Characterization of Secretory Protein Translocation: Ribosome–Membrane Interaction in Endoplasmic Reticulum

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Abstract. Secretory proteins are synthesized on ribosomes bound to the membrane of the endoplasmic reticulum (ER). After the selection of polysomes synthesizing secretory proteins and their direction to the membrane of the ER via signal recognition particle (SRP) and docking protein respectively, the polysomes become bound to the ER membrane via an unknown, protein-mediated mechanism. To identify proteins involved in protein translocation, beyond the (SRP-docking protein–mediated) recognition step, controlled proteolysis was used to functionally inactivate rough microsomes that had previously been depleted of docking protein. As the membranes were treated with increasing levels of protease, they lost their ability to be functionally reconstituted with the active cytoplasmic fragment of docking protein (DP_f). This functional inactivation did not correlate with a loss of either signal peptidase activity, nor with the ability of the DP_f to reassociate with the membrane. It did correlate, however, with a loss of the ability of the microsomes to bind ribosomes.

Ribophorins are putative ribosome-binding proteins. Immunoblots developed with monoclonal antibodies against canine ribophorins I and II demonstrated that no correlation exists between the protease-induced inability to bind ribosomes and the integrity of the ribophorins. Ribophorin I was 85% resistant and ribophorin II 100% resistant to the levels of protease needed to totally eliminate ribosome binding. Moreover, no direct association was found between ribophorins and ribosomes; upon detergent solubilization at low salt concentrations, ribophorins could be sedimented in the presence or absence of ribosomes.

Finally, the alkylating agent *N*-ethylmaleimide was shown to be capable of inhibiting translocation (beyond the SRP-docking protein–mediated recognition step), but had no affect on the ability of ribosomes to bind to ER membranes. We conclude that potentially two additional proteinaceous components, as yet unidentified, are involved in protein translocation. One is protease sensitive and possibly involved in ribosome binding, the other is *N*-ethylmaleimide sensitive and of unknown function.

THE transport of secretory proteins from their site of synthesis, the cytoplasm, to their final destination, the extracellular space, commences with the vectorial transfer of the nascent peptide across the membrane of the rough endoplasmic reticulum (ER)[1] (35). The use of in vitro systems has allowed the understanding of the first step involved in the translocation process, i.e., the recognition between the nascent chain and the membrane of the rough ER (for review see references 20 and 47). It is now well established that secretory proteins contain an NH_2-terminal signal peptide that on its emergence from the large ribosomal subunit, is recognized by a cytoplasmic receptor, the signal recognition particle (SRP) (45). SRP that is bound to the nascent chain–mRNA–ribosome complex is directed to the membrane via an interaction with a 73-kD rough ER–specific receptor, the docking protein (DP) (13, 14, 32). The synthetic machinery then becomes membrane associated and subsequently the nascent peptide is translocated across the membrane.

While the recognition process between the signal sequence and the ER membrane is fairly well characterized, very little is known about the mechanism and the components involved in the remainder of the translocation process. The involvement of other membrane proteins that aid the actual translocation of the nascent chain was proposed in the original version of the signal hypothesis (6, 7), and some evidence to support this notion has accumulated. Recently, a signal sequence (or nascent chain) receptor in the microsomal membrane was suggested (15, 36, 37). The binding of polysomes synthesizing secretory proteins to the rough ER membrane has also been postulated to play an important role in the vectorial transfer of the nascent polypeptide across the lipid

1. *Abbreviations used in this paper*: DP, docking protein; ER, endoplasmic reticulum; LSB, low salt buffer; NEM, *N*-ethylmaleimide; PMSF, phenylmethylsulfonyl fluoride; RMs, rough microsomes; SRP, signal recognition particle.

bilayer (38, 43) and indeed certain results suggest the existence of specific receptors for ribosomes in the membrane of the rough ER (8). Ribosomes are bound to the ER membrane via the nascent polypeptide chain as well as by an interaction that is salt labile (1, 42). Such binding is saturable, sensitive to proteases, and specific for the large (60S) ribosomal subunit (5, 8, 40).

Several microsomal membrane proteins have been put forward as ribosome receptors (4, 12, 23). Kreibich et al. identified two integral membrane glycoproteins with molecular masses of 65,000 and 63,000 daltons in rat liver rough microsomes (RMs) that were absent in smooth membranes (23). Several indirect lines of evidence led to the conclusion that these proteins are ribosome receptors. Upon solubilization of RMs with nonionic detergents at low salt concentrations, both proteins co-sedimented with the polysome fraction. Using bifunctional reagents to cross-link membrane proteins of RMs, these proteins were found associated with fractions containing ribosomes (24). Furthermore, a good stoichiometry exists between the number of these proteins present in rough membranes and the ribosome-binding capacity of microsomal vesicles (29). The proteins have been accordingly named ribophorin I and II (23, 24).

Proteolysis has provided a powerful tool for the inactivation and dissection of the microsomal machinery involved in protein translocation (30, 44). In this report we have used proteolytic enzymes for the selective dissociation of processes associated with the vectorial translocation of proteins that are independent of the SRP-DP–mediated recognition mechanism. Our results indicate that ribosome binding is not directly mediated by ribophorins, but involves a component having a greater sensitivity to proteases. Moreover, another component, not involved in ribosome binding, is necessary for translocation and is inactivated by alkylation with *N*-ethylmaleimide (NEM).

Materials and Methods

[^{35}S]Methionine, [5, 6-^{3}H]Uridine, ^{125}I-labeled second antibodies, and rabbit reticulocyte lysate were purchased from Amersham International, Buckinghamshire, England. Peroxidase-labeled goat anti-mouse or anti-rabbit Ig were from Dianova, Hamburg.

Proteases were obtained from the following companies: trypsin (EC 3.4.21.4), proteinase K (EC 3.4.21.14), and elastase (EC 3.4.21.11) from Merck, Darmstadt, FRG; pronase (EC 3.4.24.4) and subtilisin (EC 3.4.21.14) from Sigma Chemical Co., St. Louis, MO. Puromycin-dihydrochloride, nuclease from *Staphylococcus aureus* (EC 3.1.4.7), and aprotinin were purchased from Boehringer Mannheim, FRG. Phenylmethylsulfonyl fluoride (PMSF) and NEM were obtained from Sigma Chemical Co. Aurin tricarboxylic acid was from Serva, Heidelberg, FRG. Nikkol (octaethyleneglycol-mono-*N*-dodecyl ether) was from Nikko Chemicals Co., Ltd., Tokyo.

Antibodies

Monoclonal antibodies against ribophorins and the 68,000-dalton integral membrane protein were generated by immunization of female BALB/c mice with homogenized polyacrylamide gel pieces containing pancreatic canine ribophorins and subsequent fusion of spleen cells with Sp2-0 or NSO myeloma cells. Positive colonies were identified using the screening assay described previously (18). Ascites fluids from subcloned cell lines were produced in pristane-treated BALB/c mice. The generation and characterization of monoclonal antibodies against DP as well as the polyclonal rabbit antisera raised against canine ribophorins I and II were previously described (18, 19). Polyclonal rabbit anti-protein disulfide isomerase serum was a gift from R. Freedman and J. Paver (University of Kent, Canterbury).

PAGE and Immunoblotting Procedure

Proteins were analyzed by SDS PAGE on 10–15% gradient gels and visualized using the silver stain method of Ansorge (3). For fluorography gels were fixed in 10% trichloroacetic acid and treated with EN^{3}HANCE (New England Nuclear, Boston, MA) for 1 h. Gels were washed, dried, and placed in contact with Kodak X-Omat AR film at −80°C.

Immunoblots were performed and developed with diaminobenzidine (Sigma Chemical Co.) as described previously (18). For quantitation of immunoblots, the protocol of Burnette (10) was followed using ^{125}I-labeled second antibody at a concentration of 5×10^5 cpm/ml. Radioactively labeled proteins were cut from the nitrocellulose filters and counted in a γ counter. Background was determined by counting an equal area from an irrelevant region of the filter.

