Ribosome Binding to the Endoplasmic Reticulum: A 180-kD Protein Identified by Crosslinking to Membrane-Bound Ribosomes Is Not Required for Ribosome Binding Activity

Paula G. Collins and Reid Gilmore

Department of Biochemistry and Molecular Biology, University of Massachusetts Medical School, Worcester, Massachusetts 01655

Abstract. We have used the membrane-impermeable, thiol-cleavable, crosslinker 3,3'-dithio *bis* (sulfosuccinimidylpropionate) to identify proteins that are in the vicinity of membrane-bound ribosomes of the RER. A specific subset of RER proteins was reproducibly crosslinked to the ribosome. Immunoblot analysis of the crosslinked products with antibodies raised against signal recognition particle receptor, ribophorin I, and the 35-kD subunit of the signal sequence receptor demonstrated that these translocation components had been crosslinked to the ribosome, but each to a different extent. The most prominent polypeptide among the crosslinked products was a 180-kD protein that has recently been proposed to be a ribosome receptor (Savitz, A. J., and D. I. Meyer. 1990. *Nature (Lond.).* 346: 540–544). RER membrane proteins were reconstituted into liposomes and assayed with radiolabeled ribosomes to determine whether ribosome binding activity could be ascribed to the 180-kD protein. Differential detergent extraction was used to prepare soluble extracts of microsomal membrane vesicles that either contained or lacked the 180-kD protein. Liposomes reconstituted from both extracts bound ribosomes with essentially identical affinity. Additional fractionation experiments demonstrated that the bulk of the ribosome binding activity present in detergent extracts of microsomal membranes could be readily resolved from the 180-kD protein by size exclusion chromatography. Taken together, we conclude that the 180-kD protein is in the vicinity of membrane bound ribosomes, yet does not correspond to the ribosome receptor.

MULTIPLE proteinaceous components of the mammalian ER are recruited to function in a temporal sequence of events that is initiated when the amino-terminal signal sequence of a secretory protein emerges from the large subunit of the ribosome. The first definitive step in protein translocation is binding of the signal recognition particle (SRP)[1] to the signal sequence of the nascent polypeptide (Krieg et al., 1986; Kurzchalia et al., 1986; Walter et al., 1981). Displacement of the SRP from the ribosome-nascent chain complex is a guanine ribonucleotide–dependent reaction that occurs when the SRP interacts with the SRP receptor (or docking protein) at the microsomal membrane surface (Connolly and Gilmore, 1989; Gilmore and Blobel, 1983; Gilmore et al., 1982; Meyer et al., 1982). Membrane insertion of the signal sequence results in a direct interaction between the nascent polypeptide and several integral membrane proteins that have been identified by crosslinking studies (Kellaris et al., 1991; Krieg et al., 1989; Wiedmann et al., 1987). At this point, the nascent polypeptide is shielded from digestion by externally added protease, suggesting that the ribosome is in close contact with the membrane surface (Connolly et al., 1989). Contact between the ribosome and the membrane surface is believed to be maintained, at least in part, by a receptor for the ribosome.

Previous in vitro assays have established that 80S ribosomes will preferentially bind to membrane vesicles derived from the RER (Amar-Costesec et al., 1984; Borgese et al., 1974). The ribosome binding activity of microsomal membrane vesicles is inhibited by increased ionic strength and is sensitive to proteolytic digestion by trypsin (Borgese et al., 1974; Hortsch et al., 1986; Jothy et al., 1975). In vitro ribosome binding assays have been used to determine the affinity of ribosomes for a putative ribosome receptor, and to quantitate the number of binding sites in ribosome-stripped rough microsomal membranes from rat liver and canine pancreas (Amar-Costesec et al., 1984; Borgese et al., 1974; Hortsch et al., 1986; Yoshida et al., 1987). Rough microsomal membrane proteins have been solubilized and reconstituted into liposomes to study the characteristics of ribosome binding in a simplified system (Savitz and Meyer, 1990; Yoshida et al., 1987). This approach has permitted fractionation of the detergent-solubilized proteins and could lead to the identification of a protein that serves as the ribosome receptor in the intact membrane (Yoshida et al., 1987). One of the im-

1. *Abbreviations used in this paper*: SRP, signal recognition particle; SR, SRP receptor; SSR, signal sequence receptor; K-RM, salt extracted microsomal membranes.

plicit assumptions of the reconstitution studies is that a single protein or stable protein complex is responsible for binding of ribosomes to the intact microsomal membrane. If this assumption is valid, the properties of the reconstituted ribosome receptor should be identical to the properties of the receptor in the intact membrane with respect to the binding affinity and ribosome to receptor stoichiometry.

Although several proteins have been proposed to be the ribosome receptor, identification of a bona fide receptor for the ribosome has remained elusive. Cytochrome P_{450} was found to associate with ribosomes (Ohlsson and Jergil, 1977), but further analysis demonstrated a nonstoichiometric relationship between cytochrome P_{450} and membrane bound ribosomes; consequently the association was judged to be nonspecific (Kreibich et al., 1978*b*). Two prominent ER proteins, termed ribophorins I and II, both of which are glycoproteins, were proposed as the ribosome receptor based upon several observations (Kreibich et al., 1978*b*). The ribophorins are present in close to 1:1 stoichiometry with membrane-bound ribosomes (Marcantonio et al., 1984) and are found exclusively in the RER (Kreibich et al., 1978*b*). The ribophorins can be chemically crosslinked to membrane-bound ribosomes, indicating a physical proximity to ribosomes engaged in translocation (Kreibich et al., 1978*a*). However, data refuting a role for the participation of the ribophorins as the ribosome receptor emerged from several studies in which ribosome binding activity did not correlate with ribophorin content. Limited trypsin digestion of intact microsomal membranes abolished ribosome binding at concentrations much lower than that necessary to proteolyze the ribophorins (Hortsch et al., 1986). Furthermore, proteoliposome reconstitution experiments demonstrated that ribosomes will bind with high affinity to liposomes prepared from glycoprotein-depleted, and therefore ribophorin-depleted, detergent extracts of microsomal membranes (Yoshida et al., 1987). Recently, an integral membrane protein of ~180 kD was proposed as the ribosome receptor when proteolytic fragments of this protein were shown to interfere with ribosome binding to intact microsomal membranes (Savitz and Meyer, 1990). Purified preparations of the 180-kD protein were shown to bind ribosomes after reconstitution into liposomes (Savitz and Meyer, 1990).

Here we demonstrate that the 180-kD protein is indeed in close proximity to membrane-bound ribosomes engaged in protein translocation, based on the ability of a homobifunctional amine-reactive reagent to crosslink the 180-kD protein to the ribosome. Other proteins that were found to be crosslinked to the ribosome included ribophorin I, the SRP receptor, and the signal sequence receptor. Detergent extracts of RER proteins, either containing or lacking the 180-kD protein, were reconstituted into egg yolk phosphatidylcholine liposomes to determine whether ribosome binding activity was dependent upon the presence of the 180-kD protein. Liposomes reconstituted from both detergent extracts bound ribosomes with affinities that were experimentally indistinguishable. Furthermore, fractionation of RER proteins by gel filtration chromatography also demonstrated that ribosome binding activity could be resolved from the 180-kD protein. These two fractionation experiments indicate that the 180-kD protein is not required for binding of ribosomes to proteoliposomes prepared from RER proteins.

