared for Western blotting with immunoselected rabbit serum raised against ERp180.


mKRMs
pKRMs
T S P T S P
ERp180
lane 1 2 3 4 5 6

ERp180 is specifically localized to the ER membrane

The previous experiment demonstrates that ERp180 is associated either directly or indirectly with membrane bound ribosomes. In order to confirm the ER localization of ERp180, cell fractions derived from homogenized canine pancreas were analyzed by Western blotting for the presence of ERp180 (Fig. 2A). Thus, of the total homogenization extract (Fig. 2A, lane 1) most of ERp180 fractionated with the ER (Fig. 2A, lane 5). A small amount was recovered with a nuclear fraction, as is expected, since the outer nuclear membrane is contiguous with the ER (Fig. 2A, lane 2), and a minority fractionated with the light membranes which are probably contaminated with ER (Fig. 2A, lane 3). Most notably, no ERp180 fractionated with the cytoplasm (Fig. 2A, lane 4) as would be expected if the protein bound to all cellular ribosomes.

During the cell fractionation procedure, some polysomes which are not specifically engaged with the ER fortuitously cofractionate with microsomal vesicles. To rule out the possibility that ERp180 is associated these polysomes, microsomes were further extracted under conditions which will remove unbound polysomes (Fig. 2B). Thus, microsomes were extracted under high salt conditions, and the membranes were collected by centrifugation onto a dense sucrose cushion. Analysis of each fraction by SDS-PAGE and Commassie staining confirmed that salt-extracted proteins fractionated with the supernatant, microsome associated proteins fractionated with the membranes at the interface between the supernatant and the cushion and unbound polysomes and polysome associated proteins fractionated in the cushion (data not shown). Western blot analysis demonstrated that ERp180 fractionated exclusively with the membranes under these conditions, and thus, is likely to be a bona fide ER protein (Fig. 2B, compare lane 2 with lanes 1 and 3).

ERp180 remained membrane associated when the remaining salt-resistant ribosomes were stripped from the membrane by treatment with either high salt and EDTA (Fig. 2B, compare lane 5 with lanes 4 and 6) or high salt and puromycin (Fig. 2B, compare lane 8 with lanes 7 and 9). High salt and EDTA treatment disrupts the association between large and small ribosomal subunits such that the small ribosomal subunit becomes disengaged and stripped from the membrane; whereas, puromycin and high salt treatment causes the disengagement of the nascent chain from the translational machinery and leads to the release of both subunits from the membrane (Blobel and Sabatini, 1971). Since, ERp180 was not extracted by either of these treatments, its association with the ER membrane is independent of the presence of ribosomes. Moreover, since ERp180 is resistant to all the above treatments, it must be either an integral membrane protein or a tightly associated peripheral membrane protein.

Figure 2. ERp180 is tightly associated with the ER membrane.

(A) Homogenized canine pancreas (lane 1) was separated into nuclear (N) (lane 2), light membrane (LM) (lane 3), cytoplasmic (C) (lane 4) and ER (lane 5) fractions according to standard procedures (see Experimental Procedures). Equivalent aliquots of each fraction were prepared for analysis by Western blotting with immunoselected rabbit serum raised against ERp180. (B) RMs were extracted by treatment with buffer containing 500 mM KOAc (lanes 1 - 3), 500 mM KOAc and 25 mM EDTA (lanes 4 - 6) or 500 mM KOAc and 2 mM puromycin (lanes 7 - 9). The extracts were separated into supernatant (S) (lanes 1, 4 and 7), membrane (M) (lanes 2, 5, and 8) and cushion (C) (lanes 3, 6 and 9) fractions by centrifugation (see Experimental Procedures), and equivalent aliquots of each fraction were prepared for analysis by Western blotting with immunoselected rabbit serum raised against ERp180.

A. T N LM C ER

Lane 1 2 3 4 5

B.

