

180-kD Ribosome Receptor is Essential for Both Ribosome Binding and Protein Translocation

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Abstract. We have previously isolated a 180-kD ribosome receptor (p180) from mammalian rough ER that, when incorporated into liposomes, bound ribosomes with an affinity similar to intact membranes. To directly assess the contribution of p180 to ribosome binding as well as protein translocation, monoclonal antibodies were used to selectively deplete p180 from the detergent extracts of rough ER membranes used in the preparation of translocation-competent proteoliposomes. Proteoliposomes prepared from p180-depleted extracts showed a reduction in ribosome binding to the level of trypsin-inactivated controls as well as a loss in

their ability to cotranslationally translocate two different secretory protein precursors. When purified p180 was added back to depleted extracts before proteoliposome formation, both ribosome binding and translocation activity were restored. In addition, the monoclonal antibodies, as well as their Fab fragments, were able to inhibit ribosome binding and protein translocation when bound to intact rough microsomes. These data provide direct evidence that the 180-kD ribosome receptor is essential for ribosome binding and for the translocation of nascent proteins across the membrane of the rough ER.

THE translocation of nascent secretory proteins across the membrane of the rough ER represents the initial step of a major intracellular route of protein traffic, the secretory pathway (Palade, 1975). According to current models, translocation in mammalian systems occurs cotranslationally via a series of sequential interactions: the recognition of the signal sequence on the nascent polypeptide, the docking of the nascent polypeptide-ribosomal complex to the ER membrane, the attachment of the ribosome to the membrane, and the transport of the nascent chain to the lumen of the rough ER via a proteinaceous channel or pore (Meyer, 1991). Both cytosolic and membrane proteins have been identified that participate in this process *in vitro*. These include the signal recognition particle (SRP)¹ (Walter et al., 1981); its receptor in the ER membrane—the docking protein (Meyer et al., 1982b; Gilmore et al., 1982); and a “translocating chain-associating membrane protein” (TRAM) (Görllich, et al., 1992). Models of mammalian translocation have consistently postulated the presence of a binding factor or receptor that serves to anchor the ribosomes to the membrane during the translocation process (Blobel and Sabatini, 1971; Blobel and Dobberstein, 1975; Hortsch and Meyer, 1984; Walter et al., 1984). Since the pioneering studies of

Borgese et al. (1974), it has been known that ribosome binding *in vitro* is a salt-labile, saturable process, mediated by a proteinaceous receptor. Recently many groups have been involved in characterizing the ribosome binding reaction (Savitz and Meyer, 1990; Tazawa et al., 1991; Nunnari et al., 1991; Collins and Gilmore, 1991), and two receptor proteins have been identified (Savitz and Meyer, 1990; Ichimura et al., 1992).

One of these receptors (referred to as p180), is an abundant, rough ER-specific integral membrane protein of 180 kD apparent mol wt that, when incorporated into artificial lipid vesicles, binds ribosomes with an affinity similar to intact membranes (Savitz and Meyer, 1990). However, such data do not preclude a role for other proteins in the ribosome binding process, nor do they indicate that this or other ribosome receptors are required for translocation. With these two questions in mind, we designed experiments to directly assess the contribution of the 180-kD receptor to ribosome binding and to simultaneously test its role in translocation.

Nicchitta and Blobel (1990) have developed an assay for studying translocation into proteoliposomes derived from detergent-solubilized membrane components. Importantly, such proteoliposomes incorporate the entire repertoire of rough ER membrane proteins into bilayers of endogenous, not heterologous or synthetic, lipids. In such an assay, the participation of individual membrane proteins in translocation was determined by their depletion and/or readdition to the extracts used to form these translocation-competent proteoliposomes (Nicchitta et al., 1991; Migliaccio et al., 1992;

1. *Abbreviations used in this paper:* DP, docking protein; PC, phosphatidyl choline; PS, phosphatidyl serine; RM, rough microsomes; SRP, signal recognition particle; SSR, signal sequence receptor; TRAM, translocating chain-associating membrane protein.

Görlich et al., 1992). For example, antibodies were used to specifically deplete extracts of the docking protein, demonstrating its absolute requirement for translocation. In the same study, it was found that the signal sequence receptor protein was dispensable for reconstitution of translocation competence (Migliaccio et al., 1992). This reconstituted system is therefore ideal for studying ribosome binding and translocation since an individual component can be removed without altering the levels of the other membrane proteins.

We report here that monoclonal antibodies generated against p180 effectively and selectively depleted p180 from detergent extracts of rough microsomes. Proteoliposomes prepared from the depleted extracts were virtually unable to bind ribosomes or to translocate nascent secretory proteins. Re-addition of the 180-kD receptor to p180-depleted proteoliposomes restored both ribosome binding and translocation activity. Additionally, these monoclonals were able to bind to intact rough microsomes, profoundly diminishing both their capacity to bind ribosomes and to translocate nascent secretory protein precursors *in vitro*.

Materials and Methods

Ribosome Binding Assay

Rough microsomes (RM) were prepared from canine pancreas by the method of Blobel and Dobberstein (1975). Ribosomes were removed from RM by two rounds of treatment with 1 mM puromycin, 15 U/ml micrococcal nuclease, 500 mM KOAc, 50 mM Tris-HCl, pH 7.5, 5 mM Mg(OAc)₂, and 1 mM CaCl₂ at 24°C for 30 min (Adelman et al., 1973). After treatment, the stripped RM (RM_{SN}) were recovered by centrifugation at 100,000 g through a cushion of 500 mM KOAc, 50 mM Tris-HCl, pH 7.5, 5 mM Mg(OAc)₂, and 500 mM sucrose, and were resuspended in 0.25 M sucrose LSB (25 mM KOAc, 50 mM Tris-HCl, pH 7.5, 5 mM Mg(OAc)₂).

Tritiated ribosomes were prepared from [5,6-³H]uridine-labeled HeLa cells according to the method of Kreibich et al. (1983) and were resuspended in 0.25 M sucrose LSB. Binding assays were performed by mixing microsomes or liposomes with ribosomes in 30 μl of 0.25 M sucrose LSB at 0°C for 10 min (Borgese et al., 1974). Next, 300 μl of 2.3 M sucrose LSB was added to the assay, and steps of 2.3 ml of 1.9 M, 2.0 ml of 1.5 M, and 0.4 ml of 0.25 M sucrose LSB were overlaid.

After centrifugation at 50,000 rpm 2 h, 4°C in an SW55 rotor (all rotors are from Beckman Instruments, Fullerton, CA), the top three ml were taken as the bound fraction; the remainder of the gradient and a 1 ml water wash of the centrifuge tube were taken as the unbound fractions. These fractions were diluted with an equal volume of 0.25 mg/ml BSA and precipitated with 10% TCA. Precipitated material was collected by filtration onto glass fiber filters and counted in a liquid scintillation counter.

Trypsin-digested RM (low controls) were prepared by digestion with trypsin (10–50 μg/ml) for 30 min at 0°C. The reaction was stopped with 100 μg/ml soy bean trypsin inhibitor and 500 μM PMSF. The membranes were recovered by centrifugation through a cushion 0.5 M sucrose LSB, 220 μM PMSF, and 10 μg/ml soy bean trypsin inhibitor. The membranes were resuspended to their initial volume with 0.25 M sucrose LSB (Hortsch et al., 1986).

For ribosome binding to antibody-treated RM, the RM_{SN} were incubated with IgG or Fab' (purified as described below) in 0.25 M sucrose LSB for 30 min at 0°C before the addition of ribosomes. The remainder of the assay was performed as described above.