Materials and Methods

Preparation of K-RM, Ribosomes, and ^{125}I-labeled Ribosomes

Salt-extracted microsomal membranes (K-RM) were prepared from canine pancreas microsomal membranes as described (Walter et al., 1981). DTT-free K-RM for crosslinking experiments were prepared by two successive centrifugations and resuspensions of K-RM at 2 eq/μl (50 A_{280} U/ml = 1 equivalent/μl; Walter and Blobel, 1983) in 100 mM triethanolamine-OAc, pH 7.5, 250 mM sucrose. Canine ribosomes were isolated from rough microsomal membranes by extraction of membranes with 0.5 M KOAc (Walter and Blobel, 1980). Ribosomes were recovered from the high salt extract by a 3.5 h centrifugation at 200,000 g_{av} through a 0.5 M sucrose cushion containing 50 mM triethanolamine-OAc, pH 7.5, 500 mM KOAc, 5 mM $Mg(OAc)_2$, 1 mM DTT as described previously (Walter and Blobel, 1980; Walter and Blobel, 1983). The ribosomes were resuspended in 50 mM triethanolamine-OAc, pH 7.5, 100 mM KOAc, 2.5 mM $Mg(OAc)_2$. The concentration of ribosomes was determined using a μM extinction coefficient of 60.8 at 260 nm. The extinction coefficient was calculated using 4.5×10^6 D as the molecular mass for the 80S ribosome (Hamilton et al., 1971), and an A_{260} of 135 for a 1% solution of ribosomes (Tashiro and Siekevitz, 1965). Labeling of the ribosomes with ^{125}I was accomplished by incubating 30 pmol of canine ribosomes in 30 μl of 50 mM triethanolamine-OAc, pH 7.5, in the presence of 500 μCi of ^{125}I Bolton-Hunter reagent (Amersham Corp., Arlington Heights, IL) for 2 h on ice. After quenching the unreacted ^{125}I Bolton-Hunter reagent with 20 μl of 0.1 M Tris-Cl, pH 7.5, the iodinated ribosomes were separated from unincorporated label by sucrose density gradient centrifugation on a 5-20% sucrose density gradient in 50 mM Tris-Cl, pH 7.5, 50 mM KCl, 2.5 mM $MgCl_2$ for 1 h at 234,000 g_{av} using a SW 50.1 rotor (Beckman Instruments Inc., Palo Alto, CA).

Crosslinking of Membrane Proteins to Endogenous Ribosomes

Endogenous membrane-bound ribosomes were crosslinked to K-RM proteins using a modification of the procedure of Staros (1982). 3,3'-dithio-*bis*(sulfosuccinimidylpropionate)(DTSSP; Pierce Chemical Co., Rockford, IL) was made as a freshly prepared stock solution in 50 mM sodium phosphate, pH 7.5. The DTSSP stock solution was added at 1/10 final volume to DTT-free K-RM (2 eq/μl) to obtain the final DTSSP concentration used (see figure legends). After a 30-min incubation at 25°C with occasional agitation, unreacted DTSSP was quenched by the addition of 1/6 volume of 100 mM ethanolamine, 20 mM N-ethylmaleimide. N-ethylmaleimide was added to inhibit thiol-disulfide exchange. After quenching, the membranes were solubilized by adjustment to 10 mM Tris-Cl, pH 7.5, 500 mM KOAc, 5 mM $Mg(OAc)_2$, 1% Nikkol (octaethyleneglycol mono-N-dodecyl ether; Nikko Chemical Co., Ltd., Tokyo, Japan). The membrane concentration during solubilization was ~1 eq/μl. After 20 min on ice, 190 μl of the solubilized membranes were layered over a 50-μl, 0.5 M sucrose cushion containing 10 mM Tris-Cl, pH 7.5, 500 mM KOAc, 5 mM $Mg(OAc)_2$, 0.1% Nikkol, and the samples were centrifuged for 30 min at 30 psi at 4°C in a Beckman airfuge using an A-100/30 rotor, thereby generating a supernatant (S1) and a pellet. The pellet was resuspended in 190 μl of 10 mM Tris-Cl, pH 7.5, 500 mM KOAc, 5 mM $Mg(OAc)_2$, 0.1% Nikkol and centrifuged to yield a supernatant (S2) and a pellet. This pellet was resuspended in 190 μl of 10 mM Tris-Cl, pH 7.5, 500 mM KOAc, 5 mM $Mg(OAc)_2$, 0.1% Nikkol, 100 mM DTT to reduce the disulfide bond in the crosslinker. The samples were then incubated at 30°C for 30 min and centrifuged as before to yield a supernatant (S3) and a pellet (P3). Supernatants from each differential centrifugation step (including the cushion) were removed and precipitated with 1/5 volume of 100% TCA. The three TCA-precipitated supernatant fractions and the final pellet were each solubilized in 20 μl of 0.5 M Tris base, 12.5% SDS, reduced with DTT, and the proteins resolved on a 10–15% gradient polyacrylamide gel in SDS. All of the samples in this paper have been similarly prepared for gel electrophoresis and resolved on 10–15% gradient polyacrylamide gels in SDS. The protein molecular mass standards used were: myosin (212 kD), phosphorylase A (92.5 kD), BSA (68 kD), ovalbumin (43 kD), carbonic anhydrase (29 kD), soybean trypsin inhibitor (20.1 kD), and cytochrome c (11.7 kD).

Detergent Treatment of K-RM

Membrane proteins were solubilized using a modification of a previously described method (Yoshida et al., 1987). Briefly, 3.5 ml of K-RM were suspended at a concentration of 1 eq/μl in 50 mM Tris-Cl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 0.25 mM PMSF, and 5 mM DTT by dounce homogenization after which the nonionic detergent Nikkol was added to a final concentration of 1% and the mixture incubated on ice for 30 min. The suspension was layered over 2 ml of a 20% sucrose cushion containing 50 mM Tris-Cl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$ and centrifuged for 1 h at 125,000 g_{av}. The supernatant, including the sucrose cushion, is the Nikkol-soluble fraction, while the pellet contains ribosomes and membrane proteins that are not soluble in Nikkol at low ionic strength. Two different ionic detergent solutions were used to solubilize the membrane proteins in the pellet fraction. In the first, a Nikkol insoluble pellet derived from 7,000 eq of K-RM was resuspended by dounce homogenization in 1.8 ml of 20 mM Tris-Cl, pH 8.6, 25 mM KCl, and then adjusted to 1% sodium deoxycholate with 200 μl of a 10% stock solution. Sodium deoxycholate (Sigma Chemical Co., St. Louis, MO) was recrystalized three times from 90% acetone. In the alternate resuspension procedure, a Nikkol-insoluble pellet derived from 7,000 eq of K-RM was dounce homogenized in 1.8 ml of 50 mM Tris-Cl, pH 8.6, 500 mM KCl, 5 mM $MgCl_2$, and then adjusted to 1% sodium cholate with 200 μl of a 10% stock solution of sodium cholate (Ultrol grade; Calbiochem, San Diego, CA). Each solution was incubated for 30 min on ice before centrifugation at 150,000 g_{av} for 3.5 h. The supernatants obtained are designated the cholate extract or deoxycholate extract.

Reconstitution of Membrane Proteins into Liposomes

Egg yolk phosphatidylcholine (Sigma Chemical Co.) was dried under nitrogen to remove the chloroform:methanol, 9:1 storage solvent. Deoxycholate or cholate extracts of microsomal membranes (160–200 μl of a 1% detergent extract) were added to 1 mg of dried phospholipid. The protein content of each reconstitution varied with the type of sample, but was typically between 20 and 150 μg. Total protein concentration of detergent extracts was determined by the method of Schaffner and Weissmann (1973) using BSA as the standard. The detergent-phospholipid-protein mixtures were dialyzed for 38–48 h against several changes of 50 mM Tris-Cl, pH 7.6, 25 mM KCl, 1.0 μg/ml aprotinin, 0.1 μg/ml each of antipain, chymostatin, leupeptin, and pepstatin A.

Ribosome Binding to Reconstituted Liposomes

Ribosome binding assays were performed as described previously (Yoshida et al., 1987) with the following modifications. A constant amount of ^{125}I-labeled ribosomes (typically 0.1 pmol) was premixed with 2.1–42.7 pmol of unlabeled ribosomes as noted in the figure legends. The premixed ribosomes were incubated with the reconstituted liposomes for 30 min on ice in 50 mM Tris-Cl, pH 7.6, 25 mM KCl, 5 mM $MgCl_2$. The ribosome-liposome solution was then layered over a 4.5-ml sucrose gradient containing 50 mM Tris-Cl, pH 7.6, 25 mM KCl, 5 mM $MgCl_2$. The sucrose gradient consisted of a 3-ml linear 0.1–1.3 M sucrose gradient layered over 1.0 ml of 1.7 M sucrose and 0.5 ml of 2.4 M sucrose. The gradients were centrifuged for 4.5 h at 234,000 g_{av} in a Beckman SW 50.1 rotor. The gradients were separated into 225-μl fractions with a density gradient fractionator (ISCO, Lincoln, NE). Liposome-bound ribosomes were recovered in the linear portion of the gradient (fractions 2–12), with the location of the peak fraction dependent upon the amount of ribosomes bound to the liposomes. Unbound ribosomes were recovered in the 1.7 M sucrose cushion with the peak in fraction 15 or 16. Quantitation of the amount of bound and unbound ribosomes was done by gamma counting of the gradient fractions. Analysis of the binding data was by the method of Scatchard (1949).