500 mM	500 mM	500 mM	KOAc
–	25 mM	–	EDTA
–	–	2 mM	puromycin

Lane 1 2 3 4 5 6 7 8 9

ERp180 does not fractionate as a bona fide integral membrane protein

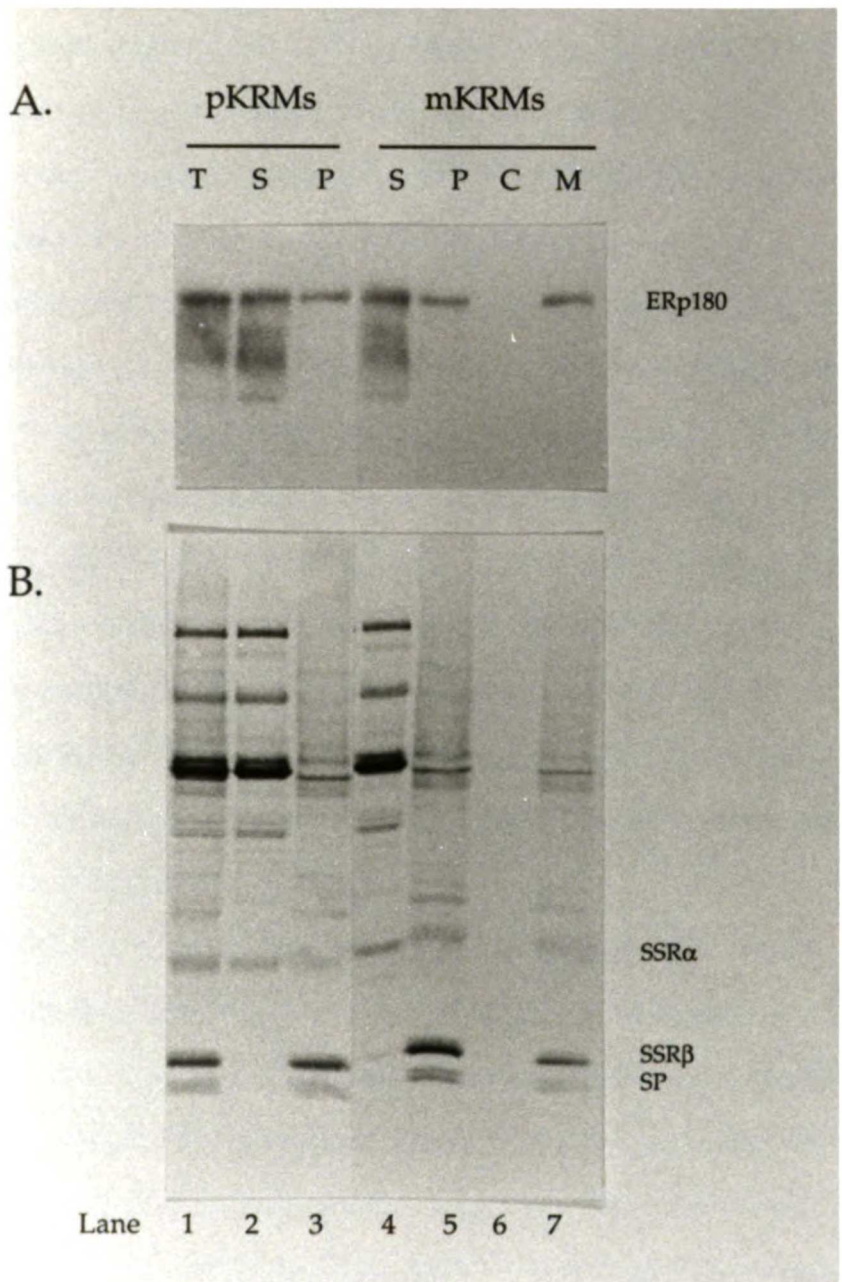
The ribosome binding site(s) which can be assayed for on microsomal membranes behaves as an integral membrane protein by classical biochemical criteria (Nunnari, J. and Walter,P., unpublished). Thus, to determine whether ERp180 is a peripheral or an integral membrane protein we treated microsomal membranes with a high pH carbonate buffer. This treatment results in the extraction of peripheral membrane proteins, while integral membrane proteins remain associated with the membrane after extraction (Fujiki *et al.*, 1982). Microsomes were diluted into an excess of carbonate buffer, and the suspension was separated into a supernatant (S) and pellet (P) fraction by centrifugation. Figure 3 shows that approximately 60% of ERp180 fractionated with the supernatant, while only 40% fractionated with the membrane (Fig. 3A, compare lanes 4 and 5 to lane 1). In contrast, signal peptidase and both subunits of SSR fractionated exclusively with the membrane as is expected for integral membrane proteins (Fig. 3B, lanes 4 and 5).