Preparation and Treatment of Microsomes

Dog pancreas was generously provided by Sandoz AG, Basel, Switzerland. RMs were prepared as described previously (7). RMs were washed with 0.5 M KCl (48) and treated with 1 mM puromycin/0.5 M KCl as described by Adelman et al. (1). Stripped microsomes were resuspended in a buffer containing 50 mM Tris/HCl, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, and 1 mM $CaCl_2$ and treated with micrococcal nuclease at a final concentration of 10 U/ml for 30 min at 20°C. The reaction was stopped by a fivefold dilution with high salt buffer (50 mM Tris/HCl, pH 7.5, 500 mM KCl, 5 mM $MgCl_2$) containing 2 mM EGTA. Stripped, nuclease-treated microsomes (RM_{SN}) were recovered by centrifugation at 37 krpm for 30 min at 4°C in a Ti 60 rotor, washed once in the high salt buffer, and finally resuspended in low salt buffer (LSB) (50 mM Tris/HCl, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$) to protein concentration of 10–20 mg/ml.

For proteolysis, 100 μl of RM_{SN} was diluted with 800 μl of protease buffer (50 mM TEA, pH 7.5, 0.25 M sucrose, 10 μM Nikkol, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 1 mM dithiothreitol). The reaction was started with 100 μl of protease solution prepared in protease buffer. After 1 h at 0°C, 50 μl of stop buffer was added (protease buffer without $CaCl_2$ containing 200 mM EGTA and 2 mg/ml PMSF). After 5 min at 0°C, another 50 μl of stop buffer was added and the membranes were pelleted by centrifugation for 1 h at 45 krpm in a Ti75 rotor through a cushion of protease buffer containing 0.5 M sucrose, 2 mM EGTA, and 10 μg/ml PMSF. In the case of trypsin, the stop buffer also contained 250 μg/ml aprotinin. The membrane pellet was finally resuspended in 200 μl LSB. Rough, salt-washed microsomes (RM_K) and trypsinized RM (RM_t) were carbonate washed according to the method of Fujiki et al. (11) with the modifications as described by Hortsch et al. (18).

Microsomes at a protein concentration of 4 mg/ml were treated with NEM in LSB for 30 min at 25°C. NEM was inactivated by the addition of 5 vol of LSB containing 50 mM dithiothreitol. Microsomes were pelleted for 1 h at 45 krpm at 4° in a Ti75 rotor and resuspended in LSB.

In Vitro Ribosome Binding

^{3}H-Labeled human ribosomes were prepared according to the protocol of Kreibich et al. (25) by labeling $5–10 \times 10^8$ HeLa cells for 2 d with 500 μCi of [5,6-^{3}H] uridine. Labeled ribosomes were isolated and resuspended in LSB to a final protein concentration of 4 mg/ml. The specific activity of the labeled ribosomes was ~4,500 cpm per μg of protein.

In vitro ribosome binding was carried out using a modification of the procedure described by Kreibich et al. (25). RM_{SN} (usually 100 μg protein) and ^{3}H-labeled ribosomes (usually 40 μg protein) were incubated in 30 μl LSB for 45 min at 0°C.

270 μl of 2.3 M sucrose in LSB were added and the sample was layered under a discontinuous sucrose gradient (1.9, 1.7, 1.5, 1.3, and 0 M sucrose in LSB with a final volume of 4 ml). After centrifugation (2.5 h at 4°C at 50 krpm in a SW60 rotor), the gradient was fractionated into five fractions plus a pellet fraction. All fractions were diluted with 1 ml water, received 2 mg of bovine serum albumin, and were precipitated with a final concentration of 10% trichloroacetic acid. Precipitated material was collected onto GF/C filters (Whatman Ltd., Maidstone, England) and counted in a liquid scintillation counter.

The top three fractions contained ribosomes bound to microsomal membranes, while the pellet and the two lower fractions contained unbound ribosomes. Based on an input of 40 μg ribosome protein, corresponding to 83 μg of ribosomes (17), the percentage of ^{3}H present in each fraction was used to determine the amount of ribosomes (in μg) bound per 100 μg of microsomal protein.

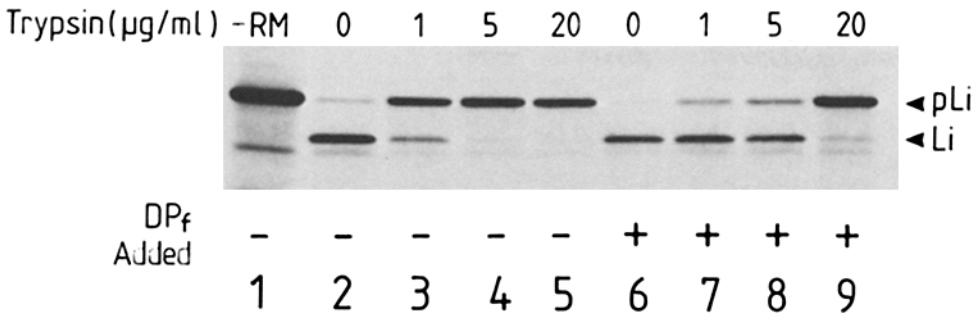

Figure 1. Inhibition of the reconstitution of light chain translocation and processing by proteolysis. Microsomes were proteolyzed and salt washed, and translations carried out as described in Materials and Methods. (Lane *1*) Translation of IgG-k mRNA in reticulocyte lysate. (Lanes *2–5*) As lane *1*, but supplemented with 4.8 A_{280}/ml rough microsomes previously trypsinized as indicated above the figure and subsequently washed with 500 mM KCl. (Lanes *6–9*) As lanes *2–5* but supplemented with DP_f (0.4 μg/ml).

Electron Microscopy

Pellets of microsomal preparations were fixed with 1% glutaraldehyde in 200 mM Pipes buffer, pH 7.0, for 1 h, impregnated with 1% OsO_4 in 100 mM cacodylate buffer, pH 7.4, and embedded in Epon. The sections were stained with uranyl acetate and lead citrate (16).

Analytical Methods

Protein was determined using the method of Bradford (9) and the RNA determination was carried out according to Schneider (41).

Cell-free Protein Synthesis

In vitro translation of purified immunoglobulin light chain mRNA with rabbit reticulocyte lysate was carried out as described previously (30). The reconstitution of trypsinized rough microsomes (RM_i) using the elastase-generated fragment of DP was performed according to Meyer and Dobberstein (30).

The rebinding of the DP fragment to RM_i was assayed using the membrane affinity factor isolation assay published by Meyer and Dobberstein (33) in conjunction with the immunoblot procedure (18). For signal peptidase activity poly(A)$^+$ mRNA from human placenta was translated in vitro and posttranslational cleavage of pre-human placental lactogen to mature human placental lactogen was carried out as described by Jackson and Blobel (22).

Results

Proteolytic Inactivation of RMs

Studies by Meyer and Dobberstein (30, 31) and Walter et al. (44) showed that pancreatic microsomes lost their ability to translocate nascent secretory proteins when treated with low concentrations of protease and high salt (see Fig. 1, lanes *1–5*). This activity was restored when the cytoplasmic domain of the docking protein (DP_f) (30, 31) was added back to the inactivated microsomes (Fig. 1, lanes *7* and *8*). The ability of DP_f to restore translocation is lost, however, when the membranes are treated with protease at a sufficiently high concentration, in this case trypsin at levels between 5 and 20 μg/ml (Fig. 1, lanes *8* and *9*). This implies that translocation requires a proteinaceous component that is independent of the signal sequence recognition system.

Three quantifiable aspects of the translocation process could be responsible for this observation. First, trypsin treatment (at concentrations in excess of 5 μg/ml) may render microsomes incapable of reassociating with DP_f; second, signal peptidase could have become inactivated; or third, the membranes may have lost the ability to bind ribosomes. We have tested all three of these possibilities with an appropriate in vitro assay.