Gel Filtration Chromatography

K-RM were solubilized at 1 eq/μl in 50 mM Tris-Cl, pH 8.5, 500 mM KCl, 5 mM $MgCl_2$, 1% sodium cholate on ice for 20 min. After centrifugation of the solubilized proteins for 2 h at 165,000 g_{av}, the supernatant was removed and this cholate extract of K-RM was applied by reverse flow at 3.0 ml/h to a 0.5 × 20 cm (4 ml) Sephacryl S-300 (Pharmacia Fine Chemicals, Piscataway, NJ) column equilibrated with the same buffer. 27 fractions of 200 μl each were collected.

Immunoblot and Con A Blot Detection of Proteins

Samples obtained in crosslinking experiments were subjected to SDS-PAGE, transferred to Immobilon membranes (Millipore Corporation, Bedford, MA), and probed with either polyclonal rabbit antisera or mAbs that recognize previously identified translocation components. Binding of mouse and rabbit antibodies to the antigens was visualized with the appropriate HRP coupled second antibody as described previously (Connolly and Gilmore, 1989). Immobilon membrane transfers of SDS polyacrylamide gels were probed for the ability to bind Con A in an HRP-coupled assay as previously described (Evans et al., 1986).

Results

Selective Crosslinking of Microsomal Membrane Proteins to Endogenous Ribosomes

Rough microsomal membranes contain endogenous protein translation and translocation activities. Although loosely associated ribosomes can be removed by extraction with a high ionic strength solution, ribosomes that were actively engaged in translocation when the membranes are isolated remain tethered to the membrane by a nascent polypeptide (Adelman et al., 1973). We have utilized a crosslinking assay to attempt to identify membrane proteins that function as a binding site for endogenous ribosomes engaged in protein translocation across the ER. The water-soluble, membrane-impermeable, thiol-cleavable, crosslinker DTSSP was selected to limit reaction to protein domains located on the cytoplasmic face of microsomal membrane vesicles.

Rough microsomal membranes were extracted with 0.5 M KOAc to remove peripheral proteins and nontranslocating ribosomes. These salt-extracted membranes (K-RM) were then suspended in a buffer lacking DTT to prevent premature reduction of the crosslinking reagent. The DTT-free K-RM were incubated at 25°C with 0.25 mM DTSSP. After a 30-min reaction, a 28-fold excess of ethanolamine was added to scavenge residual crosslinker. Solubilization of the membrane proteins with the nonionic detergent Nikkol in the presence of high salt (0.5 M KOAc) followed by airfuge centrifugation allowed the separation of ribosomes from uncrosslinked proteins. The first supernatant (Fig. 1, *S*1) contains the detergent-soluble proteins that had not been crosslinked to endogenous ribosomes. Although some reduction in staining intensity of proteins in the S1 fraction was seen after incubation of K-RM with DTSSP (Fig. 1, compare lanes *b* and *c*), most K-RM proteins were still recovered in the S1 fraction after crosslinker treatment. The S2 sample (Fig. 1, lanes *d* and *e*) contains proteins that were recovered in a supernatant fraction when the ribosomal pellet was resuspended and subsequently centrifuged under nonreducing conditions. Resuspension of the ribosomal pellet from this second centrifugation step in a detergent-high salt solution containing DTT allowed the reduction of the disulfide bond in the DTSSP. Centrifugation of the DTT-treated samples yielded a final ribosomal pellet fraction designated P3 and a supernatant fraction (S3) that contains proteins that were released from the ribosome by cleavage of the crosslinker. A subset of RER proteins with apparent molecular masses of 180, 95, 87, 74, 65, and 35 kD were observed in the S3 fraction when the K-RM had been treated with the crosslinking reagent (Fig. 1, lane *i*). A second group of low molecular weight proteins, apparently ribosomal in origin, were recovered in the S3 fraction from both control and crosslinker-treated K-RM (Fig. 1, lanes *h* and *i*). Since the S3 fraction has over three times as many membrane equivalents loaded on to the gel as the P3 frac-

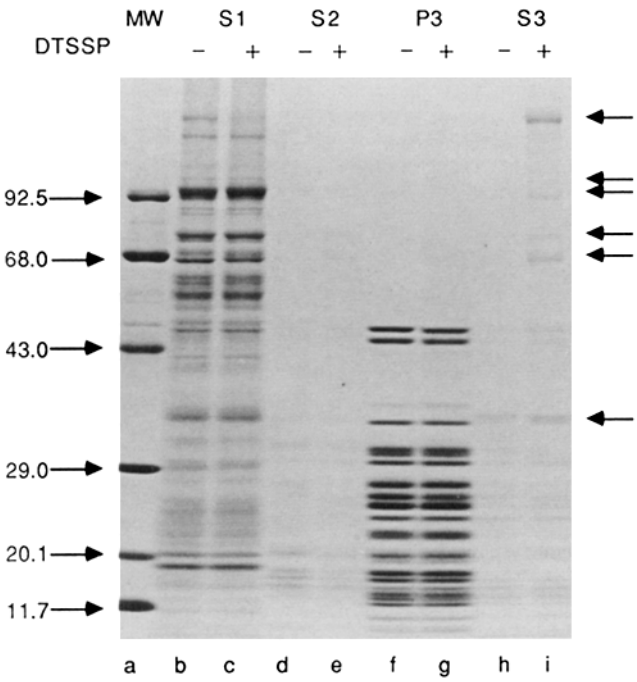

Figure 1. A subset of RER proteins can be crosslinked to membrane bound ribosomes. An SDS-polyacrylamide gel of the fractions that were obtained in a crosslinking experiment was stained with Coomassie blue. DTT-free K-RM (2 eq/μl) were adjusted to 5 mM sodium phosphate (*b*, *d*, *f*, and *h*) or to 0.25 mM DTSSP in 5 mM sodium phosphate (*c*, *e*, *g*, and *i*). After crosslinking as described in Materials and Methods, the DTSSP was quenched and the membranes were solubilized by adjustment to 1% Nikkol, 0.5 M KOAc. Detergent solubilized proteins (*S1*, lanes *b* and *c*) were separated from a ribosomal pellet containing crosslinked proteins by airfuge centrifugation as described in Materials and Methods. The pellet was resuspended in a nonreducing buffer and centrifuged under identical conditions to yield a wash supernatant (*S2*, lanes *d* and *e*). The washed pellet was resuspended, the disulfide bond in the crosslinker was cleaved with DTT, and the samples were centrifuged to separate ribosomes (*P3*, lanes *f* and *g*) from a final supernatant containing proteins that were crosslinked to the ribosome (*S3*, lanes *h* and *i*). The arrows designate proteins that appear in the S3 fraction when the crosslinker was present. Samples for electrophoresis were derived from K-RM with the following relative stoichiometry of membrane equivalents: S1, 1.0; S2, 8.0; P3, 2.5; S3, 8.0.

tion, we estimate that the ribosomal protein contamination of the S3 sample is ~1–2% of the total ribosomal protein present in the starting K-RM (Fig. 1, compare lanes *f* and *g* with lanes *h* and *i*). Unfortunately, these ribosomal proteins obscure the lower portion of the gel, so we were not able to determine whether low molecular weight proteins of nonribosomal origin were also present.