If ERp180 is tightly associated with ribosomes in the membrane then it might be pulled out of the membrane when the large hydrophilic ribosomes are stripped off the membrane by this procedure. To rule out this possibility, carbonate extraction was performed on microsomal membranes which had been previously stripped of ribosomes by treatment with puromycin (Fig. 3, lanes 1 - 3). As shown in figure 3, the same amount of ERp180 was extracted from the membrane when no ribosomes were present on the starting membranes (Fig. 3, compare lanes 2 and 3 to lanes 4 and 5). Thus, ERp180 apparently does not behave like a classical integral membrane protein as was suggested by Savitz and Meyer (Savitz and Meyer, 1990). Savitz and Meyer concluded that ERp180 is an integral membrane protein by doing a similar extraction; however, they analyzed only the pellet fraction and did not account for the 60% of the protein which is extacted by carbonte treatment.

Since ERp180 does not fractionate exclusively with the supernatant after carbonate extraction, it was important to determine whether the 40% of ERp180 that fractionated with the membrane was actually associated with the lipid bilayer and did not pellet with the membranes simply because it was insoluble. Thus, the carbonate extracted membranes (Fig. 3, lane 5) were resuspended in a buffer containing 2 M sucrose and were floated by centrifugation to an interface of buffer containing 1.5 M sucrose. The floated membranes were collected from the sucrose interface (Fig. 3, lane 7), and the supernatant and 2 M sucrose fractions were pooled (Fig. 3, lane 6). All of ERp180 which pelleted with the membranes after carbonate extraction subsequently floated with the membranes (Fig. 3A, lane 7); thus, 40% of ERp180 behaves as expected for an integral membrane protein. When carbonate extracted membranes are isolated and are reextracted with carbonate buffer all of ERp180 which had been associated with the membrane remains associated with the membrane, indicating that the association of 40% of ERp180 with the mmembrane is stable (data not shown). These results suggests that some of ERp180, though not an integral membrane protein itself, is tightly associated with an integral membrane protein in the ER. Alternatively, a population of ERp180 might be anchored to the membrane by a post-translational modification.

Figure 3. ERp180 does not fractionate as a bona fide integral membrane protein.

(A) mKRMs (lanes 4 - 7) or pKRMs (lanes 1 - 3) were diluted into carbonate buffer as described (see Experimental Procedures) and were separated into supernatant (S) and pellet (P) fractions by centrifugation. The remainder of the pellet fraction from the mKRMs was resuspended in a buffer containing 2 M sucrose and was separated into a membrane (M) (lane 7) and cushion (C) fraction (lane 6) by centrifugation as described (see Experimental Procedures). Aliquots of each fraction were prepared for analysis by Western blotting (A) and for blotting with [^{14}C]ConA (B). Signal peptidase (SP) and both subunits of SSR (SSRα and SSRβ).


A.
pKRMs
mKRMs
T S P S P C M
ERp180
B.
SSRα
SSRβ
SP
Lane 1 2 3 4 5 6 7

Protein sequencing reveals some homology to myosin heavy chain

In order to obtain protein sequence information for ERp180, the purified protein was subjected to proteolysis with either trypsin or CNBr, and five HPLC purified peptides were sequenced by automated Edman degradation. The amino acid sequences are shown in figure 4. Four of the peptide sequences share no significant homology to any known protein sequences. However, peptide 1 shows striking homology to myosin heavy chain in the carboxy terminus.

Myosin heavy chains typically form homodimers which assemble into hexomeric complexes with two light chains. The heavy chains which are about 230 kD each consist of two globular heads joined by an extended alpha helical coiled-coil tail. The head structures, formed from the amino terminal half of each subunit have the actin binding sites and actin-activated ATPase activity, while the carboxy-terminal halves of the heavy chains assemble into the filamentous coiled-coil rod structure (Warrick and Spudich, 1987). Thus, peptide 1 is homologous to myosin in the filamentous tail indicating that ERp180 might have the coiled-coil structural motif characteristic of myosin and other intermediate filaments (Bourne, 1991; McKeon *et al.*, 1986; Traub and Piez, 1971).