Cell-free Protein Synthesis and Translocation Assay

mRNA encoding preprolactin (ppL) was synthesized *in vitro* by T7 RNA polymerase from the plasmid pGEMBP1 (Connolly and Gilmore, 1986; the generous gift of Christopher Nicchitta, Rockefeller University, NY). mRNA encoding nonglycosylated prepro-α-factor (ppαf) was transcribed using Sp6 RNA polymerase (Promega Corp., Madison, WI) from the plasmid pSP64αfΔCHO. *In vitro* translation/translocation reactions (Meyer and Dobberstein, 1980) contained 6 μl nuclease-treated reticulocyte lysate (Promega Corp.), 3 μl compensation buffer (470 mM KOAc, 2.5 mM

Mg(OAc)₂, 0.5 μl amino acids (1 mM) without methionine, 0.5 μl 0.05% Nikkol (Nikko Chemicals, Tokyo, Japan), 0.25 μl 100 mM ATP, 2 μl ³⁵S-methionine (New England Nuclear, Boston, MA), microsomes or liposomes (0.5–5 μl), and H₂O to 25 μl. After 40 min at 25°C, the reactions were transferred to ice, and 12.5 μl was mixed with 4 μl 2 mg/ml proteinase K for 60 min at 0°C. To stop the proteolysis, 1.2 μl 110 mM PMSF was added for 5 min at 0°C. An equal volume of sample buffer was added, and the samples were heated at 95°C for 4 min after which 1/6 volume of 500 mM iodoacetamide was added. Samples were separated on 14% SDS-PAGE gels which were subjected to fluorography as previously described (Hortsch et al., 1986) and to direct radioanalytical analysis with an AMBIS Radioanalytic Imaging System (Automated Microbiology Systems, San Diego, CA).

For translocation assays carried out on antibody-treated membranes, the purified antibodies or Fab' (see below) were incubated with RM_{EX} (EDTA- and salt washed-microsomes) in 0.25 M sucrose LSB for 15 min at 25°C. A 300-μl cushion of 0.5 M sucrose LSB was underlaid, and the membranes were pelleted at 100,000 g for 40 min at 4°C. Microsomes were resuspended in 0.25 M sucrose LSB to their original volume. To determine antibody binding, an aliquot of each sample was separated on a 13% SDS-PAGE gel which was transferred to nitrocellulose for immunoblotting with an anti-mouse secondary antibody. The remainder was used in translocation assays described above.

Construction of Nonglycosylated Prepro-α-factor

The plasmid pSP64-XβM (Krieg and Melton, 1984) was modified by site directed mutagenesis (Kunkel et al., 1987) to incorporate an Eco RV site in the vector (at the 3' end of the globin cDNA). The three oligosaccharide-accepting asparagine residues at positions 23, 57, 67 in prepro-α-factor were mutated to glutamines simultaneously using two primers and site-directed mutagenesis (Kunkel et al., 1987). The correct clone was verified by DNA sequencing. Then, PCR was used with the mutated prepro-α-factor cDNA to add an Nco I site to the 5' end of prepro-α-factor. This construct was inserted into the modified pSP64-XβM vector, previously digested with EcoRI and NcoI to excise globin sequences, to form the plasmid pSP64αfΔCHO. The correct clone was isolated and linearized by Eco RI for *in vitro* transcription.

Monoclonals, IgG Purification, and Fab' Preparation

Mouse mAbs were raised against p180, purified as previously described (Savitz and Meyer, 1990). Monoclonal antibodies were generated according to Hortsch et al. (1985) and were screened for reactivity to p180 on immunoblots. Two p180-reactive mAbs from different primary culture wells were isolated. To test for specificity, RM were separated on a 10–15% SDS-polyacrylamide gel and transferred to nitrocellulose. Immunoblots were stained with the monoclonal antibodies followed by secondary anti-mouse antibodies conjugated to alkaline phosphatase. Both mAbs were determined by serological methods to have γ₁ heavy and κ light chains. For the various experiments, the nonimmune control mAb (raised against a cytosolic protein from *Schizosaccharomyces pombe*) was determined not to possess reactivity to any proteins in canine pancreatic microsomes. The docking protein mAb was isolated previously (Hortsch et al., 1985).

To purify IgG, the ascites fluid was precipitated by the slow addition of an equal volume of saturated ammonium sulfate and mixing for 6 h at 4°C. The suspension was centrifuged at 3,000 g for 30 min. The pellet containing the IgG was dissolved in 5 mM sodium phosphate buffer, pH 6.5, and subsequently dialyzed against two changes of 1,000 vol of 5 mM phosphate buffer. The dialysed material was mixed with equilibrated DEAE-Sepharose (Pharmacia, Uppsala, Sweden) for 60 min at 4°C. 2 vol of DEAE-Sepharose were used per vol of ascites fluid. The matrix was pelleted at 500 g, and the unbound material was taken as the purified IgG fraction (Harlow and Lane, 1989).

Fab' fragments were purified from the previously-purified IgG. The antibodies were dialyzed against three changes of 100 mM sodium citrate buffer, pH 3.5. Proteolysis was then carried out by the addition of 5 μg pepsin per mg of IgG and incubating at 37°C for 16 h. The reaction was stopped by adding 0.2 vol of 2 M Tris base and 1/200 vol. 110 mM PMSF. The fragments were separated by the addition of DTT to 1 mM and incubation at 37°C for 10 min, followed by the addition of iodoacetamide to 50 mM (Harlow and Lane, 1989). The purified IgG and Fab' fractions were dialyzed against 1,000 vol of 0.25 M sucrose LSB for use in ribosome binding and translocation experiments. The quantities of the IgGs and Fab's were normalized for protein concentration (A₂₈₀), and their purity was analyzed on 15% SDS-polyacrylamide gels stained with Coomassie blue.

Polyclonal antisera used in these studies were obtained as follows: rabbit anti-p180 was prepared from purified p180 as described by Savitz and Meyer (1990); rabbit anti-docking protein was described in Meyer et al. (1982a); rabbit anti-signal sequence receptor (α and β subunits) were the gift of Tom Rapoport (Max Delbrück Center, Berlin); rabbit antibodies against ribophorins I and II were described in Hortsch and Meyer (1985).

Solubilization of Microsomes

Detergent extracts were produced essentially as described by Migliaccio et al. (1992) with modifications as follows. Rough microsomes were diluted with 3 vol of 500 mM KOAc, 25 mM EDTA, 250 mM sucrose, and centrifuged (80,000 rpm, 20 min, 4°C, TLA 100.2 rotor) to remove the ribosomes. The membranes (RM_{EX}) were resuspended to 80 A₂₈₀ U/ml in solubilization buffer (450 mM KCl, 20 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, 0.5 mM EGTA, 400 mM sucrose). To solubilize the membranes, 900 μ l RM_{EX} were mixed with 67.5 μ l 10% sodium cholate (Calbiochem Corp., La Jolla, CA), 1 μ l 100 mM ATP, 1 μ l 100 mM GTP, 1 μ l 110 mM PMSF, 1 μ l protease inhibitor cocktail (10 mg/ml chymostatin, leupeptin, antipain, pepstatin in DMSO [Sigma Chemical Co., St. Louis, MO]), and 0.8 μ l 500 mM DTT, followed by incubation at 0°C for 15 min with occasional mixing. The extract was centrifuged (75,000 rpm, 20 min, 4°C, TLA 100.2 rotor), and the supernatant was taken as the detergent extract and was stored at -80°C.

Antibody Columns and Depletion of Extracts

Mouse ascites fluid containing the nonimmune mAb (for use in mock depletions), the two anti-p180 mAbs (which were combined), and the anti-docking protein mAb were bound to protein A-agarose (Schleicher and Schuell, Keene, NH) by incubation at 4°C for 6-18 h (Schneider et al., 1982). After washing the matrix with 15 vol of 0.2 M TEA (triethanolamine-HCl, pH 8.2), the matrix was resuspended in 25 mM dimethyl pimelidate (Sigma Chemical Co.) in 0.2 M TEA and incubated at 24°C for 45 min. After centrifugation, the matrix was resuspended in 20 mM ethanalamine, pH 8.2, for 5 min at 24°C. The matrix was sequentially washed with 15 vol of PBS, 15 vol of solubilization buffer, and 5 vol of solubilization buffer containing 0.8% sodium cholate and 1 mg/ml lipids. The lipids were purified from microsomes derived from bovine pancreas according to the method described by Nicchitta et al. (1991).