Proteins recovered in the S3 fraction are not necessarily directly crosslinked to the ribosome. An examination of the DTSSP concentration dependence of crosslink formation should allow the determination of which proteins deserve further scrutiny. Proteins that are recovered in the S3 fraction at low crosslinker concentrations are likely to be directly crosslinked to the ribosome, whereas proteins that are recovered in the S3 fraction only at a very high concentration may be linked to the ribosome via an intermediate protein. Titration of DTSSP between 0.01 and 2.0 mM indicated that many proteins were not crosslinked to the ribosome even at high concentrations (Fig. 2, lanes *b–g*). Inspection of the S3 fractions revealed that there was a diversity in the concentration of crosslinker required to link a significant amount of the proteins identified in Fig. 1 to the ribosome (Fig. 2, lanes *i–n*). Relatively constant amounts of ribosomal proteins (10–33 kD) were recovered in the S3 fraction at all crosslinker concentrations, thus confirming their appearance in this fraction as artifactual. High DTSSP concentrations were required to crosslink a 35- and a 65-kD product to the ribosome. In both cases, the majority of these proteins remained uncrosslinked in the S1 fraction (Fig. 2, lane *g*). Efficient crosslinking with DTSSP probably requires close contact as well as a favorable disposition of lysyl residues in both the target protein and the ribosome. In contrast, essentially all of a 180-kD protein was crosslinked to the ribosome at 0.5 mM DTSSP (Fig. 2, compare lanes *b–d* with lanes *e–g*). Of the major proteins recovered in the S3 fraction, the 180-kD protein was the most readily crosslinked to the ribosome. The apparent increase in molecular weight that this protein shows at 2.0 mM DTSSP is probably a result of extensive modification of the protein with the crosslinking reagent.

Identification of Several Crosslinked Proteins as Known and Proposed Translocation Components

Samples from a typical crosslinking experiment were analyzed by protein immunoblotting to determine whether any of the abundant proteins in the S3 fraction could be positively identified (Fig. 3). S1, P3, and S3 fractions were prepared after treatment of K-RM with 0.0, 0.2, or 2.0 mM DTSSP. Immunoblotting using an mAb to ribophorin I (Yu et al., 1990) demonstrated that the 65-kD protein in S3 fractions of Figs. 1 and 2 comigrated with ribophorin I (data not shown). Furthermore, the immunoblot demonstrated that a high concentration of crosslinker was required before there was a noticeable decrease in the amount of ribophorin I in the S1 fraction (Fig. 3, lane *c*) and a significant quantity of the protein in the S3 fraction (Fig. 3, lane *i*). Antibodies to the α and β subunits of the signal recognition particle receptor (SRα and SRβ) (Tajima et al., 1986) revealed that this protein complex is more readily crosslinked to the ribosome than ribophorin I. The majority of SRα and SRβ was depleted from the S1 fraction after reaction with 2.0 mM DTSSP (Fig. 3, lane *c*). The receptor subunits were recovered in both the P3 fraction (lane *f*) and the S3 fraction (lane *i*), presumably due to incomplete cleavage of the disulfide bond in the crosslinking reagent. Further reduction of the samples before electrophoresis reversed these residual crosslinks. SRα migrated between the major 65- and 74-kD proteins in the S3 fraction when the blot was compared to the Coomassie-stained gel (data not shown). Protein immunoblotting with antibody against the 35-kD subunit of the signal sequence receptor (αSSR) (Hartmann et al., 1989) demonstrated that the 35-kD polypeptide observed in Figs. 1 and 2 comigrated with αSSR (data not shown). Although αSSR was more readily crosslinked to the ribosome than ribophorin I, we estimate that no more than 25% of this protein was linked to the ribosome after treatment with 2.0 mM DTSSP.

The two subunits of SSR do not dissociate in nonionic detergent-high ionic strength solutions (Görlich et al.,

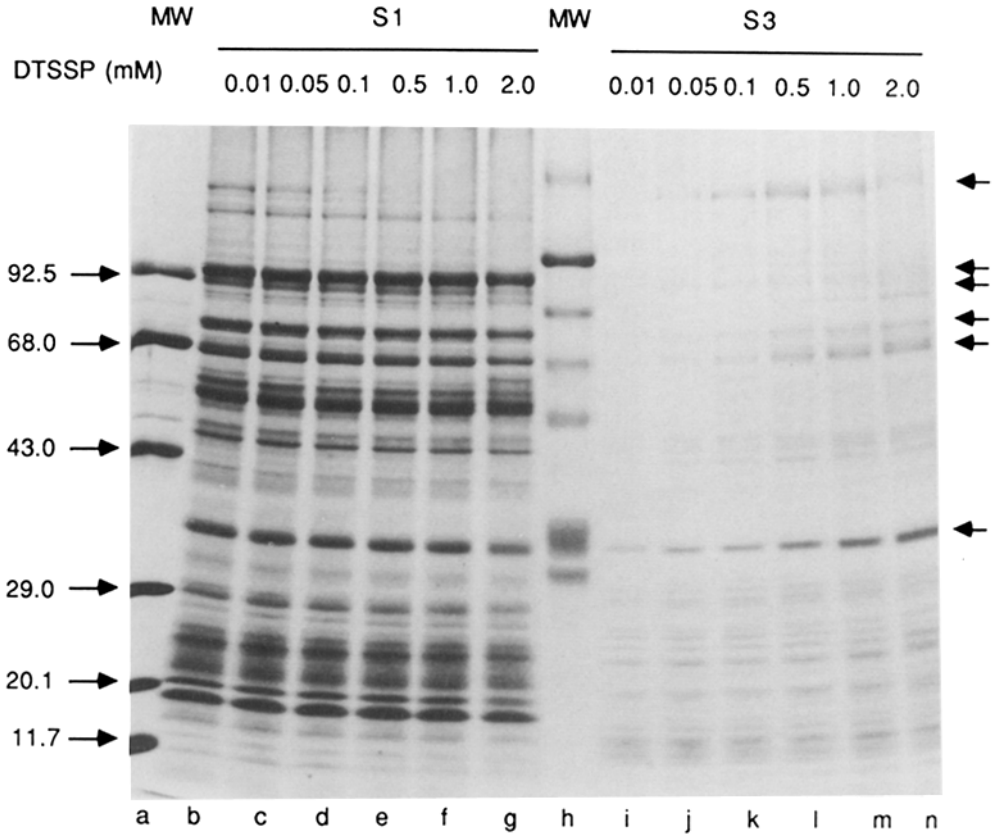

Figure 2. Titration of DTSSP shows diverse crosslinking efficiency of membrane proteins to the ribosome. DTT-free K-RM (2 eq/μl) were incubated with DTSSP at a final concentration between 0.01 and 2.0 mM. Supernatant fractions containing noncrosslinked proteins (*S1*, lanes *b–g*) and proteins crosslinked to the ribosome (*S3*, lanes *i–n*) were generated as in Fig. 1. The polypeptides were resolved by PAGE in SDS and stained with Coomassie blue. The arrows designate proteins that were recovered in the S3 fraction as the crosslinker concentration was raised and correspond to the proteins designated by arrows in Fig. 1. The prestained protein molecular weight markers in lane h migrated anomalously as compared to the standards in lane a. The S3 samples were derived from eight times as many membrane equivalents as S1 samples.

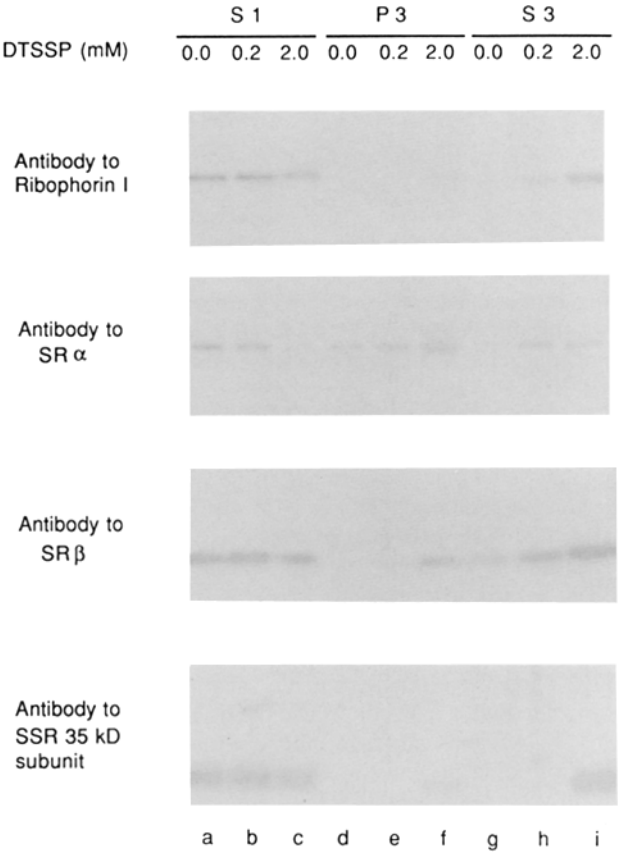

Figure 3. Identification of translocation components in the crosslinked samples by protein immunoblotting. The S1, P3, and S3 fractions for immunoblotting were prepared as in Fig. 1 using final DTSSP concentrations of 0.0 mM (lanes *a*, *d*, and *g*), 0.2 mM (lanes *b*, *e*, and *h*) or 2.0 mM (lanes *c*, *f*, and *i*). Each sample was divided into four aliquots; one each for ribophorin I, the 35-kD subunit of the signal sequence receptor (*SSR*), one for a combination immunoblot of the α and β subunits of the SRP receptor (*SR*) and one for the Con A blot in Fig. 4. Proteins resolved by PAGE in SDS were transferred to Immobilon membranes and probed with antibodies as described in Materials and Methods. The stoichiometry of the various samples is such that P3 has 2.5 times and S3 has 20 times as many membrane equivalents loaded into each lane as S1.