The coiled-coil motif is characterized by long proline-free stretches of heptad repeats, in which the side chains of the first and fourth amino acid residues are hydrophobic. This pattern of side chains can form uninterrupted stretches of α helices with a hydrophobic streak on one side of the helix, and these helices can then form coiled-coil structures with similar α helices. Thus, coiled-coil proteins are characterized as having the ability to form rod-like homodimeric complexes.

Figure 4. Peptide sequence analysis of ERp180.

(A) The amino acid sequences derived from HPLC purified trypsin and CNBr generated peptide fragments of ERp180 are shown in one letter amino acid code. X denotes places where the amino acid could not be determined, and a slash between amino acids indicates that at that position it was not possible to discriminate between the two amino acids indicated. (B) The homology of peptide 4 to rabbit myosin heavy chain (amino acids 1801 to 1817) is shown (":" denotes identity and "." denotes similarity according to the Dayhoff rules). Note that there is 68.8% identity over a 16 aa overlap. Over the sequence of myosin hydrophobic residues (h) conforming to the coiled-coil motif are shaded.

A.

Peptide 1	E	S/V	A	P	N	Q	G	K								
Peptide 2	E	Q	E	I	T	A	V/N	Q	A	K						
Peptide 3	E	A	N/T	L	E	D	S	Q	X	L	R					
Peptide 4	A	E	L	D	S	K	L	Q	X	X	E	A	E	V	K	S	K
Peptide 5	V	Q	V	P	P	V	A	A	K	A	G	X	P	A		

B.

Coiled-Coil Motif			*h*	*x*	*x*	*x*	*h*	*x*	*x*	*h*	*x*	*x*	*x*	*h*			
Rab. Myosin HC	K	E	L	K	S	K	L	Q	E	M	E	G	A	V	K	S	K
		:	:	.	:	:	:	:			:	.	.	:	:	:	:
Peptide 4	A	E	L	D	S	K	L	Q	X	X	E	A	E	V	K	S	K

Purified ERp180 is a dimer with an elongated structure

The amino acid homology between ERp180 and myosin indicated that ERp180 might be a member of the growing family of proteins with the coiled-coil motif. If ERp180 were a member of this family then it should exist as a dimer with a highly elongated structure. To test whether ERp180 exists as a dimer we used the bifunctional crosslinking reagent DSS to determine the form of the purified protein.

When purified ERp180 (Fig. 5A, lane 2) was crosslinked with DSS all of it was crosslinked as expected for a homodimer (Fig. 5B). Thus, in the absence of DSS, ERp180 migrated as 180 kD on a 5% denaturing SDS-PAGE gel (Fig. 5B, lane 3). The same was true when DSS was added and immediately quenched by addition of excess ammonium acetate (Fig. 5B, lane 4). However, after crosslinking with activated DSS, all of ERp180 migrated on an SDS gel with an apparent molecular weight expected for a dimer (Fig 5B, lane 5). The same crosslinked products appeared when the salt was raised to 250 mM or 500 mM KOAc (Fig. 5, lanes 6 and 7). Thus, the dimer form of the purified protein behaves as a stable structure.