Table I. Rebinding of DP_f to Rough Microsomes

Sample	Translocation activity in presence of DP_f	% of DP_f added associated with RM (pellet)	Activity of DP_f*
No microsomes	–	0	–
Trypsinized RMs (5 μg/ml trypsin)	+	40	48
Trypsinized RMs (100 μg/ml trypsin)	–	49	53
Trypsinized RMs (5 μg/ml trypsin) treated with 40 mM NEM	–	48	61

RMs treated as shown above were incubated with an excess of DP_f, sedimented, and DP_f in both supernatants and pellets was assayed as described in Materials and Methods.
* Measured as the ability to release an SRP-induced translation arrest, expressed as % of release relative to intact microsomes. Release was measured as described previously (13).

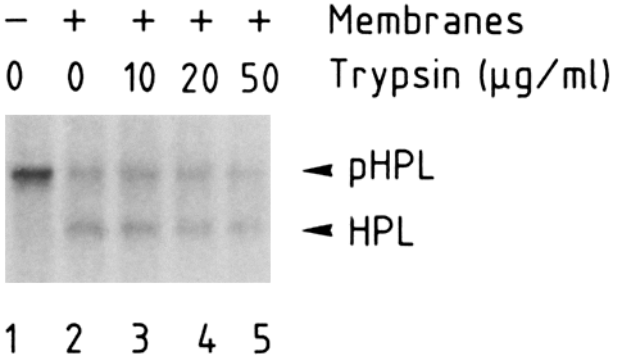

Figure 2. Signal peptidase activity of trypsinized rough microsomes. Poly (A)$^+$ RNA from human placenta was translated in vitro and posttranslational assays were carried out as described by Jackson and Blobel (22). RM_K were trypsinized at the concentrations indicated above the figure and subsequently washed with 500 mM KCl. Lane *1* is the translation product incubated without solubilized membranes. HPL and pHPL refer to human placental lactogen and its precursor, respectively.

To determine the ability of proteolyzed microsomes to reassociate with the active fragment of docking protein, DP_f, we used an immunoassay to determine the amount of DP_f that will co-sediment with membranes under saturating levels of DP_f. As can be seen in Table I, the membranes

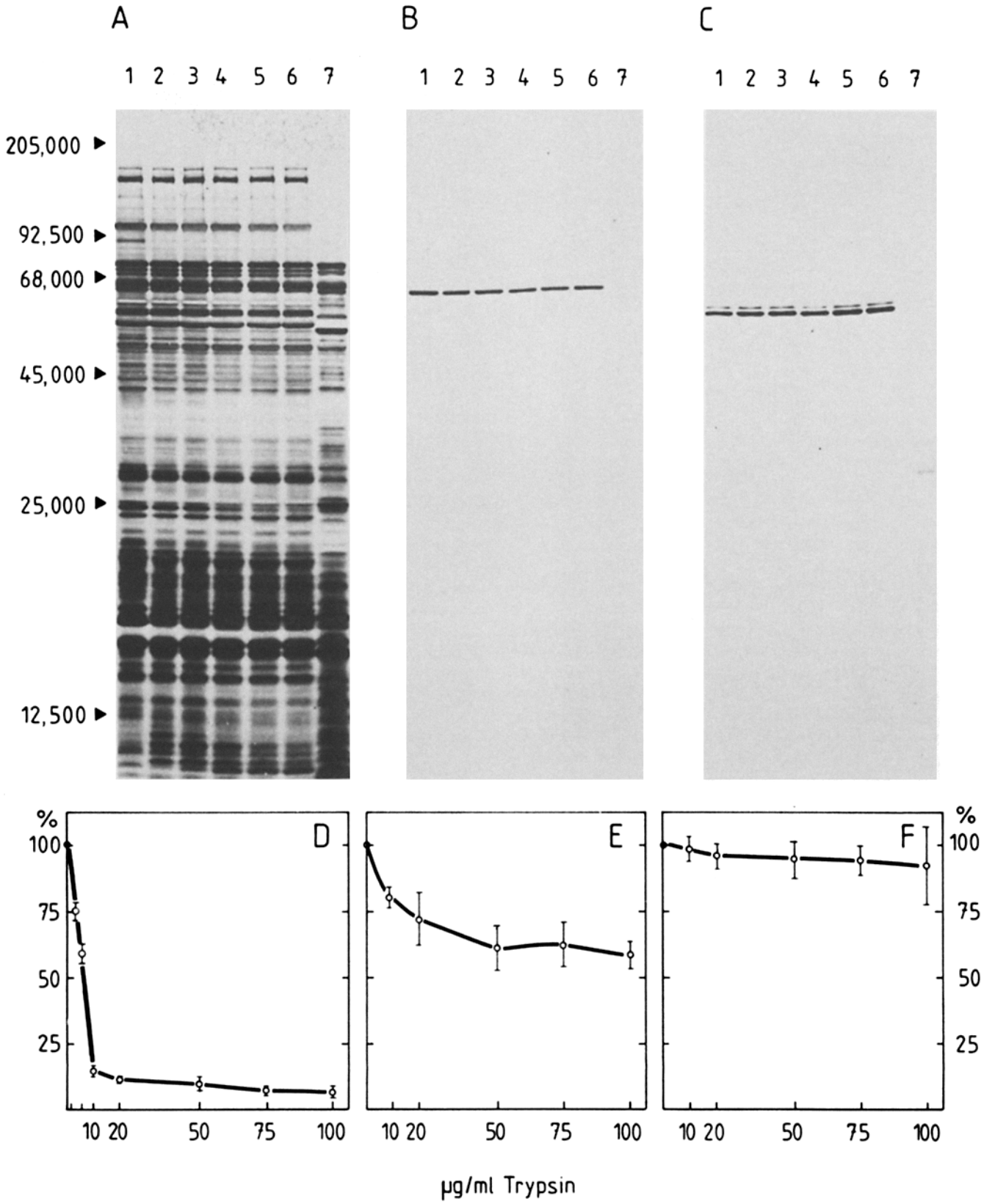

Figure 3. Trypsin abolishes ribosome binding but does not degrade ribophorins. RM_{SN} were treated with increasing concentrations of trypsin (0, 10, 20, 50, 75, 100 µg/ml and 100 µg/ml in the presence of 0.2% Nikkol, lanes *1–7*, respectively). *A* shows a silver-stained polyacrylamide gel; *B* and *C* are immunoblots incubated with monoclonal anti-canine ribophorins I and II, respectively, and developed with peroxidase-labeled second antibody. *D* depicts the ribosome-binding capacities of the microsomal preparations shown above (lanes *1* to *6*) as well as microsomes treated with trypsin at 1 and 5 µg/ml. Ribosome binding to untreated microsomes was taken as 100%. *E* and *F* show a quantitation of the amount of ribophorins in these microsomal preparations. Immunoblots containing amounts of microsomal proteins that were in the linear range of the assay were incubated with monoclonal anti-ribophorins, followed by ^{125}I-labeled anti-mouse