1990), suggesting that the S3 fraction should contain βSSR in addition to αSSR. To obtain a more direct confirmation for the presence of the glycosylated β subunit of SSR in the S3 fraction, aliquots of the S1, P3, and S3 fractions that were analyzed above by protein immunoblotting were also probed with Con A after transfer to Immobilon sheets (Fig. 4). The majority of the RER glycoproteins were recovered in the S1 samples (Fig. 4, lanes *a–c*) both in the absence and presence of the crosslinking reagent. Several glycoproteins of the RER were unique to the S1 fractions (Fig. 4, *asterisks*, lanes *a–c*) and hence did not crosslink to the ribosome under the conditions used. The major RER glycoproteins that were crosslinked to the ribosome correspond to ribophorin I (65 kD) and both the α (35 kD) and β (24 kD) subunits of SSR (*arrows* on the right, top to bottom, respectively). The less abundant glycoprotein that migrates immediately beneath βSSR in the S1 fraction is tentatively identified as the 22/23-kD subunit of the signal peptidase complex (see Fig. 3 from Evans et al., 1986). Several glycoproteins that migrated

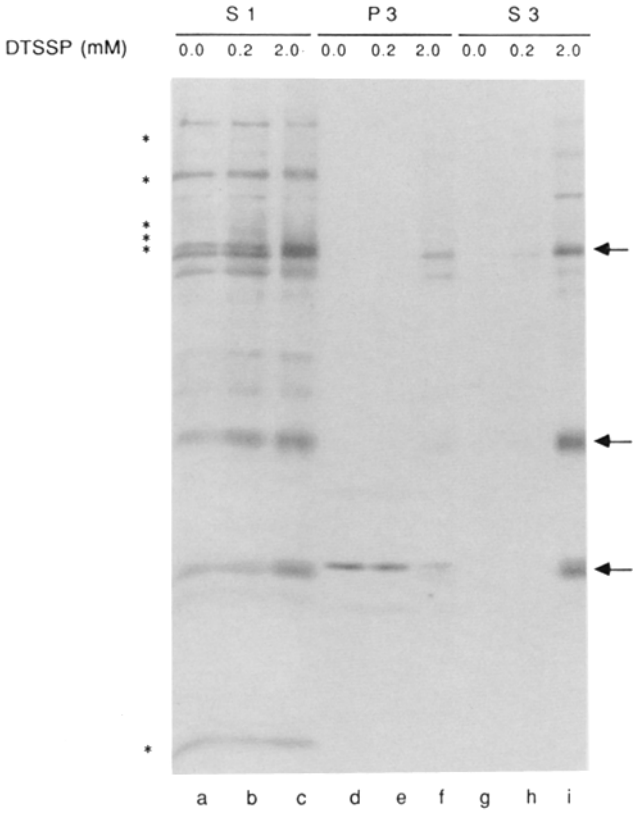

Figure 4. A subset of RER glycoproteins can be crosslinked to the ribosome. The S1, P3, and S3 fractions for this blot were generated as described in Figs. 1 and 3. The top arrow on the right designates ribophorin I (65 kD), as identified in Fig. 3. The lower two arrows on the right indicate the α (35 kD) and β (24 kD) subunits of the SSR. The asterisks on the left indicate several RER glycoproteins that do not crosslink to the ribosome and hence only appear in S1. Polypeptides that appear in lanes d and e are abundant ribosomal proteins that apparently bind Con A in a nonspecific manner.

more slowly than ribophorin I were also observed in the S3 fraction. None of these latter polypeptides comigrated with the 180-kD protein detected in Figs. 1 and 2 (data not shown), and none were crosslinked to the ribosome with a comparable efficiency; hence we concluded that the 180-kD protein is not a glycoprotein. K-RMs were treated with sodium carbonate (pH 11) to determine whether the 180-kD protein was an integral membrane protein (Fujuki et al., 1982). Although several other high molecular mass proteins were extracted by alkaline treatment, the 180-kD protein sedimented with the membrane pellet, as is characteristic for an integral membrane protein (data not shown). While this work was in progress, a 180-kD nonglycosylated integral membrane protein from canine pancreas rough microsomal membranes was proposed to be the RER ribosome receptor (Savitz and Meyer, 1990). A comparison of Figs. 1 and 2 with the Coomassie blue–stained polyacrylamide gel of the ribosome receptor purification (see Fig. 4 from Savitz and Meyer, 1990) indicated that the protein designated as the 180-kD protein corresponds to the most slowly migrating major polypeptide present in both membrane preparations. Based upon the criteria of identical mobility on polyacrylamide gels, lack of glycosylation, and retention in the membrane after alkaline extraction, we conclude that the 180-kD protein identified here is identical to the polypeptide that was recently proposed to be the ribosome receptor.

Differential Solubilization Separates the 180-kD Protein from Ribosome Binding Activity

The 180-kD protein could be quantitatively crosslinked to the ribosome by low concentrations of DTSSP. Interestingly, this protein appeared to be comparable in abundance to membrane-bound ribosomes based upon staining intensity with Coomassie blue. Although these observations indicated that the 180-kD protein seemed like a reasonable candidate for the ribosome receptor, functional studies of the ribosome binding activity of the 180-kD protein were required to more directly address this possibility. Previously reported data indicate that intact rat liver microsomal membranes that have been stripped of endogenous ribosomes will bind 40–75 nmol of 80S ribosomes per gram of microsomal membrane protein when assayed under low ionic strength conditions (Amar-Costesec et al., 1984; Borgese et al., 1974; Yoshida et al., 1987). Scatchard analysis of these binding data yielded K_d values that ranged between 3 and 16 nM. Ribosome-stripped canine pancreas microsomal membranes bind 120–152 nmol of 80S ribosomes per gram of microsomal membrane protein with a K_d of 19 nM (Hortsch et al., 1986). Yoshida et al. (1987) developed a proteoliposome reconstitution assay to measure the ribosome binding activity in detergent extracts of microsomal membranes. Extraction of microsomal membranes first with a nonionic detergent (Emulgen 913) at low ionic strength, followed by extraction of the membrane residue with deoxycholate, yielded a fraction that contained ribosome binding activity. The binding affinity of the reconstituted receptor for 80S ribosomes (K_d of 1 nM) was somewhat higher than that reported for the intact rat liver membrane (Yoshida et al., 1987).

We used a modification of the extraction and reconstitution procedure to prepare two detergent extracts that differed markedly in the content of the 180-kD protein. To prepare both types of extracts, microsomal membrane proteins were initially treated with 1% Nikkol at low ionic strength. Centrifugation of the detergent-treated membranes through a sucrose cushion yielded a Nikkol-insoluble residual membrane pellet consisting of a subset of RER membrane proteins and ribosomes. When this pellet was resuspended with a 1% sodium deoxycholate-low salt solution as described (Yoshida et al., 1987) and then centrifuged, we obtained a detergent-insoluble pellet that was enriched in ribosomes (Figure 5 *A*, lane *a*) and a deoxycholate extract containing a subset of RER proteins (Fig. 5, lane *b*). Coomassie blue staining of the SDS-polyacrylamide gel of these fractions indicated that the 180-kD ribosome receptor was not present in the deoxycholate extract (Fig. 5, lane *b*), but was recovered entirely in the ribosome-enriched pellet (Fig. 5, lane *a*). Alternatively, when the Nikkol-insoluble membrane residue was resuspended with 1% sodium cholate at high ionic strength (0.5 M KOAc) and centrifuged as above, the fractionation behavior of some proteins was markedly altered. Most significantly, some of the 180-kD protein was now recovered in the cholate extract (Fig. 5, lane *c*), while the balance of the protein still sedimented with the ribosomes (Fig. 5, lane *d*).