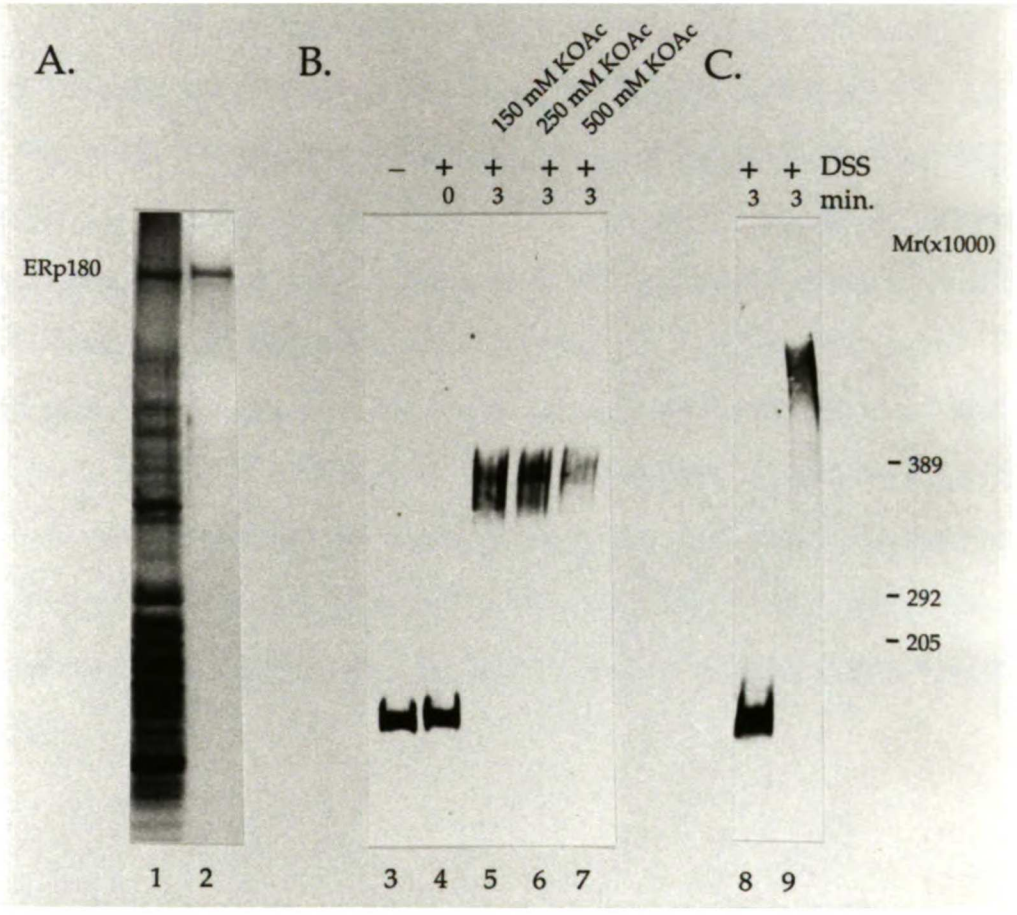
In order to determine the structure of native ERp180 in the membrane, intact microsomes (Fig. 5A, lane 1) were treated with DSS, and the crosslinked form of ERp180 was analyzed by Western blotting (Fig. 5C). Thus, crosslinking of the native protein resulted in a crosslinked product with a molecular weight greater than that of a dimer (Fig. 5C, lane 9). There are two possible explanations for this result. In microsomal membranes ERp180 either exists in a higher order form or is tightly associated with one or more other microsomal proteins.

The expected molecular weight for a homodimer of ERp180 is approximately 360 kD. A globular protein of this molecular weight would be expected to migrate on a sucrose gradient with a sedimentation coefficient of approximately 15 S. However, ERp180 migrates with an apparent sedimentation coefficient of approximately 6 S

indicating that it has an elongated structure (data not shown). Moreover, preliminary EM analysis confirmed that ERp180 has an elongated structure (data not shown).

Figure 5. Purified ERp180 is a dimer.

(A) EKRMs (lane 1) or purified ERp180 (lane 2) were substrates for crosslinking by DSS in (B) and (C). (B) ERp180 was incubated in the absence (lane 3) or presence (lanes 4 - 7) of 0.5 mM DSS as described in Experimental Procedures. The samples were prepared for analysis by Western blotting with immunoselected rabbit serum raised against ERp180. (C) EKRMs were incubated in the absence (lane 8) or presence of 1 mM DSS (lane 9). The protein standards (Mr) indicated apply to panels (B) and (C).