In depletion studies, 0.8 ml of detergent extract was loaded onto a column containing 1 ml of immunomatrix at a flow rate of 1-2 ml/h. The column was washed in solubilization buffer with 0.8% cholic acid and 1 mg/ml lipids (Migliaccio et al., 1992). Fractions of 0.5 ml were taken, and the three fractions with the highest protein concentration, as determined by Coomassie blue-stained SDS-PAGE gels, were combined. In the case of mock- and p180-depletions the combined fractions were reapplied to the washed column and the fractions with the highest protein concentrations were combined (~750 μ l).

To obtain bound material, columns were washed with 7 vol of solubilization buffer containing 0.8% CHAPS at 5 ml/h, and the bound proteins were eluted with 5 vol of 0.2 M glycine-HCl, pH 2.2, 250 mM KOAc, 10% glycerol, 0.8% CHAPS at 3 ml/h. The eluted fractions were neutralized with 2 M Tris base.

Reconstitution of Translocation into Proteoliposomes

p180 was purified by the method described by Savitz and Meyer (1990). A fraction that eluted from a DEAE-Sepharose column at ~250 mM KOAc in 50 mM Tris-HCl, pH 7.5, 5 mM Mg(OAc)₂, 10% glycerol, 1% octylglucoside (p180 buffer) was used for the readdition experiments and was greater than 95% pure.

For reconstitution, 150 μ l of depleted (or mock-depleted) extract was mixed with 150 μ l p180 buffer. In studies where p180 was restored to depleted extracts, 150 μ l of purified p180 were mixed with 150 μ l of p180-depleted detergent extract. In the case of restoration of an 0.5 \times aliquot, 75 μ l of p180 buffer and 75 μ l of purified p180 were mixed with 150 μ l p180-depleted detergent extract. To form the proteoliposomes, the supplemented detergent extracts were incubated with 200 mg SM2 Bio-beads for 2-4 h at 4°C. The Bio-beads were pretreated with methanol, 2 vol of water, and 2 vol of solubilization buffer (Görllich et al., 1992). The solution was removed from the beads, mixed with an equal volume of 0.6 M KCl, 50 mM Tris-HCl, pH 7.5, and centrifuged (75,000 rpm, 20 min, 4°C, TLA 100.1 rotor). The liposomes were resuspended in 50 μ l 0.25 M sucrose LSB, 2.0 mM DTT, 100 μ M PMSF, and 10 μ g/ml protease inhibitor cocktail. The

trypsin proteoliposomes were produced from detergent extracts of RM_{EX} that had been digested with 50 μ g/ml trypsin for 30 min at 0°C. For ribosome binding and translocation assays, the liposomes were normalized for protein concentration. To analyze their protein composition, proteoliposomes were separated on 10% SDS-PAGE gels which were either directly stained with silver according to Anson (1985) or transferred to nitrocellulose for immunoblotting. The immunoblot was incubated with polyclonal antibodies against both p180 and docking protein, followed by an anti-rabbit secondary antibody conjugated to alkaline phosphatase, and visualized by chemiluminescence (according to the instruction manual of the supplier, Tropix Inc., Bedford, MA).

Results

Monoclonal Anti-p180 Antibodies Bound to Intact Microsomes Inhibit Ribosome Binding and Translocation

To deplete p180 from extracts used to prepare translocation-competent proteoliposomes (see below), we raised anti-p180 monoclonal antibodies. Two IgG-secreting hybridoma lines were found to be specific for p180 by immunoblotting (Fig. 1). To demonstrate their potential use as affinity ligands, we first examined their ability to bind to rough microsomes. Intact canine pancreatic microsomes were incubated with anti-p180 IgG prepared from ascites fluid. In contrast to control mouse IgG, both anti-p180 monoclonals were recovered by centrifugation together with microsomes from the incubation mixtures (Fig. 2 A). This finding not only indicated that the monoclonals would be useful in depletion experiments but provided us with a unique opportunity to investigate the role of p180 in ribosome binding and translocation in intact microsomes.

The effect of anti-p180 antibodies on ribosome binding was determined in a standard *in vitro* assay where ribosomes, radiolabeled *in vivo*, were allowed to bind to ribosome-free (stripped) membranes, followed by reisolation of the membranes by flotation in a sucrose density gradient (Borgese et al., 1974; and Materials and Methods). Before the addition of labeled ribosomes, stripped rough microsomes were first incubated either with buffer, with IgG fractions derived from an irrelevant monoclonal antibody (nonimmune), with an anti-docking protein monoclonal (Hortsch et al., 1985), or with the two anti-p180 monoclonals. As can be seen in Fig. 2 B, ribosomes were bound with high efficiency to membranes treated with buffer, the nonimmune IgG or anti-docking protein IgG. In marked contrast, the treatment of stripped membranes with both anti-p180 IgGs diminished ribosome binding to levels comparable to membranes pretreated with trypsin, a potent inhibitor of ribosome binding *in vitro* (Jothy et al., 1975; Hortsch et al., 1986). The ability of the antibody to diminish ribosome binding to intact membranes is further characterized by the antibody titration curve shown in Fig. 2 C. The fact that the binding of an anti-docking protein monoclonal had no effect on ribosome binding, but did affect translocation (see below), indicates that the mere binding of an antibody to the surface of the microsomal membrane is not sufficient to inhibit ribosome binding.

Despite considerable research into how ribosomes are associated with the rough ER, the involvement of ribosome binding or of a ribosome binding protein in the translocation process has never been directly demonstrated (Kreibich and Sabatini, 1992). The inactivation of ribosome binding by

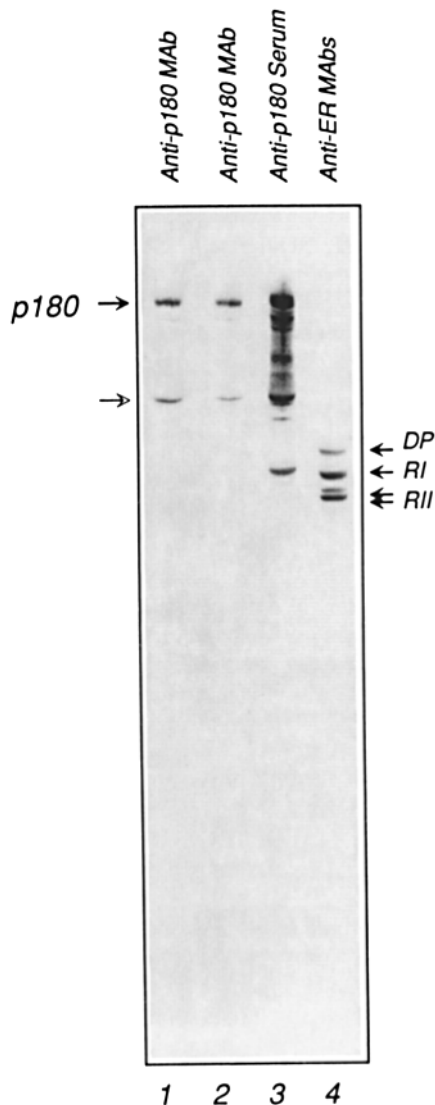


Figure 1. Immunoblot of rough microsomes stained with anti-p180 antibodies. Canine pancreatic rough microsomes were isolated and prepared for immunoblotting as described in Materials and Methods. Lanes 1 and 2 are stained with mouse monoclonal anti-p180 antibodies. Lanes 3 and 4 represent identical samples of rough microsomes, stained with a polyclonal anti-p180 serum and a mixture of anti-ER monoclonals, respectively. *DP*, docking protein; *RI*, ribophorin I; *RII*, ribophorin II.