The deoxycholate and cholate extracts were reconstituted

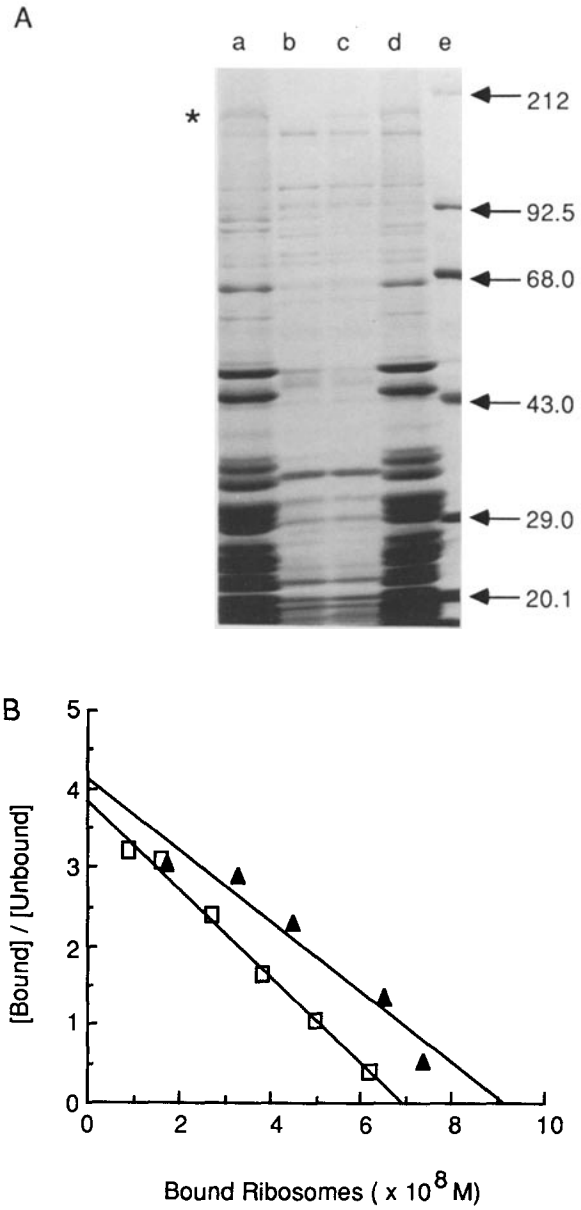

Figure 5. Ribosome binding to reconstituted liposomes: lack of correlation to the 180-kD protein. (*A*) A Coomassie blue-stained polyacrylamide gel indicates the protein composition of fractions used to prepare liposomes. An aliquot of K-RM was solubilized in a low salt, 1% Nikkol buffer and then centrifuged. The pellet was resuspended in either low salt, 1% sodium deoxycholate (lanes *a* and *b*) or high salt, 1% sodium cholate (lanes *c* and *d*). Both resuspended samples were centrifuged as described in Materials and Methods to obtain detergent extracts (lanes *b* and *c*) and ribosomal pellets (lanes *a* and *d*). The asterisk indicates the position of the 180-kD protein. (*B*) Liposomes reconstituted from either a deoxycholate extract (110 μg of protein each) or a cholate extract (60 μg of protein each) were incubated with 0.10 pmol of ^{125}I-labeled canine ribosomes and between 2.1 and 42.7 pmol of unlabeled ribosomes in 200 μl of 35 mM triethanolamine-OAc, pH 7.5, 70 mM KOAc, 3.5 mM $Mg(OAc)_2$. Ribosomes bound to liposomes were separated from unbound ribosomes by centrifugation and fractionation as described in Materials and Methods. Scatchard analysis of the binding data indicated a K_d of 18 nM for liposomes prepared from the cholate extract (□) and a K_d of 22 nM for liposomes prepared from the deoxycholate extract (▲).

into egg yolk phosphatidylcholine liposomes using a detergent dialysis method (Yoshida et al., 1987). The efficiency of the reconstitution procedure was evaluated by separating the reconstituted proteoliposomes from unincorporated proteins by sucrose density gradient centrifugation. When the reconstituted proteoliposomes were analyzed by PAGE in SDS, the staining intensity of the 180-kD protein was equivalent to that present in the detergent extract before reconstitution (data not shown). This analysis demonstrated that the 180-kD protein was not proteolyzed during dialysis, and that the 180-kD protein was efficiently incorporated into the proteoliposomes. The ribosome binding activity of the reconstituted liposomes was quantitated by incubating the liposomes with a constant amount of ^{125}I-labeled ribosomes in the presence of increasing amounts of unlabeled ribosomes. The ability of the unlabeled ribosomes to bind and compete with the labeled ribosomes was determined as described in Materials and Methods. As shown in Fig. 5 *B*, liposomes reconstituted from the cholate extract (Fig. 5, □) as well as the deoxycholate extract (Fig. 5, ▲) bound ribosomes in a saturable manner. When normalized to the quantity of microsomal membranes from which the extracts were prepared, liposomes reconstituted from the cholate extract bound roughly comparable (only 1.6-fold more) ribosomes than liposomes prepared from the deoxycholate extract. Scatchard analysis (1949) of the binding data indicated that the affinity of ribosomes for the liposomes prepared from the cholate and deoxycholate extracts was experimentally indistinguishable (K_d of 18 and 22 mM, respectively). As described above, these dissociation constants are similar to those obtained by previous investigators in studies using intact membranes (Amar-Costesec et al., 1984; Borgese et al., 1974; Hortsch et al., 1986; Yoshida et al., 1987).

Gel Filtration Separates the 180-kD Protein from Ribosome Binding Activity

The preceding experiment did not indicate a positive correlation between the presence of the 180-kD protein and measurable ribosome binding activity. However, both extraction methods used in Fig. 5 yielded detergent extracts that contained <50% of the ribosome binding activity reported for intact canine pancreatic microsomal membrane vesicles (Hortsch et al., 1986). The low recovery could be due to a number of factors, including an incomplete extraction or partial inactivation of the ribosome receptor during sequential detergent extraction. To determine whether the majority of the ribosome binding activity present in microsomal membrane vesicles could nonetheless be ascribed to the 180-kD protein, intact membranes were solubilized with 1% sodium cholate under high ionic strength conditions to obtain a total cholate extract. The total cholate extract was resolved into 27 fractions by gel filtration chromatography and the protein constituents of the individual fractions were disclosed by Coomassie blue staining after PAGE in SDS (Fig. 6 *B*). It was observed both visually on the gel and quantitatively by densitometric scanning (Fig. 6 *A*, ■) that the 180-kD protein was most abundant in fraction 12 and absent from all fractions after 15. Aliquots of fractions 11–20 were tested individually for ribosome binding activity after reconstitution into egg yolk phosphatidylcholine liposomes. Our results indicate that ribosome binding was maximal in fraction