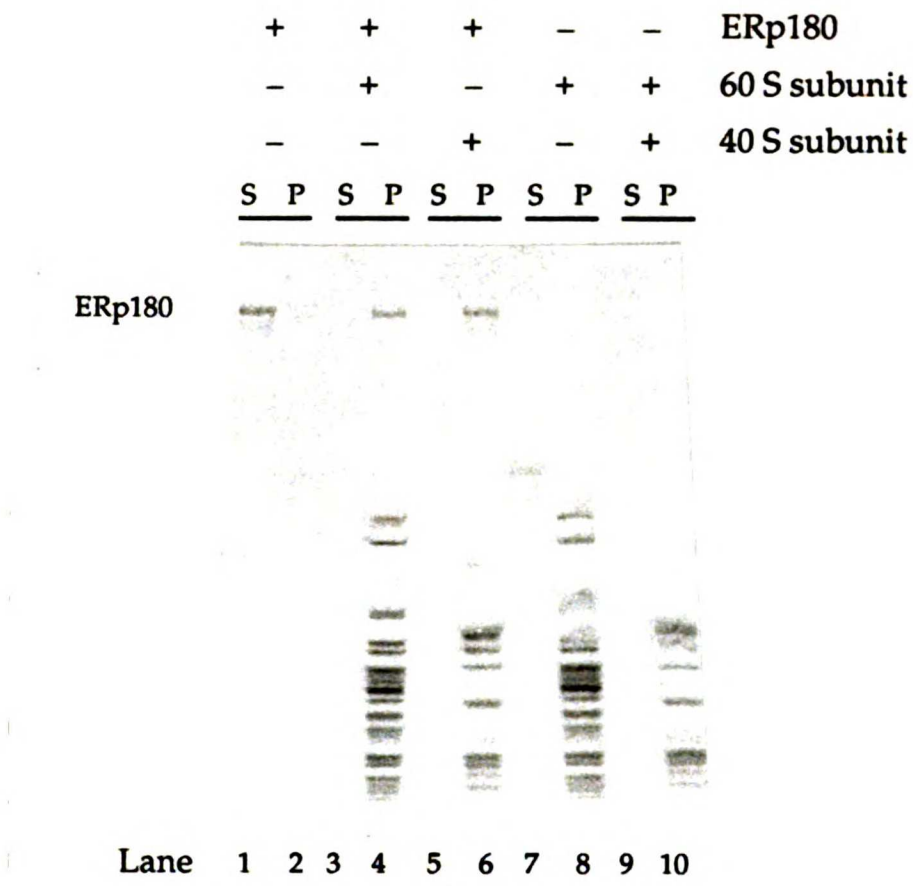

A.
B.
C.
150 mM KOAc
250 mM KOAc
500 mM KOAc
– + + + +
0 3 3 3
+ + DSS
3 3 min.
ERp180
Mr(x1000)
389
292
205
1 2 3 4 5 6 7 8 9

ERp180 binds to both ribosomal subunits

Ribosome binding to the ER membrane is believed to be mediated by the large ribosomal subunit (Adelman *et al.*, 1973). Thus far, ERp180 has not been shown to bind specifically to either subunit. Moreover, the ribosome preparations typically used for ribosome binding assays utilize crude preparations of ribosomes which are contaminated with nonribosomal proteins. In order to demonstrate that ERp180 binds directly to ribosomal proteins, we have tested its binding with purified ribosomal subunits. Thus, ERp180 was incubated with purified 60 S and 40 S ribosomal subunits and the suspensions were centrifuged under conditions where the ribosomal subunits will pellet (Fig. 6, lanes 8 and 10) but ERp180 alone will not (Fig. 6, lane 2). If ERp180 binds tightly to either subunit, it will fractionate in the pellet when that subunit is present. After incubation with an approximately 2 fold molar excess of either 40 S or 60 S ribosomal subunits, ERp180 was recovered in the pellet fraction with the ribosomal proteins (Fig 6, lanes 4 and 6). The salt sensitivity of binding to both subunits is apparently indistinguishable (data not shown). Thus, it remains to be determined whether the binding interaction between ribosomes and ERp180 is specific.

Figure 6. ERp180 binds to both 40 S and 60 S ribosomal subunits.

Samples which contained purified ERp180 (lanes 1 - 6) in the absence (lanes 1 and 2) or presence of either 60 S ribosomal subunits (lanes 3 and 4) or 40 S ribosomal subunits (lanes 5 and 6) were separated into supernatant (S) and pellet (P) fractions after centrifugation and were analyzed by SDS-PAGE. Control samples which contained 60 S subunits alone (lanes 7 and 8) or 40 S subunits alone (lanes 9 and 10) are shown. All samples were prepared for analysis by SDS-PAGE and coomassie staining.