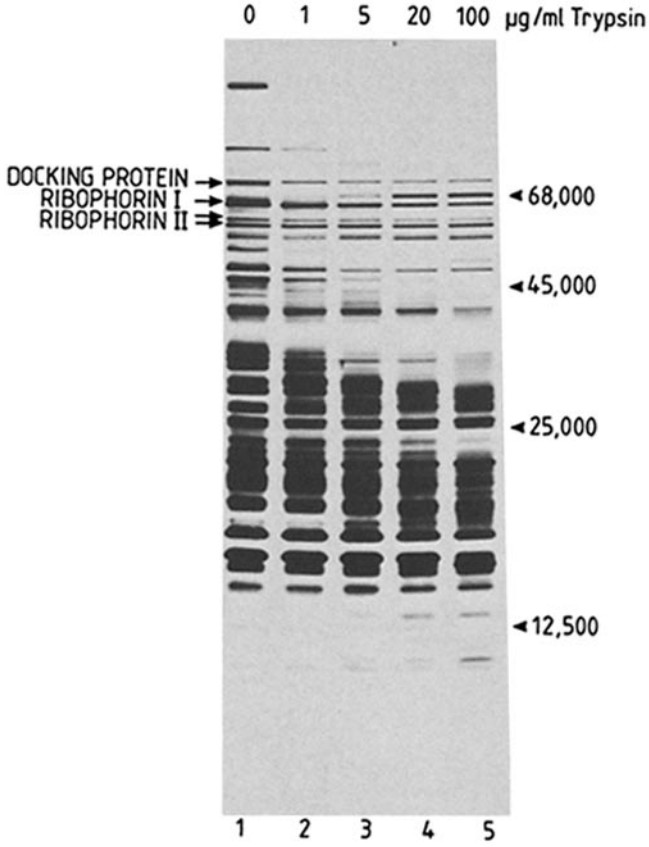

Figure 4. Degradation of the integral membrane proteins of RMs by trypsin. Intact microsomes were proteolyzed and subsequently carbonate washed (pH 11) as described in Materials and Methods. Shown above is a silver-stained SDS polyacrylamide gel. Trypsin concentrations used are indicated on the top of the figure.

that have been irreversibly inactivated with trypsin (i.e., addition of DP_f cannot restore their translocation activity) have slightly more DP_f associated compared to those that can be reactivated by DP_f. The DP_f which had co-sedimented with these membranes was still in a functional state as determined by its ability to release SRP-induced translation arrests. From this experiment, we conclude that a lack of functional DP_f reassociation is not responsible for the irreversible inactivation microsomes resulting from trypsinization.

Our assay for translocation depends on cleavage of the signal sequence of pre-IgG light chain. The observed results could be due to proteolytic inactivation of the enzyme catalyzing this reaction, signal peptidase. We therefore assayed this activity in a soluble system where deoxycholate extracts of microsomes were allowed to act on pre-human placental lactogen. Shown in Fig. 2 are results that indicate that signal peptidase activity is not affected by proteolysis.

What was, however, affected by proteolysis was the ability of the microsomes to bind ribosomes. Using the ribosome-binding assay described by Kreibich et al. (25), it is clear that a very close correlation exists between the amount of trypsin required to functionally inactivate DP_f-depleted RMs and

Table II. Removal of Membrane-bound Ribosomes from Canine Pancreatic Microsomes

Treatment	A_{260}/A_{280}	µg RNA/µg protein
500 mM KCl wash	1.97	0.185
Puromycin + 500 mM KCl wash	1.68	0.108
Nuclease + Puromycin + 500 mM KCl wash	1.04	0.048

Procedures for salt washing, puromycin, and nuclease treatment of rough microsomes are described in Materials and Methods.

their ability to bind ribosomes (compare Fig. 3 *D* with Fig. 1). On the basis of these data, it appears likely that at least one cause of the inability to restore the translocation activity of microsomes (treated with trypsin at levels of 20 µg/ml or higher) with DP_f resides in their loss of ribosome-binding activity. To see if any gross changes in protein integrity had occurred that might correlate with the observed loss in ribosome binding, the integral membrane protein composition of trypsinized microsomes was examined. The profiles of trypsinized and subsequently carbonate washed (pH 11) microsomes are shown in Fig. 4. Docking protein was destroyed, as expected (30), by trypsin at concentrations as low as 1 µg/ml. Several other integral membrane proteins, in all molecular mass ranges, were sensitive to trypsin, some at lower and some only at higher concentrations. This indicates that several candidates exist whose involvement in ribosome binding is suggested by their behavior during proteolysis. Most interestingly, the amounts of ribophorins I and II do not appear to decrease proportionally.

Protease Sensitivity of Ribophorins

Ribophorins have been postulated to be directly involved in the association of ribosomes with the microsomal membrane. It was logical, therefore, to examine more closely the integrity of these proteins after proteolytic treatments that abolish the membranes' ability to bind ribosomes. To be certain that ribosome binding in our system (canine pancreatic microsomes) was identical to that of rat liver microsomes, from which ribophorins were originally identified and isolated, we first characterized this binding process in detail.

The fact that ribosomes can be efficiently detached from pancreatic microsomes is shown in Table II. The most effective treatment involves a combination of not only puromycin and high salt (500 mM KCl) (1) but also micrococcal nuclease to eliminate polysomes. In this way, the majority of RNA normally associated with RMs was removed. Electron micrographs of native and stripped microsomes (Fig. 5) demonstrate the effectiveness of this stripping procedure.

The ability of stripped microsomes to rebind radiolabeled ribosomes was examined. As can be seen in Fig. 6, ribosome binding was a saturable phenomenon (panel *A*) and Scatchard analysis (panel *B*) indicated an affinity constant of $5.2 \times 10^7\ M^{-1}$. This value is similar to the $8 \times 10^7\ M^{-1}$ obtained by Borgese et al. (8) for rat liver. Per gram of membrane pro-

Ig antibody, and quantitated as described in Materials and Methods. Ribophorin content of untreated microsomes was taken as 100% after subtraction of background. Values represent the mean of three determinations; bars indicate the range.

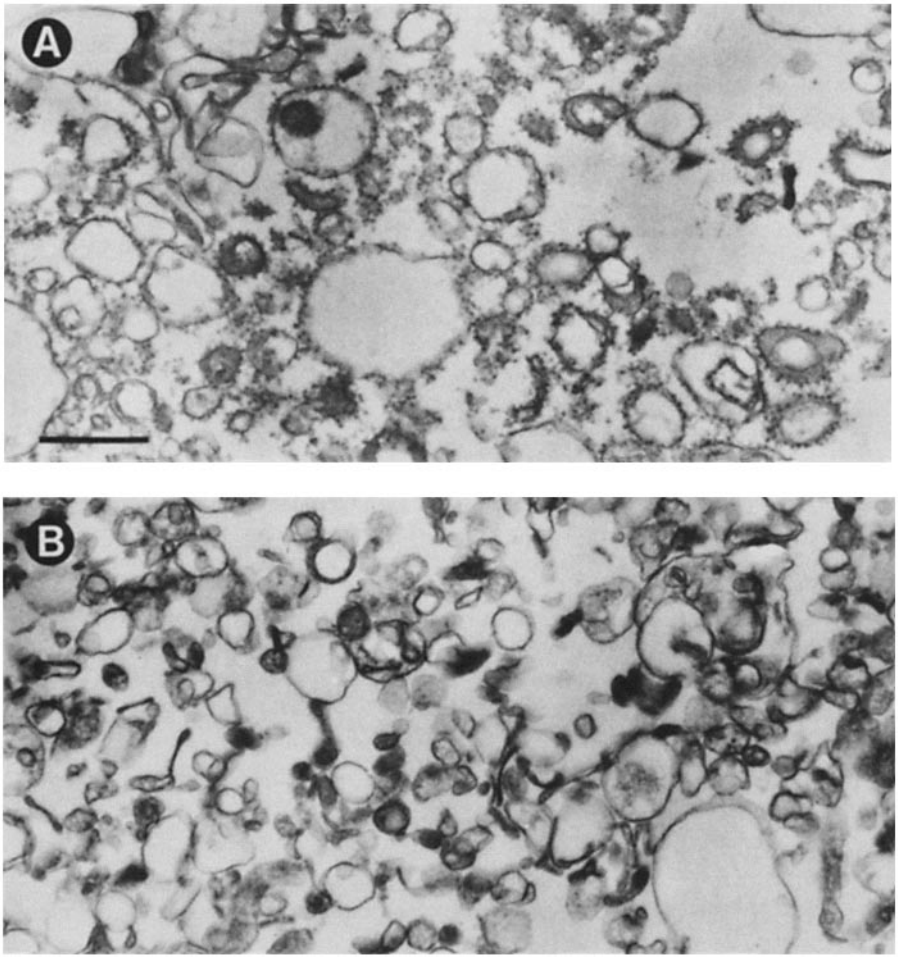

Figure 5. Electron microscopic analysis of canine pancreatic RMs before and after a puromycin/high salt/nuclease treatment. Aliquots of membranes were pelleted, fixed, and processed for electron microscopy as described in the Materials and Methods. Pictures were magnified 22,000×. (*A*) A representative field of salt-washed RMs from canine pancreas. (*B*) Membrane pellet after treatment with puromucin/high salt/micrococcal nuclease. Bar, 500 nm.

tein, 16.1 × 10^{-8} moles of ribosomes are bound, a value about three times higher than for rat liver (8). This is reflected in the higher RNA/protein ratio in native pancreatic microsomes (25). It could also be explained by a higher proportion of bona fide RMs in pancreatic preparations compared to rat liver. Finally, ribosome binding is salt labile in our system, as in rat liver (8); increased levels of KCl in the binding assay results in decreased ribosome binding (panel *C*). Therefore, we concluded that our assay system, with which we examined the integrity of ribophorins, appeared identical in all aspects to the system from which these proteins were originally derived.