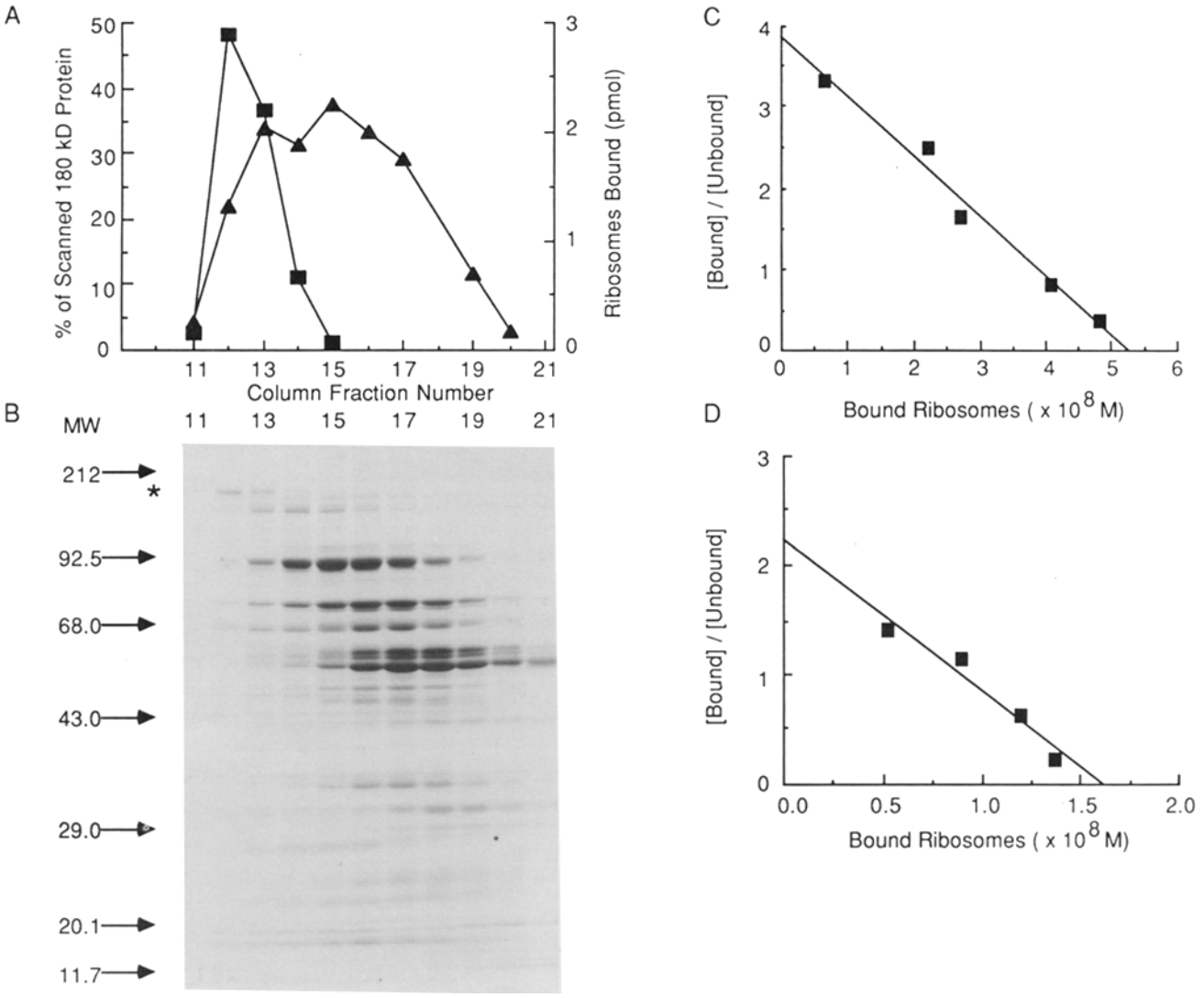

Figure 6. Ribosome binding activity and the 180-kD protein can be resolved by gel filtration chromatography. K-RMs were solubilized in high salt, 1% sodium cholate and centrifuged to obtain a ribosome-free extract which was applied to a 4-ml Sephacryl S-300 column. (*B*) The protein composition of fractions 11 through 21 was analyzed by Coomassie blue staining after 60 μl of each fraction was resolved by SDS-PAGE. The 180-kD protein is designated by an asterisk. (*A*) The distribution of the 180-kD protein in the column eluate was determined by densitometric scanning of the gel in *B* (■). Aliquots (50 μl) of the indicated column fractions were reconstituted into liposomes as described in Materials and Methods in a total volume of 200 μl. The reconstituted liposomes were incubated at 0°C with 0.08 pmol of labeled ribosomes and 6.4 pmol of unlabeled ribosomes. The quantity of liposome-bound ribosomes (▲) was determined as described. (*C*) 250 μl (250 eq) of the cholate extract was reconstituted into liposomes in a total volume of 1 ml. Five aliquots of the reconstituted liposomes were incubated at 0°C with 0.12 pmol of labeled ribosomes and the following quantities of unlabeled ribosomes: 2.1, 8.6, 12.8, 21.4, or 42.7 pmol. The quantity of liposome-bound ribosomes was determined as described. Scatchard analysis of the binding data indicated a K_d of 13 nM for liposomes prepared from the cholate extract of K-RM. (*D*) 400 μl of column fraction 17 was reconstituted into liposomes in a total of 800 μl. Four aliquots of the reconstituted liposomes were incubated at 0°C with 0.12 pmol of labeled ribosomes and the following quantities of unlabeled ribosomes: 2.1, 4.3, 8.6, or 21.4 pmol. The quantity of liposome-bound ribosomes was determined as described. Scatchard analysis of the binding data indicated a K_d of 7 nM.

15, with significant amounts of binding in fractions 13 through 17 (Fig. 6 *A*, ▲). Clearly, ribosome binding activity did not cofractionate with the 180-kD protein (Fig. 6 *A*). Scatchard analysis of the ribosome binding activity of the total cholate extract yielded a K_d of 13 nM (Fig. 6 *C*) which, again, was in excellent agreement with previously reported affinity constants for ribosome binding activity. The reconstituted liposomes contained 260 fmol of ribosome binding sites per equivalent of starting K-RM. This value can be converted to 108 nmol of binding sites per gram of membrane protein based on a protein concentration of 2.4 mg/ml for ribosome-stripped K-RM. After consideration of the likely nonasymmetric reconstitution of ribosome receptors into the liposomes, we conclude that this value is in reasonable agreement with the value of 120–152 nmol of binding sites per gram of membrane protein reported for intact puromycin, high salt-stripped canine pancreas microsomal membranes (Hortsch et al., 1986). Scatchard analysis of the ribosome

binding activity present in fraction 17 (Fig. 6 *D*), a sample that contained no detectable 180-kD protein, revealed readily detectable ribosome binding activity with an affinity constant (K_d = 7 nM) that is comparable to the unfractionated extract. We conclude that the 180-kD protein is dispensable for ribosome binding activity.

Based upon the possibility that cholate or deoxycholate might inactivate the 180-kD protein, we solubilized K-RM with octyl glucoside. The ribosome binding activity that was reconstituted from octyl glucoside extracts of K-RM had an affinity (K_d = 9 nM) that was comparable to that obtained from the total cholate extract. In results that confirm those shown above using gel filtration chromatography, it was found that the ribosome binding activity did not cofractionate with the 180-kD band when the octyl-glucoside extract was resolved using a Mono-Q cation exchange column (data not shown).

Discussion

We have used a chemical crosslinking reagent in an effort to identify proteins that are in the vicinity of the endogenous membrane-bound ribosomes of the ER. The crosslinking reagent that was selected for this study is a membrane-impermeable, primary amine reactive reagent with a crosslinking span of 12Å. Polypeptides that are anticipated to be within 12Å of a membrane-bound ribosome would include proteins that participate directly in nascent polypeptide transport, protein modification enzymes such as signal peptidase, or proteins involved in ribosome targeting and attachment to the ER.

Several experimental limitations of the crosslinking procedure were apparent, and deserve further consideration. Proteins that lack a lysyl residue within 12Å of a complementary lysyl residue on the ribosomal surface will not be detected by this procedure. Consequently, proteins that are not crosslinked to the ribosome by DTSSP may still be in direct contact with the ribosome. A second limitation of the crosslinking procedure concerned the detection of proteins with molecular masses less than 35 kD. Contamination of the S3 fraction with ribosomal proteins prevented the identification of novel proteins in this molecular weight range. Although we were able to confirm the presence of both subunits of SSR by immunoblotting and Con A blotting, other proteins of less than 35 kD were not readily detectable. A third limitation of the crosslinking procedure is the potential for indirect linkage of a protein to the ribosome via an intermediate protein. Complex products consisting of multiple crosslinked proteins are more likely to arise upon treatment of K-RM with high concentrations of crosslinker.