anti-p180 IgG allowed us to test whether ribosome binding is necessary for protein translocation. Accordingly, salt-washed, EDTA-treated, translocation-competent RM were incubated with anti-p180 IgG and recovered by centrifugation through a sucrose cushion (as shown in Fig. 2 A). Anti-p180-treated membranes showed a marked reduction in translocation activity (Fig. 3, A and B). Fig. 3 A is a fluorogram of the translocation of a variant of prepro- α -factor from which putative glycosylation sites had been removed by site-directed mutagenesis (see Materials and Methods). In addition to the translocation defect, we routinely observed that the overall level of *in vitro* translation was lower in the presence of membranes that had been treated with anti-docking protein or anti-p180 antibodies (Fig. 3 A). A radioanalytical

imaging quantification of translocation, where the actual amount of pro- α -factor translocated is measured as a percentage of total prepro- α -factor synthesized, is shown in Fig. 3 B. Control membranes, treated with buffer or nonimmune IgG, were equally competent in the translocation of prepro- α -factor, whereas translocation dropped to roughly 20% of this value by treating membranes with either anti-docking protein or anti-p180 IgG. This level of translocation competence paralleled that of membranes inactivated with 10 μ g/ml trypsin, a treatment which has been shown previously to remove both p180 and docking protein from RM (Hortsch et al., 1986).

The use of large probes such as IgG which are not only bulky, but multivalent, could lead to the nonspecific inhibition of function due to steric hindrance or cross-linking of antigens on the membrane surface. This obstacle is best overcome by the use of smaller, monovalent derivatives of IgG such as Fab or Fab' fragments. Accordingly, we prepared Fab' fragments from the two monoclonal anti-p180 IgGs as well as from nonimmune controls (Fig. 4). Ribosome binding assays were carried out with the Fab' anti-p180 and ribosome binding was found to be diminished (Fig. 5 A), although to a lesser extent than with intact IgG. This was the case with either anti-p180 monoclonal. The 40% reduction in ribosome binding (compared to controls) observed in the presence of Fab' anti-p180 was due in large part to a significant drop in the affinity to the ribosomes for the membrane (Fig. 5 B). Two interpretations would be consistent with these data. One is that the Fab' partially obstruct the access of the ribosomes to p180, leaving a residual low-affinity binding. The other possibility is that high affinity, p180-mediated ribosome binding is completely blocked by the Fab', leaving a residual, low-affinity binding activity mediated by a different membrane protein. Results presented below tend to rule out the latter alternative.

The obvious question that arises is whether the order of magnitude difference in affinity for ribosomes brought about by anti-p180 Fab' is sufficient to affect the translocation process. Results from translocation assays conducted with membranes that had been treated with control and anti-p180 Fab' are shown in Fig. 6. With anti-p180 Fab' substantial decreases in translocation of secretory protein precursors were observed (70 and 80% inhibition, respectively) in comparison to identical samples treated with nonimmune Fab'.

From these data on intact rough microsomes, we tentatively concluded that p180 plays a role in both *in vitro* ribosome binding and protein translocation. Moreover, it seemed likely that the monoclonal anti-p180 antibodies were appropriate reagents for depleting p180 from detergent extracts of microsomes before their reassembly into functional proteoliposomes.

The Ribosome Binding Activity of Proteoliposomes Depends upon the Presence of the 180-kD Ribosome Receptor

Proteoliposomes prepared by the method of Nicchitta and Blobel (1990) have been shown to bind ribosomes *in vitro* and are competent for the translocation of nascent polypeptides. The advantage of using this system is that a total solubilization of rough microsomal proteins is achieved before reconstitution into proteoliposomes. This allows a func-

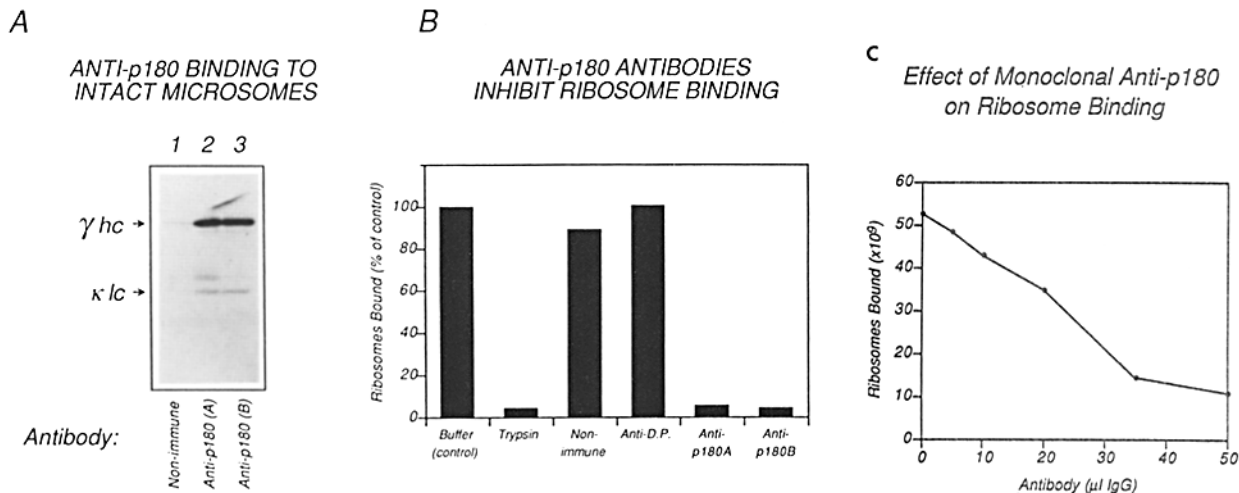


Figure 2. Anti-p180 monoclonal IgGs bind to rough microsomes and inhibit ribosome binding. IgG fractions derived from nonimmune, anti-p180 (two monoclonals), or anti-docking protein (*D.P.*) ascites fluid were incubated with either EDTA/KCl-washed membranes (RM_{EK}) or puromycin/KCl/nuclease-stripped membranes (RM_{SN}). (A) Antibodies bind to intact rough microsomes. RM_{EK} were sedimented through a sucrose cushion to separate bound from unbound IgG. The pellets were analyzed by immunoblotting with alkaline phosphatase-conjugated goat anti-mouse antibody. γhc , gamma heavy chain; κlc , kappa light chain. (B) Anti-p180 monoclonals, bound to intact microsomes, inhibit ribosome binding. RM_{SN} were incubated with the monoclonal IgG fractions (as in A) before the addition of ribosomes. Saturation levels of ribosome binding are shown. Ribosome binding is expressed as percent of control, where controls reflect the number of ribosomes bound to RM_{SN} preincubated with 0.25 M sucrose in LSB (see Materials and Methods). Trypsin (lane 2) refers to RM_{SN} incubated with 10 $\mu g/ml$ trypsin at 0°C for 30 min and re-isolated by centrifugation. The samples of microsomes were normalized to contain equal amounts of membrane protein. (C) Titration of inhibition of ribosome binding by anti-p180 monoclonals. Varying amounts of anti-p180 IgG or buffer were added to equal amounts of RM_{SN} before the addition of ribosomes in a standard binding assay (See Materials and Methods). Concentration of anti-p180 IgG = 1.2 mg/ml.

tional assessment of selectively depleting a specific component, within the context of all remaining ER proteins.

Using this system, we were able to determine the influence of p180, as well as the remainder of other ER membrane proteins, on ribosome binding and translocation.

As ribosome binding has not been extensively characterized in proteoliposomes (Nicchitta et al., 1991; Migliaccio et al., 1992), it was first necessary to confirm that saturation levels and affinity constants are comparable to what has been observed in intact ER vesicles. As can be seen in Fig. 7 A, the saturation kinetics of ribosome binding to stripped rough microsomes and proteoliposomes were virtually identical. Scatchard analysis (Fig. 7 B) indicated that ribosomes bind to proteoliposomes with an affinity of 2.0×10^{-8} M, which is in good agreement with the values observed for intact membranes (Borgese et al., 1974; Hortsch et al., 1986; Savitz and Meyer, 1990).