Ribophorins from canine sources have been shown previously to comprise polypeptides of similar molecular mass to those from rat liver (28). We have confirmed that canine pancreatic ribophorins I and II are immunologically identical to their rat liver counterparts with specific antibodies (Fig. 7). Based on these studies, canine ribophorin I has an apparent molecular mass of 65,000 daltons, while ribophorin II is comprised of two proteins of 61,000 and 63,000 daltons, respectively. These proteins have also been shown to be integral membrane proteins (Fig. 7) and fractionation of canine liver indicates that they are rough ER specific (M. Hortsch, unpublished). Sensitivity to endoglycosidase H indicates that both canine ribophorins are glycoproteins (data not shown); ribophorin II is only partially glycosylated, thus explaining the two species seen in SDS gels. Monoclonal antibodies, used in subsequent experiments, were raised against these same antigens and demonstrated to bind them specifically (data not shown).

Ribophorins are resistant to concentrations of trypsin that eliminate ribosome binding. Membranes were treated with trypsin at levels up to 100 μg/ml, and ribophorins were examined by quantitative immunoblotting using monoclonal anti-canine ribophorin I and II antibodies. The results of these studies are shown in Fig. 3. In panel *A*, the protein composition of intact, trypsinized microsomes is visualized by silver staining. Panels *B* and *C* show peroxidase-stained immunoblots of identical samples treated with monoclonal anti-canine ribophorins I and II, respectively. In the presence of detergents (lanes *7*) these proteins are easily degraded, however in intact membranes they are very resistant (lanes *1–6* in *A*, *B*, and *C*). A quantitation of identical samples on immunoblots using ^{125}I-labeled second antibody (panels *E* and *F*) showed ribophorin I to be >80% and ribophorin II virtually 100% resistant to a trypsin concentration (10 μg/ml) that effectively abolished ribosome binding (panel *D*). At higher protease concentrations, ribophorin I was degraded to a somewhat greater extent (by as much as 40%), whereas ribophorin II maintained its resistance almost completely. On the basis of data obtained with trypsin, we have found no correlation between ribosome binding and ribophorin integrity.

To further characterize this protease resistance and extend

the observations made with trypsin, we examined the effects of four other proteases on ribophorins and ribosome binding. High concentrations (200 μg/ml) of trypsin, protease K, elastase, pronase, and subtilisin were used. In all cases (see Table III), ribosome binding was diminished by at least 60% (for elastase) or as much as 97% (for trypsin). A parallel decrease in either ribophorin I or II (Fig. 8 *A* and *B*, respectively) was not observed. The addition of detergent always rendered the ribophorins protease labile. These results thus confirm our results with trypsin.

Ribophorin Association with Ribosomes

The principle argument for the participation of ribophorins in ribosome binding was based on the fact that ribophorins co-sedimented with ribosomes in low salt, detergent-solubilized RMs (23). To determine if this sedimentation of ribophorins was indeed due to an association with ribosomes, we solubilized and fractionated native and ribosome-free (stripped) microsomes. Such membranes were solubilized in 1% Nikkol in either low (20 mM) or high (500 mM) concentrations of NaCl. They were then centrifuged to obtain soluble (supernatant) and insoluble (pellet) fractions. The data presented in Fig. 9 indicates that at low salt, the bulk of the ribophorins (panels *C* and *D*) appeared in the pellet (insoluble) fraction regardless of the presence or absence of ribosomes. Moreover, this was true also for other rough ER-specific proteins such as the DP (panel *E*) and a rough ER-specific protein of ~68 kD (panel *F*). Thus the observed behavior of ribophorins appears to have more to do with their rough ER specificity (19) than with any association with ribosomes (23). In this same assay, protein disulfide isomerase, a peripheral protein of the lumen of the rough and smooth ER (26, 34), was always present solely in soluble (supernatant) fractions (panel *B*).

NEM Sensitivity of the Translocation Process and Ribosome Binding

NEM has been effectively used to inhibit translocation (21). Since then it has been determined that the activity of SRP (46) as well as DP (31) can be affected by this alkylating agent. To determine if the alkylation of proteins can affect processes independent of SRP-DP-mediated recognition, we treated DP_f-depleted, but reactivatable, microsomes with NEM at concentrations of 1 and 40 mM. Similar to using increasing concentrations of trypsin (refer to Fig. 1), NEM could be used to inactivate microsomes so that translocation activity was not restored when the recognition function was reconstituted by the addition of DP_f (Fig. 10). Again, this effect could be due to a number of factors and we examined the obvious ones: reassociation of DP_f with the membrane via an immunoassay; signal peptidase activity; and ribosome binding.

Reassociation of functional DP_f with the membrane was not affected by NEM treatment (Table I). Signal peptidase activity was normal in intact or proteolyzed microsomes after treatment with either 1 or 40 mM NEM (Fig. 11), as was the ability of NEM-treated membranes to bind ribosomes (Table IV). It appears, therefore, that yet another protein-associated activity, of undetermined function, plays a role in translocation. It is susceptible to inactivation by alkylation indicating the requirement for a free sulfhydryl group in this process.

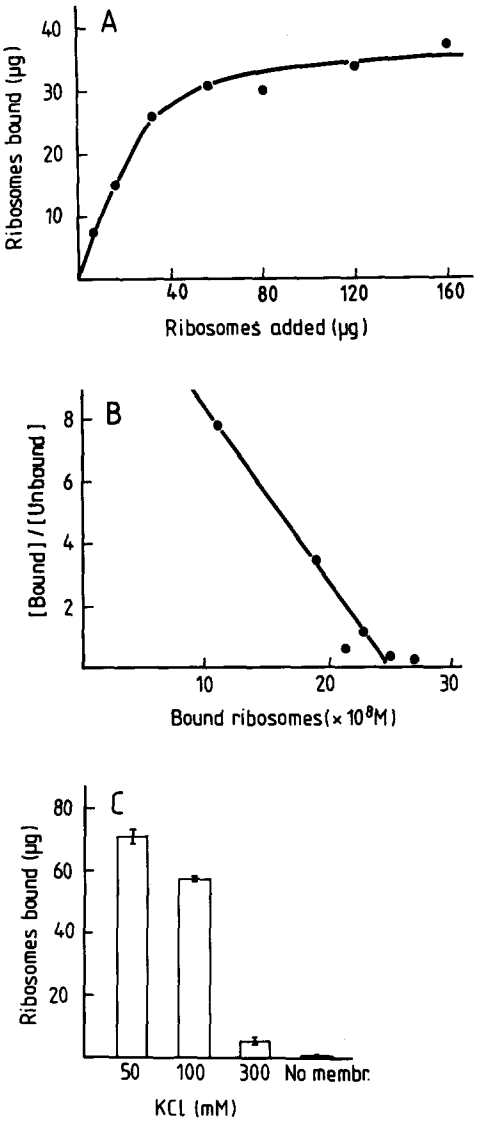

Figure 6. Characterization of ribosome binding to membranes, Scatchard analysis, and dependence on salt concentration. (*A*) RM_{SN} (50 μg protein) were incubated with various amounts of ^3H-labeled ribosomes in LSB for 45 min at 0°C. Bound ribosomes were separated from unbound material on a discontinuous sucrose gradient as described in Materials and Methods. (*B*) Scatchard plot of the data in *A*. The points were fitted by a least-square linear fit program. The affinity constant calculated from the slope $K = 5.2 \times 10^7\ M^{-1}$. (*C*) Binding of ribosomes (80 μg) to RM_{SN} (100 μg protein) at the indicated KCl concentration. The sucrose gradients contained the appropriate salt concentration as indicated below the figure. Values represent the mean of two determinations.