A restricted subset of RER proteins was reproducibly crosslinked to the ribosome using DTSSP. The identity of several of these polypeptides was confirmed by protein immunoblotting. Based upon the role of the SRP receptor in targeting of SRP-ribosome complexes to the membrane surface (Gilmore et al., 1982; Meyer et al., 1982; Walter and Blobel, 1981), it was not surprising that the receptor can be crosslinked to membrane-bound ribosomes. We cannot determine which subunit of the receptor was crosslinked directly to the ribosome, since the SRP receptor does not dissociate into subunits under the ionic strength conditions used in the crosslinking procedure (Tajima et al., 1986). The signal sequence receptor (SSR) was also found to be crosslinked to the ribosome with DTSSP. SSR was initially identified as an integral membrane glycoprotein that can be crosslinked to the signal sequence of a nascent polypeptide undergoing translocation across the ER (Wiedmann et al., 1987). Further research demonstrated that SSR (or mp39) is in direct contact with nascent polypeptides at early and late stages of the transport process (Kireg et al., 1989). The results presented here provide additional, albeit indirect, support for these previous conclusions, by indicating that SSR is in the immediate vicinity of the ribosome. As in the case of the SRP receptor, we do not know which subunit of SSR has been crosslinked to the ribosome, since the two subunits do not dissociate in nonionic detergent, high ionic strength solutions (Görlich et al., 1990; Hartmann et al., 1989). The signal peptidase complex did not appear to be readily crosslinked to the ribosome by DTSSP. Although the Coomassie blue–stained gels did not provide definitive results on this point, the glycosylated 22/23-kD subunits of signal peptidase appeared to be in the S1 rather than in the S3 fraction by Con A blotting. To date, the sequences of three of the five subunits of the canine signal peptidase complex have been determined (Greenberg et al., 1989; Shelness and Blobel, 1990; Shelness et al., 1988). The proposed membrane orientation of the 18-, 21-, and 22/23-kD subunits of the signal peptidase complex places the bulk of the polypeptide mass of each protein within the RER lumen (Greenberg et al., 1989; Shelness and Blobel, 1990; Shelness et al., 1988). The short cytoplasmic domains of these three polypeptides contain a total of two lysyl residues (Greenberg et al., 1989; Shelness and Blobel, 1990; Shelness et al., 1988); consequently crosslinking of these polypeptides to the ribosome with DTSSP is likely to be unfavorable.

Previous research has shown that ribophorins I and II could be crosslinked to the ribosome using several different crosslinking reagents (Kreibich et al., 1978*a*). This observation was confirmed here for ribophorin I by protein immunoblotting. Although the ribophorins are dispensable for ribosome binding (Hortsch et al., 1986; Yoshida et al., 1987), recent results demonstrate that antibodies that recognize the cytoplasmically exposed domain of ribophorin I inhibit targeting of SRP-ribosome complexes to the RER membrane (Yu et al., 1990). The authors suggested two possible explanations for this result: (*a*) ribophorins directly participate in targeting of SRP-ribosome complexes to the membrane; or (*b*) ribophorins are so close to the ribosome binding site that the antibodies sterically interfere with the SRP-SRP receptor interaction. Although the original crosslinking experiments contributed to the premature conclusion that the ribophorins were the ribosome receptor, both the crosslinking data and the antibody inhibition data support a role for the ribophorins at some stage during the protein translocation and assembly process.

Interactions between 80S ribosomes and an RER ribosome receptor have been quantitated using intact microsomal membranes and reconstituted liposomes (Amar-Costesec et al., 1984; Borgese et al., 1974; Hortsch et al., 1986; Jothy et al., 1975; Savitz and Meyer, 1990; Yoshida et al., 1987). In vitro ribosome binding assays measure the attachment of a nontranslating ribosome to the membrane surface under subphysiological ionic strength conditions (25–50 mM KOAc). Consequently, nonspecific ionic interactions between the ribosome

and cytoplasmic domains of RER proteins may contribute to both the measured affinity and the quantity of ribosome binding sites. This problem may be exacerbated when ribosome binding to proteoliposomes is assayed, because nonasymmetric reconstitution of membrane proteins will lead to the exposure of luminal protein domains on the exterior surface of the liposome. Therefore, results obtained in the proteoliposome reconstitution assays must be scrupulously compared to the results of binding studies conducted with intact membranes. The stoichiometry between bound ribosomes and the reconstituted ribosome receptor should not exceed the stoichiometry between in vivo bound ribosomes and the receptor in the intact membrane. If this latter condition is not met, substantial concern about nonspecific ribosome–protein interaction is justified. In addition, the binding affinity, the number of binding sites solubilized and reconstituted, the salt sensitivity, and the protease sensitivity of the ribosome receptor in intact membranes are parameters that should be replicated by the reconstituted proteoliposome. Adherence to these criteria would suggest that a putative ribosome receptor is responsible for ribosome binding to intact RER membranes.

A central assumption in the quest for the ribosome receptor is that the in vitro ribosome binding assay is diagnostic of the ribosome–membrane junction that is assembled during a protein translocation reaction. Unfortunately, the validity of this assumption has not been rigorously examined. Deletion or selective inactivation of the ribosome receptor should lead to a disruption of translocation if the ribosome receptor is an essential component of the protein translocation apparatus. The inactivation of the ribosome receptor by trypsin digestion may not be sufficiently selective due to the trypsin sensitivity of the α subunit of the SRP receptor. Perhaps a more direct experimental approach would be to assemble translocation-competent proteoliposomes from sodium cholate–solubilized rough microsomes (Nicchitta and Blobel, 1990). Immunodepletion of a putative ribosome receptor from the cholate extract prior to reconstitution into liposomes should lead to a readily detectable loss in ribosome binding and translocation competence if the ribosome receptor is an essential component of the translocation apparatus. We assert that the demonstration of a correlation between ribosome binding activity and an essential role in translocation is a criteria that must be met by the ribosome receptor.

Recently, a 34-kD nonglycosylated protein was proposed to be the ribosome receptor (Tazawa et al., 1991). RER-derived fractions that were enriched in the 34-kD protein bound ribosomes after reconstitution into liposomes (Tazawa et al., 1991). Proteolytic digestion of the reconstituted liposomes severed the 34-kD protein with a concentration dependence that resembled the loss of ribosome binding activity present in the reconstituted liposomes (Tazawa et al., 1991). Although the DTSSP crosslinking and proteoliposome reconstitution studies we have described here do not address the ribosome binding properties of the 34-kD polypeptide, we feel that several of the criteria outlined above remain to be met before the 34-kD protein can be designated as the ribosome receptor for the ER. Specifically, further research will be required to determine whether liposomes containing a homogeneous preparation of the 34-kD protein bind ribosomes with an affinity and a stoichiometry that is comparable to that of intact rough microsomal membranes. Once these criteria have been met, a correlation between ribosome binding activity and a role in protein translocation remains to be established.

In addition to previously described translocation components, we observed that a major band of 180 kD was quantitatively recovered in the S3 fraction after membranes were treated with low concentrations of crosslinking reagent. While this work was in progress, the 180-kD protein was proposed to be the ribosome receptor (Savitz and Meyer, 1990). Proteolytic fragments derived from the 180-kD protein were shown to inhibit binding of ribosomes to puromycin-high salt-stripped microsomal membrane (Savitz and Meyer, 1990). Furthermore, purified preparations of the 180-kD protein bound ribosomes after reconstitution into phospholipid vesicles (Savitz and Meyer, 1990). In an effort to confirm that the 180-kD protein is the ribosome receptor of the ER, we measured the ribosome binding activity of proteoliposomes prepared using subpopulations of RER-derived proteins. Several different fractionation methods yielded complementary results that cast considerable doubt on the role of the 180-kD protein as the ribosome receptor. Ribosome binding activity was readily detectable in RER-derived fractions that were devoid of the 180-kD protein. Gel filtration chromatography revealed no correlation between the content of the 180-kD protein and ribosome binding activity. A similar conclusion concerning the ribosome binding activity of the 180-kD protein has been reached by other investigators who have used different fractionation methods from those described here to resolve ribosome binding activity from the 180-kD protein (Nunnari et al., 1991). The data we have presented here does not, by itself, rule out a function for the 180-kD protein at some stage during protein translocation. Like ribophorin I and II, the role of the 180-kD protein must await the development of an assay to measure the appropriate function.

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