To selectively remove p180 from detergent extracts used to generate proteoliposomes, affinity columns of monoclonal anti-p180 IgG were prepared (see Materials and Methods). The efficacy of the affinity column in the depletion of specific proteins from total extracts of rough microsomes is shown in Fig. 8. Even on stained gels (A), it can be seen that p180 was effectively depleted (compare lanes 1 and 2). This was corroborated by immunoblotting with rabbit anti-p180 which demonstrated that levels of intact as well as any breakdown products of p180 were virtually eliminated (B, compare lanes 1 and 4). A second passage over the anti-p180 affinity column eliminated the remaining detectable p180 in the extract (as shown in Fig. 9 B, lane 2).

Identical extracts were also passed over two control columns. One consisted of monoclonal IgG directed against

an irrelevant protein from yeast as a negative control ("Mock depleted"), while a positive control (for later studies) comprised an affinity column of monoclonal anti-docking protein (DP) IgG. Two cycles of mock depletion had little effect on the composition of the extracts (Fig. 8 A, lane 4 and Fig. 8 B, lane 2). In contrast, one pass over the anti-docking protein column selectively removed docking protein, without affecting p180 (compare lanes 1 and 3 in Fig. 8 B). The low abundance of docking protein precludes its visualization in stained gels of total microsomal proteins. Using a panel of other available antibodies, we determined that the content of ribophorin I, ribophorin II, and signal sequence receptor (SSR) α and β subunits was unaffected by passage of extracts over anti-p180 affinity columns, while virtually all (>90%) of the material bound to the affinity column was composed of anti-p180-reactive polypeptides (not shown).

To ensure that the p180 and DP composition of the extracts was reflected in that of the actual proteoliposomes, immunoblotting was used to examine proteoliposomes produced from control and depleted extracts. As an important experiment involved the readdition of p180 to depleted proteoliposomes, our ability to reconstitute exogenous p180 was evaluated as well. Our p180 purification scheme was previously shown to produce p180 that is greater than 95% homogeneous (Savitz and Meyer, 1990), and the material used in these studies is shown in the silver-stained gel in lane 5 of Fig. 9, A. Proteoliposomes from control and depleted extracts as well as those to which purified p180 had been restored were examined by silver staining as well as immunoblotting (Fig. 9, A and B). Proteoliposomes generated from extracts passed twice over an anti-p180 column had no detectable p180 (compare lanes 1 and 2). By titrating varying

ANTI- p180 ANTIBODIES INHIBIT
TRANSLOCATION INTO INTACT MICROSOMES

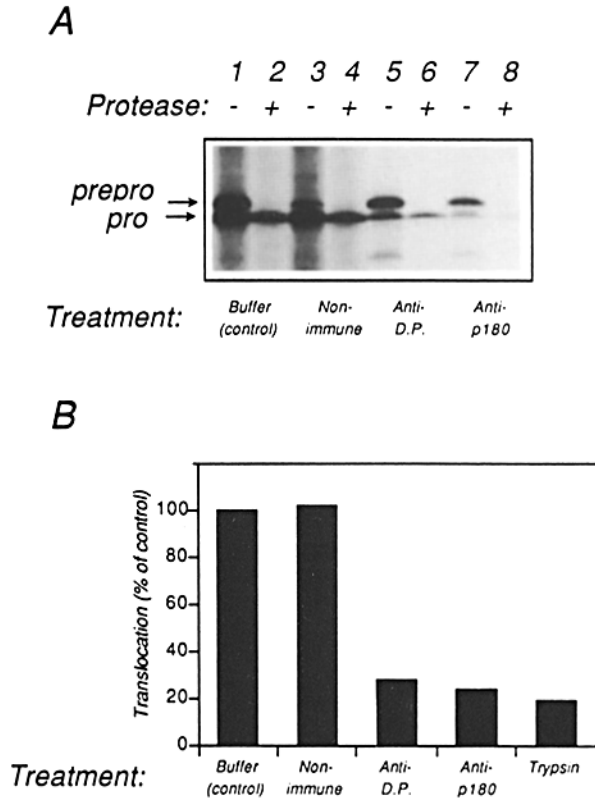


Figure 3. Anti-p180 monoclonal IgGs inhibit in vitro translocation into intact microsomes. Nonimmune, anti-p180, or anti-docking protein (D.P.) monoclonal IgG, derived from ascites fluid, was incubated with RM_{EK} which were subsequently sedimented through a sucrose cushion and resuspended. These microsomes were tested for the translocation of a nonglycosylated form of prepro- α -factor in a cell-free system. To determine translocation, half of each sample was treated with 0.5 mg/ml proteinase K at 0°C for 1 h. (A) Fluorograph of the translocation reactions is shown. (B) Translocation activity determined directly by radioanalytical imaging of SDS-polyacrylamide gels. The translocation activity was calculated as: protease-protected α -factor \div total α -factor-specific translation products. Histogram values are expressed as percent of control, where the control value is the translocation activity of RM_{EK} treated with 0.25 M sucrose in LSB instead of antibodies. Trypsin refers to RM_{EK} treated with 10 μ g/ml trypsin (0°C, 30 min) and reisolated by centrifugation. *prepro*, prepro- α -factor; *pro*, pro- α -factor.

amounts of purified p180 into depleted extracts, we generated proteoliposomes in which roughly 40% and 80% of control levels of p180 were restored (lanes 3 and 4).

Depletion of p180 resulted in a considerable decrease in ribosome binding to proteoliposomes in comparison to either intact rough microsomes or proteoliposomes prepared from mock-depleted extracts (Fig. 10). The low level of ribosome binding observed was comparable to that of proteoliposomes prepared from extracts derived from inactive rough microsomes (obtained by proteolysis with 50 μ g/ml trypsin). Worth noting is the apparent lack of contribution by the remainder of microsomal proteins to ribosome bind-

Isolation of Fab' Fragments

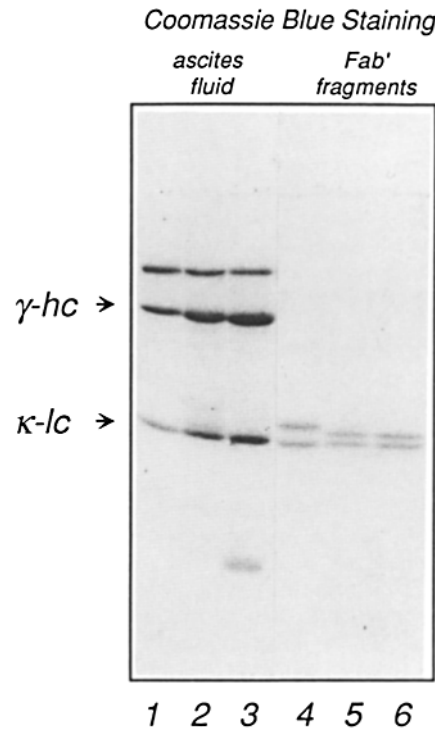


Figure 4. Isolation of anti-p180 Fab' fragments from monoclonal antibodies. Fab' fragments were isolated from mouse anti-p180 IgG as described in Materials and Methods. Lanes 1 and 4: nonimmune ascites; lanes 2 and 5, and lanes 3 and 6 represent anti-p180 ascites from two monoclonal antibodies respectively. Polyacrylamide gels were stained with Coomassie blue.

ing, as evidenced by the minimal amount of residual activity when p180 was depleted. Further direct evidence linking p180 to ribosome binding came from experiments in which p180 was restored to depleted extracts. Preliminary findings indicated that increasing amounts of readded p180 led to parallel increases in ribosome binding, and in the case presented here where p180 was incorporated into proteoliposomes to about 80% of control levels (Fig. 9, A and B, lane 4), a marked restoration of ribosome binding, to about 70% of control activity, was achieved (Fig. 10).