Discussion

The results of this study represent three important findings relevant to protein translocation across the membrane of the ER. First, there is a function, beyond that of SRP-DP-mediated recognition, that is protease sensitive. The loss of translocation activity brought about by proteolysis correlated well with a loss in the ability of the proteolyzed membranes to bind ribosomes. Second, ribosome binding to canine pancreatic microsomes is not directly mediated by an association with the rough ER-specific glycoproteins known as ribophorins. Finally, yet another aspect of translocation has

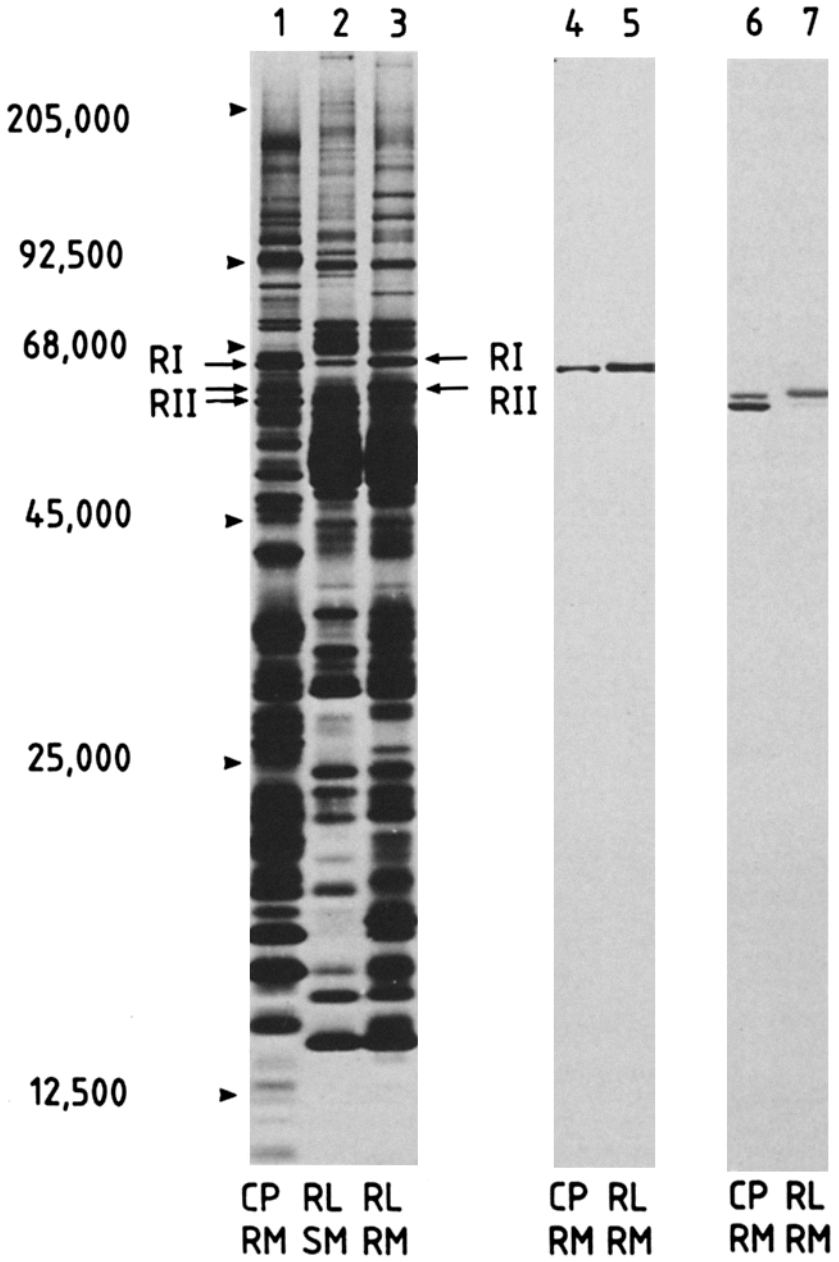

Figure 7. Immunochemical identity of ribophorins from rat liver and canine pancreas. Canine pancreatic and rat liver microsome fractions were prepared as described previously (19). Lanes *1–3* represent silver-stained patterns of integral membrane proteins from canine pancreas rough microsomes (*CPRM*), rat liver smooth (*RLSM*), and rat liver rough microsomes (*RLRM*), respectively. Lanes *4* and *5* represent immunoblots of pancreatic (lane *4*) and liver (lane *5*) RMs developed with rabbit anti-canine ribophorin I antibodies. Lanes *6* and *7* are identical to lanes *4* and *5*, only using rabbit anti-canine ribophorin II antibody. RI and RII denote the positions of ribophorin I and ribophorin II, respectively.

been discovered that is sensitive to alkylation, but not involved in either signal sequence recognition, nor in ribosome binding.

Protease Sensitivity of Translocation

In this study we demonstrate that further proteolysis of RMs, i.e., digestion of functionally competent membranes from which DP has already been proteolytically removed, results in their inactivation. This loss of translocation persisted even in the presence of fresh, active DP_f. There are two obvious questions: which function is being destroyed, and which protein is being degraded that is involved in this aspect of the translocation process?

Translocation can be divided into three individual steps or stages. First is the recognition of proteins to be translocated, second the actual translocation across the lipid bilayer of the membrane, and third the various modifications known to occur co-translationally such as signal sequence cleavage, disulfide bond formation, or N-linked glycosylation (for review see reference 20). Recognition is, at least in part, mediated by SRP and its receptor, the docking protein. This process will allow the translation machinery to engage the rough ER, but ultimately translocation is probably initiated by an interaction of the signal peptide with the membrane (49). A recent report suggests that such an interaction occurs in a hydrophilic rather than a hydrophobic environment (15). If such an environment was created by proteinaceous components, it might be inactivated by proteolytic digestion from the cytoplasmic side. The activity of this putative signal sequence–binding function has not yet been challenged by proteases, nor has the identity of its relevant membrane components been discovered.

The isolation of the cytoplasmic domain of the docking

Table III. Ribosome Binding to Microsomal Membranes Treated with Various Proteases

Protease	Concentration	Ribosomes bound	% of Control
	μg/ml	*μg/100 μg membrane protein*	
None	0	61.2 ± 7.2	100.0
Trypsin	200	0.9 ± 0.1	1.5
Proteinase K	200	18.7 ± 0.7	30.6
Elastase	200	24.0 ± 2.3	39.2
Pronase	200	1.5 ± 0.0	2.5
Subtilisin	200	6.1 ± 0.9	10.0
Aurintricarboxylic acid	3×10^{-4} M	4.5 ± 0.2	7.4
No membranes	–	0.5 ± 0.2	0.8

Membranes were prepared and proteolyzed and ribosome binding was carried out as described in Materials and Methods. Aurintricarboxylic acid has been shown to inhibit ribosome binding to microsomes (8).

protein (DP_f) was crucial to its identification and characterization (31). Because it can recombine with the membrane from which it has been removed, one has assumed that it recombines with either a receptor, its own membrane anchor, or membrane lipids, possibly electrostatically. This interaction, whatever its nature, is undisturbed by proteolysis. As can be seen from the immunoassay, active DP_f reassociates with the membrane even after extensive trypsin treatment (Table I). This would mean that the "DP_f receptor," if it is a protein, is very resistant to proteases, or is in fact the lipid bilayer.