+ + + – – ERp180
– + – + + 60 S subunit
– – + – + 40 S subunit
S P S P S P S P S P
ERp180
Lane 1 2 3 4 5 6 7 8 9 10

DISCUSSION

We have identified an ER protein, termed ERp180, which binds efficiently to ribosomes. ERp180 was identified as a potential ribosome binding protein, because under physiological salt conditions it could be solubilized from microsomal membranes stripped of ribosomes by the addition of puromycin. This type of selective solubility might be expected for membrane proteins which bind either directly or indirectly to ribosomes. ERp180 was independently identified by Savitz and Meyer as a potential ribosome receptor in the ER, because soluble, proteolytic fragments derived from ERp180 could inhibit ribosomes from binding to the membrane (Savitz and Meyer, 1990). However, they never demonstrate that ERp180 binds directly to purified ribosomes.

Our findings are the first demonstration that ERp180 does bind directly to ribosomes. Moreover, we have demonstrated that the purified protein binds efficiently to both 40 S and 60 S ribosomal subunits. Thus, it is possible that ERp180 binds in the membrane to both subunits of the ribosome. Alternatively, ERp180 might bind to the RNA or there is some nonspecific component contributing to the binding that we observe. However, taking into consideration that the concentration of ERp180 on the membrane is approximately equimolar with that of membrane bound ribosomes (Savitz and Meyer, 1990) and that ERp180 has been shown to be in close proximity to ribosomes on the membrane (Collins and Gilmore, 1991), it is likely that ribosome binding is a biologically relevant function of ERp180. It is possible, for example, that in the context of the membrane ribosome binding to ERp180 is modulated by other factors or by its association with the membrane. In this regard we have observed by crosslinking that the purified protein which exists

as a dimer differs from the membrane bound form which migrates on an SDS-PAGE gel as a higher molecular weight complex after crosslinking.

ERp180 was identified by us as a potential ribosome binding protein using biochemical criteria. Thus far, there is no functional assay for the role of ERp180. Savitz and Meyer demonstrated that proteolytic fragments of ERp180 can inhibit ribosomes from binding to the endogenous ribosome binding sites of the ER membrane, but they do not demonstrate that ERp180 in the membrane contributes to the endogenous binding sites (Savitz and Meyer, 1990). Moreover, we have shown that ERp180 can be fractionated away from these sites after solubilization and reconstitution over microsomal vesicles (Nunnari *et al.*, 1991). Partial protein sequencing revealed that the protein is homologous to some extent to myosin in the tail region and thus might have regions with a coiled-coil structural motif. Thus, it is interesting to speculate that ERp180 may play a structural role in maintaining the organization or the ER. Consistent with this idea are our observations that ERp180 is purified as a homodimer, and appears to have a highly elongated shape.

Electron microscopy of cell sections has revealed that ribosomes are not randomly distributed on the RER membrane (Palade, 1975). Thus, there could easily be at least two types of ribosome-binding proteins associated with the ER membrane, those that anchor the ribosome to the membrane and those that organize ribosomes on the membrane. The first class of ribosome-binding proteins are expected to be part of the "translocon" which facilitates the vectorial movement of the nascent chain across the membrane, while the second class of ribosome-binding proteins is expected to be extrinsic to the translocon and probably not required for the translocation process per se. It is interesting to speculate that ERp180 is a member of this second class of ribosome binding proteins. This would be consistent with our previous finding that ERp180 does not account for the ribosome binding sites which can be quantitatively assayed for on microsomal membranes, as these binding sites

are expected to be of the first class of ribosome receptors (Nunnari *et al.*, 1991). In addition, we have previously demonstrated that this protein is probably not required for protein translocation (Zimmerman and Walter, 1991).

Interestingly the ribophorins have characteristics of intermediate filament proteins, and thus are thought to have a role in maintaining the structure of the ER (Harnik-Ort *et al.*, 1987). However, it has recently been shown that these proteins have the oligosaccharyl transferase activity which catalyzes the N-linked glycosylation of asparagine residues on nascent polypeptide chains in the ER lumen (Kelleher *et al.*, 1992). Thus, there is precedence for ER membrane proteins to have characteristics of structural proteins as well as defined activities in ER function. Thus, ERp180 may ultimately prove to have an important functional role in protein targeting and translocation. For example, it might be involved in the regulation of polysome assembly and maintenance on the membrane. Future studies on the function of ERp180 should prove valuable in understanding the structure of the ER membrane both and may provide a link between the structural organization of the ER and the functional organization of the translocation machinery.

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