180-kD Ribosome Receptor (p180) is Required for Protein Translocation into Proteoliposomes

Both the Blobel and Rapoport groups have used this proteoliposome system to establish the participation of a particular protein in the translocation process. In this way, it was shown that docking protein (Migliaccio et al., 1992) and the TRAM (Görlich et al., 1992) are required for translocation in vitro, whereas the SSR protein is not (Migliaccio et al., 1992). We first confirmed that such proteoliposomes were competent in the translocation of our reporter protein, the nonglycosylated form of prepro- α -factor (Fig. 11 A, lanes 1-3). Similar to results obtained by other groups using this system, we also observed a variable amount of translocated (protease-protected) prepro- α -factor in these proteoliposomes. A partial uncoupling of translocation from signal

Effect of Anti-p180 Fab' on Ribosome Binding

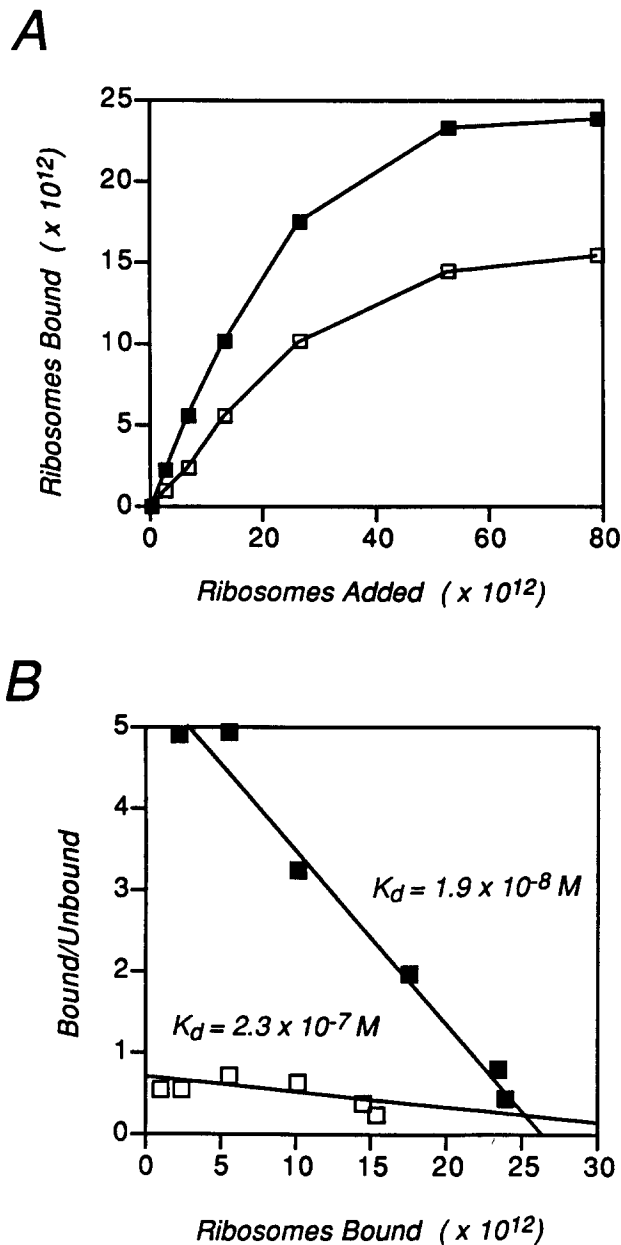


Figure 5. Anti-p180 Fab' inhibits ribosome binding. Ribosome binding assays were carried out in the presence of anti-p180 Fab' as described in Materials and Methods. (A) Saturation curves of ribosome binding. (B) Scatchard analysis. (Closed symbols) Non-immune Fab'; (open symbols) anti-p180 Fab'.

peptidase activity would account for protease-protected precursors. As can be seen by examining the protease-protected forms of prepro- α -factor shown in Fig. 11 A, proteoliposomes depleted of p180 exhibited greatly diminished levels of translocation activity (lane 6), similar to proteoliposomes lacking the DP (lane 4). Again, as was observed for ribosome binding activity, the re-addition of purified p180

Anti-p180 Fab' Inhibits Translocation

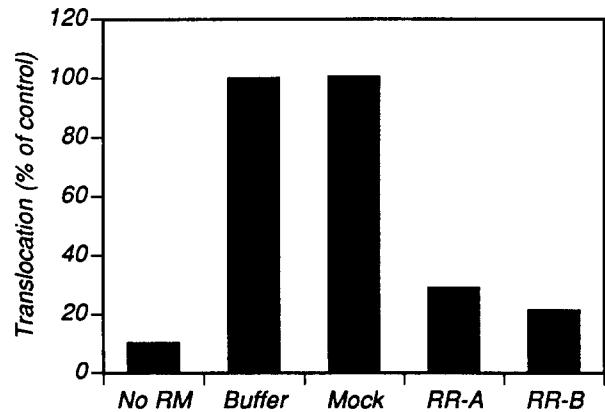


Figure 6. Anti-p180 Fab' inhibits translocation. Translocation assays using prepro- α -factor as a substrate were carried out and quantified by radioanalytic imaging as described in Materials and Methods. *RM*, rough microsomes; *Mock*, nonimmune Fab'; *RR-A/RR-B*, two anti-p180 monoclonal Fab'. Controls (buffer) were carried out on identical samples incubated in the buffer used to dialyze Fab' before their inclusion in the assay. Translocation is defined as the ratio of protease-protected forms of α -factor to total prepro- α -factor translated $\times 100$.

restored much of the translocation activity that was lost through the depletion of p180 (lane 7).

The data shown in Fig. 11 A are probably the most significant in terms of demonstrating a role for p180 in the translocation process. As Görlich et al. (1992) found that different preproteins were translocated to different extents in TRAM-depleted proteoliposomes, we confirmed the validity of our prepro- α -factor results by extending them to include a commonly-used mammalian preprotein, preprolactin. Data obtained from quantifications using radioimaging of both prepro- α -factor and preprolactin translocation are shown in Fig. 11 B. The depletion of either p180 or DP resulted in reductions in translocation activity of 80% or more. Restoration of p180 to depleted extracts used to make the proteoliposomes resulted in a recovery of translocation activity in a p180-dependent fashion; the more p180 that was re-added to p180-depleted proteoliposomes (see immunoblots in Fig. 9 B, lanes 2-4), the higher the translocation activity (Fig. 11 B). The ability of re-added p180 to restore the translocation competence of proteoliposomes made from p180-depleted extracts (as depicted in Fig. 11) roughly paralleled its ability to restore ribosome binding in these same vesicles (as shown in Fig. 10).