Ribosome binding to stripped microsomes correlated nicely with proteolytic inactivation of translocation. That ribosome binding to membranes is sensitive to proteases has been known for some time (8, 12, 42). It has not until now, been experimentally correlated to its postulated function, translocation of nascent secretory proteins.

As can be seen in Fig. 4, there is a number of proteins that are sensitive to tryptic digestion. This implies that, topologically, several rough ER-specific proteins have cytoplasmically disposed domains that are accessible to the protease. This would be a logical prerequisite for a ribosome receptor.

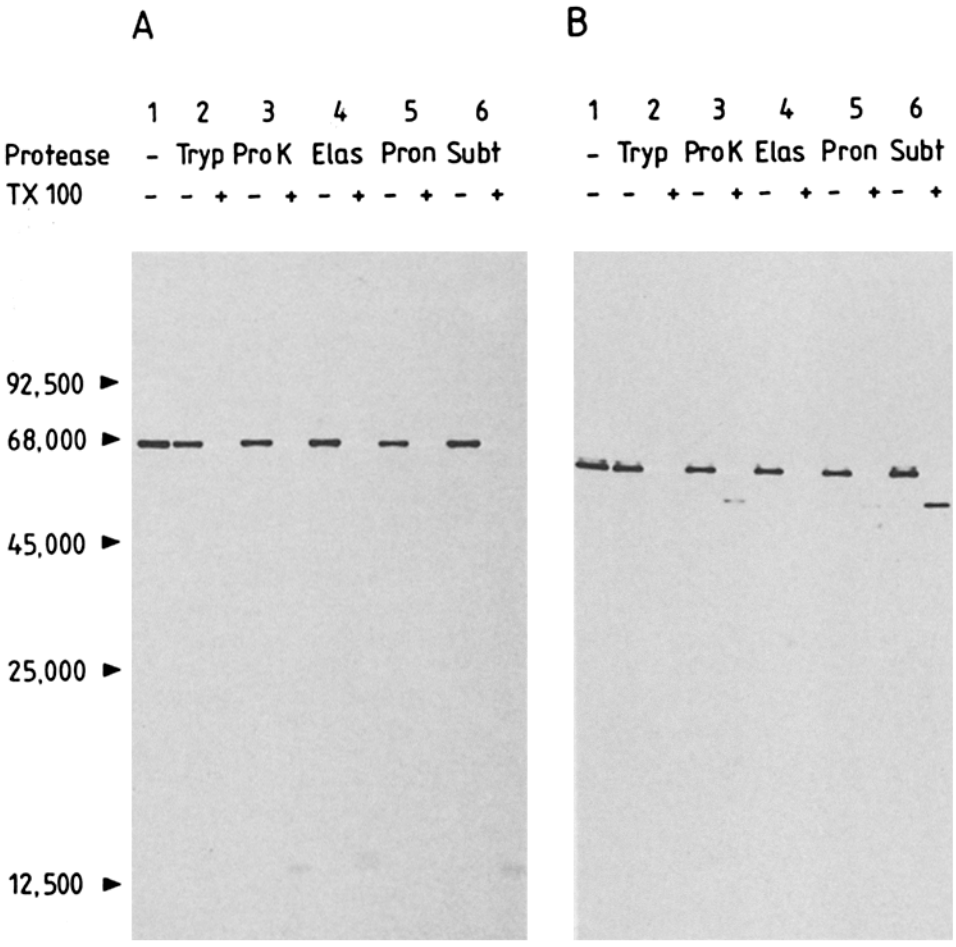

Figure 8. Resistance of ribophorins in intact RMs to various proteases. Stripped, nuclease-treated RMs were treated for 1 h at 0°C with 200 μg/ml of one of the following proteases: (lanes 2) trypsin, (lanes 3) proteinase K; (lanes 4) elastase; (lanes 5) pronase, (lanes 6) subtilisin either in the absence or in the presence of 0.5% Triton X-100 as indicated above the panels. Lanes *1* show microsomes that were incubated in the absence of any protease. *A* is an immunoblot incubated with the monoclonal anti-canine ribophorin I antibody whereas *B* shows a duplicate using the monoclonal anti-canine ribophorin II antibody. Both blots were developed with peroxidase-labeled second antibody.

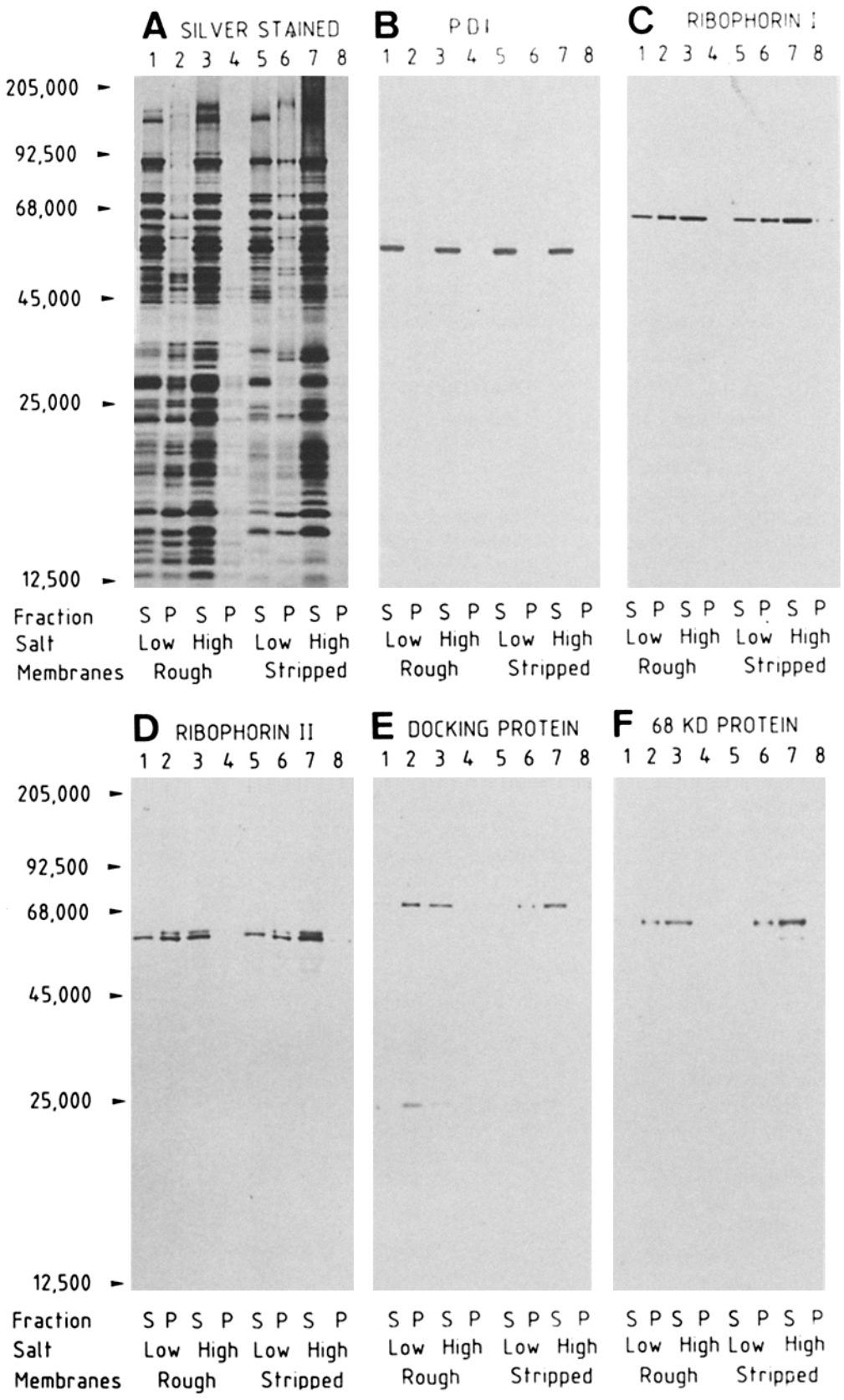

Figure 9. Solubilization of microsomal membrane proteins by the nonionic detergent Nikkol at low and high salt concentrations. Intact rough or stripped ribosome-free dog pancreas microsomes were solubilized with 1% Nikkol at either low (20 mM NaCl) or high (500 mM NaCl) salt concentrations. Insoluble material was pelleted by centrifugation in an airfuge for 1 h at 24 psi. *S* refers to solubilized material and *P* to the pellet fractions. Aliquots were loaded on SDS gels and analyzed by silver staining (*A*) or immunoblotting with the following antibodies: (*B*) rabbit anti-protein disulfide isomerase (*P.D.I.*), (*C*) monoclonal anti-canine ribophorin I, (*D*) monoclonal anti-canine ribophorin II, (*E*) monoclonal anti-DP, and (*F*) monoclonal anti-68-kD protein.