Discussion

Using an appropriate in vitro system, i.e., one in which both ribosome binding and translocation could be measured as a function of the presence or absence of a given protein, we have determined an important role for the 180-kD ribosome receptor in both processes. We have demonstrated that the selective removal of p180 from translocation-competent proteoliposomes results in a loss of their ability to bind ribo-

Ribosome Binding to Proteoliposomes

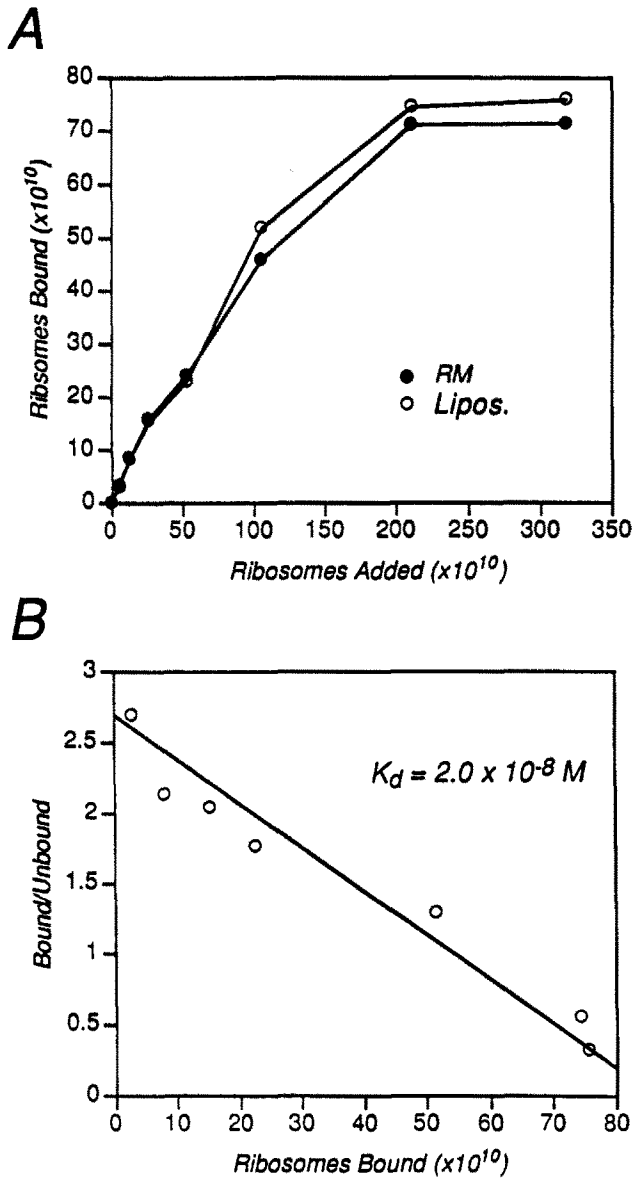


Figure 7. Proteoliposomes and stripped rough microsomes show similar kinetics of ribosome binding. RM_{SN} or mock-depleted proteoliposomes were mixed with increasing amounts of ribosomes in a constant volume to derive a saturation curve of ribosome binding. From this curve, Scatchard analysis was carried out to determine the affinity constant for ribosome binding of control proteoliposomes. The slope of the line was determined by least-squares analysis. (A) Saturation kinetics. (B) Scatchard analysis. The RM_{SN} and the proteoliposomes were normalized to equal protein concentrations. *RM*, rough microsomes; *Lipos*, proteoliposomes.

somes with high affinity and to translocate two different presecretory proteins. A strict dependence upon the presence of p180 for both activities was shown in experiments where purified p180 was added back to extracts from which it had been depleted before the formation of proteoliposomes. Moreover, only minimal ribosome binding activity

DEPLETION OF SPECIFIC PROTEINS FROM ROUGH MICROSOMAL EXTRACTS

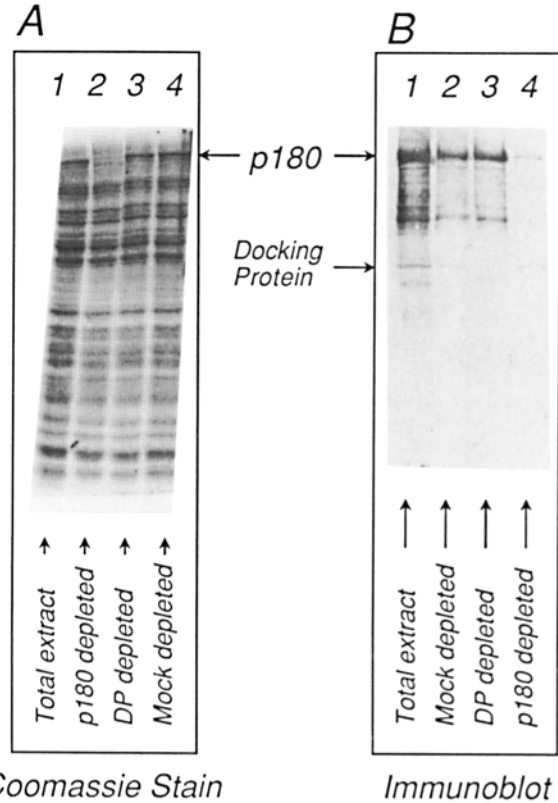


Figure 8. Monoclonal antibody affinity columns deplete specific proteins from detergent extracts of rough microsomes. RM_{EK} were solubilized by sodium cholate to derive a detergent extract of microsomal proteins and lipids. These extracts were applied to columns of IgG (nonimmune, anti-p180 and anti-docking protein) cross-linked to protein A-agarose. (A) Unbound material, visualized by Coomassie blue staining. (B) Unbound material analyzed by immunoblotting with a combination of rabbit anti-p180 and anti-docking protein antibodies. Starting material (cholate extract) is depicted in lane 1 of A and B. The type of affinity column used to obtain the fractions analyzed is shown at the bottom of each panel.

was found among the population of proteins which remained after the selective depletion of p180. The same monoclonal anti-p180 antibodies used to generate extracts depleted of p180 were found to inhibit ribosome binding and translocation when bound to intact microsomes. Taken together, these data provide direct evidence to support our hypothesis that the 180-kD ribosome receptor is necessary for most, if not all, of the ribosome binding measurable in vitro.

These results extend those of our previous study in which we demonstrated that p180 was sufficient to enable the reconstitution of high-affinity ribosome binding in artificial lipid vesicles composed of phosphatidyl serine (PS) and phosphatidyl choline (PC) (Savitz and Meyer, 1990). Since then, several groups have also found that fractions of ER membrane proteins have ribosome binding activity when incorporated into liposomes. In these cases, either a 34-kD membrane protein, or heterogeneous fractions of membrane proteins, which lacked intact p180, were found to have the ability to bind ribosomes when incorporated into liposomes

Composition of Proteoliposomes

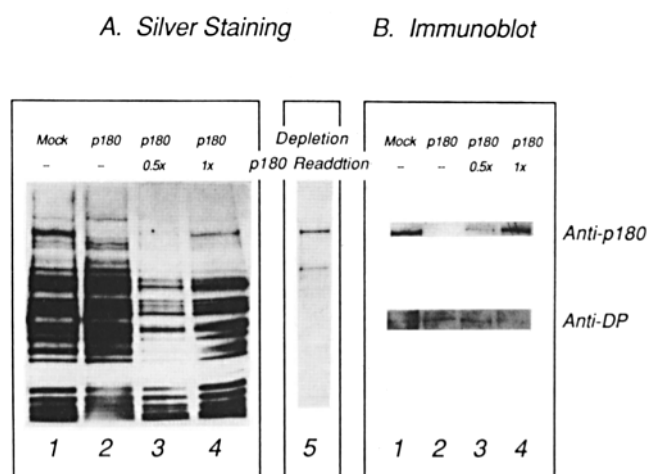


Figure 9. Composition of proteoliposomes used for ribosome binding and translocation studies. Proteoliposomes were prepared from various detergent extracts as described in Materials and Methods. (A) Protein profiles of proteoliposomes and purified p180 visualized by silver staining. (B) Immunoblot of proteoliposomes stained with anti-p180 and anti-docking protein antibodies. Liposomes were reconstituted from the following extracts: Mock depleted (lanes 1); p180 depleted (lanes 2); p180 depleted + purified p180 (0.5 aliquot, lanes 3); p180 depleted + purified p180 (1.0 aliquot, lanes 4). Lane 5 shows a silver stained profile of the fraction of p180 used in the re-addition experiments. 100-kD band represents the major breakdown product of p180 (see immunoblot, Fig. 1).

Ribosome Binding to Proteoliposomes

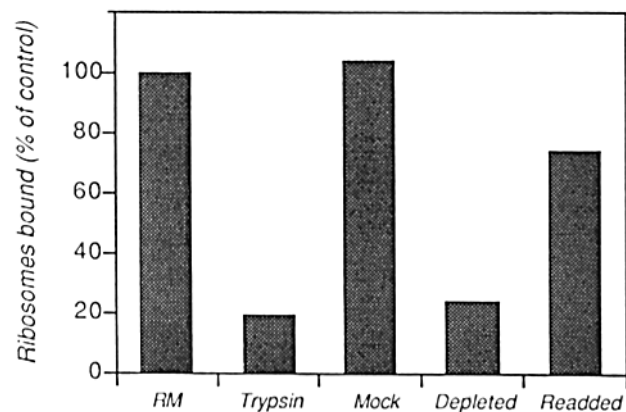


Figure 10. Depletion of p180 reduces and its re-addition restores ribosome binding to proteoliposomes. Proteoliposomes were reconstituted from mock-depleted detergent extracts or from p180-depleted detergent extracts to which p180 had been restored in varying amounts (see Fig. 9). As an additional control, liposomes were also prepared from extracts of inactive (50 $\mu\text{g/ml}$ trypsin-treated) RM. The proteoliposomes were normalized for protein concentration and assayed for ribosome binding activity. Saturation levels of ribosome binding are shown. The percent of control is based on ribosome binding to intact RM_{SN} . RM, stripped rough microsomes; Trypsin, liposomes prepared from RM treated with 50 $\mu\text{g/ml}$ trypsin; Mock, liposomes shown in lanes 1 of Fig. 9; Depleted, liposomes shown in lanes 2 of Fig. 9; Readded, proteoliposomes depicted in lanes 4 of Fig. 9.

Translocation in p180-depleted Proteoliposomes

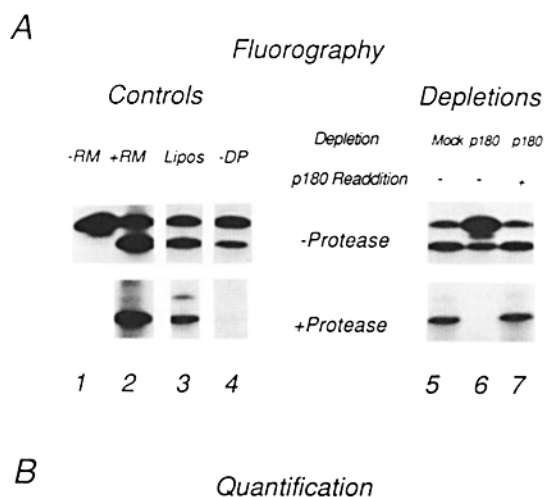


Figure 11. Proteoliposomes depleted of p180 are reduced in translocation activity. Liposome preparation and translocation assays were carried out as described in Materials and Methods. The liposomes were normalized for protein concentration and were added to cell-free translocation assays. The ability of the proteoliposomes to translocate two different preproteins, preprolactin and nonglycosylated prepro- α -factor, was tested. (A) Fluorogram of translocation of nonglycosylated prepro- α -factor. (Upper portion) translocation assay prior to proteolysis (exposure time: 24 h); (Lower portion) translocation assay following treatment with protease K at 500 $\mu\text{g/ml}$, for 60 min, at 0°C (exposure time: 68 h). (B) Quantification of translocation of prepro- α -factor and prolactin. Translocation assays were quantified by radioanalytic imaging as described in Materials and Methods. Translocation is defined as the ratio of protease-protected forms of α -factor to total prepro- α -factor translated $\times 100$. RM, rough microsomes; DP, docking protein; Mock, liposomes described in Fig. 9, lanes 1.

(Nunnari et al., 1991; Collins and Gilmore, 1991; Ichimura et al., 1992). We have recently conducted a series of investigations to reconcile these discrepancies and found a profound influence of lipid composition on the ability to incorporate p180 into artificial lipid vesicles. Just as acidic phospholipids are required for the reconstitution of prokaryotic protein translocation (Lill et al., 1989), and for ribosome binding to intact membranes (Jothy et al., 1975), this class of phospholipids were found necessary to enable the incorporation of purified p180 into liposomes. Purified p180 was successfully incorporated into liposomes composed of PS/PC or pancreatic microsomal phospholipids, but not into

ones composed only of PC (Savitz and Meyer, manuscript in preparation). Except for the studies from our group, all of the other published studies on reconstitution of ribosome binding into liposomes have used pure phosphatidyl choline as the lipid source (Nunnari et al., 1991; Collins and Gilmore, 1991; Ichimura et al., 1992). To specifically rule out any differences in ribosome binding that may arise from the use of artificial lipid vesicles, the studies described here exclusively made use of the proteoliposome system of Nicchitta and Blobel (1990) in which vesicles are reconstituted from the endogenous, microsomal cohort of phospholipids.

Our results cannot rule out the possibility that ER membrane proteins other than p180 mediate or participate in ribosome binding. For a contaminating protein to account for the high affinity ribosome binding that we have observed in this and previous studies, it would have to have the following properties: It would have to be 180 kD in size or undetectable on silver stained gels (Savitz and Meyer, 1990). As our fractions of p180, that are greater than 95% homogeneous, have been calculated to bind one ribosome per molecule of receptor (Savitz and Meyer, 1990), a putative contaminant would either have a molecular weight of 9 kD or, if larger, bind multiple (up to 20) ribosomes per molecule to exhibit comparable activity. Such a contaminant would have to be tightly bound to p180 in order to enable its co-purification on monoclonal anti-p180 antibody affinity columns, or share an epitope with p180 that allows its recognition. Moreover, a contaminant-p180 interaction would have to be stable in both 1% octyl glucoside/700 mM KOAc (Savitz and Meyer, 1990), or in 0.8% sodium cholate/400 mM KCl (see Materials and Methods) to accompany p180 through the purification and the depletion steps, respectively. We therefore consider that the ribosome binding that we observe cannot be accounted for by putative contaminants.

Our studies with proteoliposomes indicate a requirement for p180 in both ribosome binding and translocation. On the basis of such studies, we cannot unequivocally conclude that ribosome binding is required for protein translocation, merely that depletion of p180 from proteoliposomes profoundly affects both. A role for ribosome binding in translocation is further supported, however, by our studies on intact membranes, where anti-p180 monoclonals inhibited both processes. Final resolution of this question could come from studies on genetically manipulable organisms in which the effect of mutations in a ribosome receptor could be correlated with both ribosome binding and translocation in an intact membrane.

Traditional ribosome binding assays measure the rebinding of ribosomes to stripped membranes. Based on models of how translocation occurs, ribosomes are not only bound to membranes, but after termination of translation, dissociate and return to cytosolic pools (Blobel and Dobberstein, 1975). Irrespective of the precise mechanistic details of such a process, the association and dissociation of ribosomes with the ER membrane must be regulated in some fashion. Along these lines, it is most interesting that p180 has been recently shown to be an ATP binding protein which can be efficiently labeled by 8-N₃ATP, a photoaffinity ATP analog (Zimmerman and Walter, 1991). Our preliminary studies show that the cross-linking of 8-N₃ATP to stripped rough microsomes increases their affinity for ribosomes in the *in vitro* assay, whereas the inclusion of ADP in the binding reaction

decreases both saturation levels and the overall affinity of the ribosome-membrane interaction (C. Sütterlin, A. Savitz and D. Meyer, unpublished observations). A more precise analysis of p180 function and regulation will be possible once a sequenced cDNA clone is available.

In our previous study, we removed p180 from its context within the ER membrane and demonstrated its capability to bind ribosomes when purified and incorporated into lipid vesicles (Savitz and Meyer, 1990). In this study, we have performed the complementary analysis, showing the inability of membranes or proteoliposomes to perform the functions of ribosome binding and translocation in the absence of functional p180. We, as well as other contributors in the translocation field (Nicchitta et al., 1991; Migliaccio et al., 1992; Görlich et al., 1992), consider this rigorous type of "biochemical knockout" conclusive in demonstrating a requirement for a specific component in the translocation reaction. Based on the validity of the approach, and on the data presented in this and our previous study, we conclude that p180 is essential for both ribosome binding and protein translocation into ER membranes.

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