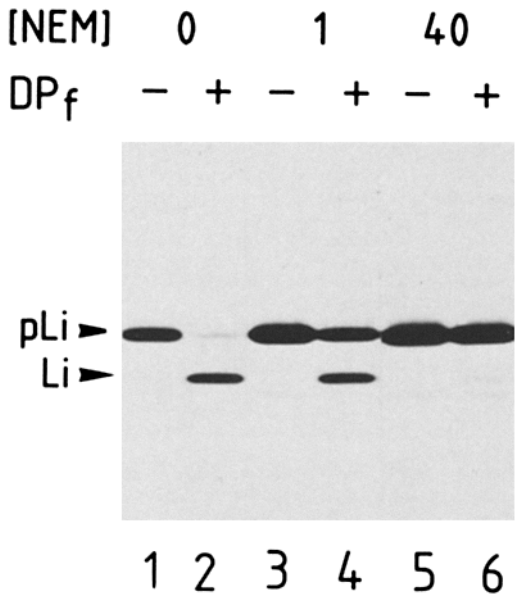

Figure 10. Effect of NEM treatment on the reconstitution of recognition-incompetent microsomes. RM_K were trypsinized (5 μg/ml) and washed with 500 mM KCl to remove DP_f followed by a treatment with NEM at concentrations indicated above the figure. The ability of such membranes to be reconstituted was then examined by the addition of fresh DP_f prepared from control membranes. *pLi* refers to IgG-k light chain precursor, *Li* to mature IgG-k light chain.

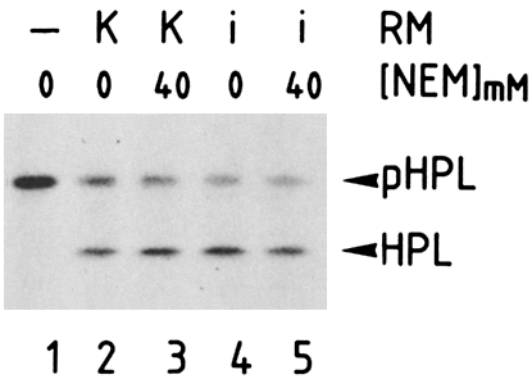

Figure 11. Signal peptidase activity of NEM-treated RMs. Poly $(A)^+$ RNA from human placenta was translated in vitro and post-translational assays were carried out as described by Jackson and Blobel (22). *K* refers to salt-washed microsomes and *i* to those inactivated by protease-high salt. *HPL* and *pHPL* refer to human placental lactogen and its precursor, respectively.

To identify such a protein, more careful analysis of the changes in integral membrane protein composition resulting from proteolysis must be carried out in conjunction with functional and ribosome-binding assays. An interesting finding is that neither ribophorin I nor II seem particularly sensitive to levels of protease that totally eliminated both translocation and ribosome binding.

Ribophorins and Ribosome Binding

Kreibich and co-workers have presented considerable evidence suggesting that ribophorins I and II serve as the ribosome receptors in rough ER (23, 24). The data supporting this postulation are based on two types of experiments. In the first, an association between ribophorins and ribosomes was inferred from their similar solubilization properties and co-isolation in the ultracentrifuge. The second type of experimental approach involved the ability to reversibly cross-link ribosomes to rough ER proteins, notably to ribophorins I and II. Why then, in light of these findings, is there reason to doubt their involvement in ribosome binding?

When ribosome-free (stripped) membranes were used, the results were similar to those obtained using intact microsomes. In the absence of ribosomes, ribophorins sedimented when membranes had been solubilized with non-ionic detergents at low salt concentrations (Fig. 9). Moreover, using bifunctional reagents, we have found that numerous rough ER–specific proteins, including DP and ribophorins can be cross-linked into aggregates in situ (Avossa, D., and D. Meyer, unpublished results). This means that an interaction with ribosomes cannot be concluded from these types of studies.

Kreibich et al. (23) realized that another interpretation of their data was possible. ". . . The ribophorins either directly provide binding sites for the ribosomes or contribute, in association with other membrane components, to the integration of supramolecular assemblies which act as ribosome receptors" (23). The accumulated body of evidence supports the second alternative. It now seems clear that certain proteins are generally ER-specific and are found in roughly equal amounts in rough and smooth microsomes (19). Beyond this is a certain subset of proteins that is present only in rough membranes (19, 27). This class of proteins seems to differ in its solubility characteristics from the bulk of ER proteins. Such proteins, including ribophorins and DP, interact with one another electrostatically and need either ionic detergents or high salt with nonionic detergents to render them soluble. Thus, the interpretation most consistent with all findings is that a matrix or network exists within the ER that among other things contains the ribophorins (2), and is involved in the only as yet defined function of rough ER: protein translocation. One of the obvious capabilities of this specialized membrane domain would be the binding of ribosomes synthesizing nascent secretory and membrane proteins.

NEM-sensitive Components

In addition to a protease-sensitive component that may be involved in ribosome–membrane interaction, we have presented data demonstrating the presence of a functional aspect of translocation that is inhibited by alkylation. As alkylation is not a very specific sort of inhibition—any free sulfhydryl

Table IV. Ribosome Binding to Microsomal Membranes Is Not Inhibited by NEM

NEM	Ribosomes bound
mM	*μg/100 μg membrane protein*
0	76.0 ± 2.5
1	79.5 ± 0.1
40	76.8 ± 0.4
No membranes	0.5 ± 0.2

Stripped microsomes were treated with NEM and ribosome binding was measured as described in Materials and Methods.

should be sensitive to NEM – any number of functions may be affected. Since we have inactivated DP_f-depleted membranes, an affect on the SRP-DP-mediated signal sequence recognition can be ruled out. We have shown that these NEM-treated membranes can rebind both DP_f and ribosomes (Tables I and IV) and that signal peptidase remains active (Fig. 11).

A number of possible functions may have biochemical correlates that are sensitive to NEM. The aforementioned aqueous compartment involved in signal sequence (or nascent chain) binding (15), as well as the postulated pore or tunnel for translocation, would be potential targets for NEM inactivation. Although a functional involvement of ATPases, electrochemical gradients or membrane potential has never been demonstrated for translocation in ER, such components have been shown to be present in this membrane system, and inhibitable by NEM (39). To isolate the component being inactivated by NEM, as well as those that are protease sensitive and/or involved in ribosome binding, more precise dissection and analysis of the protein components of rough ER, in conjunction with functional reconstitution, is necessary.

The discovery of an NEM-sensitive component is also important from a conceptual point of view. Of the various models that have been proposed in order to explain the mechanism of protein translocation, a prominent difference between them has been whether or not proteins are required for the actual translocation step. The evidence for the involvement of proteins in recognition, in ribosome binding, and in co-translational processing is convincing, whereas their participation in translocation across the bilayer has been speculative. These results support those hypotheses that include protein-mediated translocation since a protein-mediated activity other than recognition, ribosome binding, and signal peptidase was effectively lost through alkylation.

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