Identification and Characterization of a Membrane Component Essential for the Translocation of Nascent Proteins across the Membrane of the Endoplasmic Reticulum

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ABSTRACT   When rough microsomes are subjected to limited proteolysis and high salt, a soluble fraction can be separated from the membrane. Neither fraction alone is capable of vectorially translocating nascent peptides. When the soluble extract is recombined with the residual membrane fraction, translocating activity is restored. Standard biochemical techniques were used to identify and characterize the active component derived by treating rough microsomes with elastase and high salt.

The active factor is a peptide fragment with an apparent molecular weight of 60,000. It represents the cytoplasmic domain of a larger membrane protein. The fragment is basic and has at least one accessible sulfhydryl group. These characteristics facilitated its purification and identification as a membrane component required for translocation of nascent peptides across microsomal membranes.

Secretory proteins are discharged vectorially across the membrane of the endoplasmic reticulum during their synthesis (1). This process can be separated into several steps which must occur sequentially for proper functioning of protein translocation. Protein synthesis is initiated on cytoplasmically located ribosomes. For secretory proteins, this complex must become specifically attached to the membrane of the endoplasmic reticulum (2). This takes place not only through the interaction of the ribosome with specific binding sites on the membrane, but probably through a recognition of the signal sequence as well (3, 4). During or shortly after translocation of the nascent peptide across the membrane, the signal sequence is removed by a specific protease (4, 5). Certain proteins are further modified subsequently by glycosylation, hydroxylation, or disulfide bond formation (6–10). Chain termination is followed by the dissociation of the ribosome from the membrane (2, 11).

We assume that such a sequence of events is mediated by a specific set of membrane proteins. However, membrane components that are functionally involved in this process are poorly understood. Kreibich et al. (12, 13) have identified transmembrane glycoproteins, referred to as ribophorins, which are physically associated with membrane-bound ribosomes. Several groups are involved in the isolation and characterization of signal peptidases of procaryotic (14) and eucaryotic origin (15–17). In contrast to the purification of signal peptidase(s), whose activity can be assayed in solution, the characterization of the molecules involved in the earlier events requires the intactness of the entire membrane. Therefore, a system that can be reconstructed from component parts, as described in the companion paper (18), is ideally suited for the characterization of a component necessary for translocation. This component, derived from the cytoplasmic face of rough microsomes in a water-soluble form, can be functionally reconstituted with the remaining membrane (19, 20). Identification and purification of this active factor would then provide the means whereby the membrane protein translocating system from which it derives can be characterized. In this study, standard biochemical techniques in conjunction with an in vitro assay system have led to the characterization and isolation of the proteolytically derived membrane component that functions during vectorial translocation of nascent secretory proteins across microsomal membranes.

MATERIALS AND METHODS

Isolation of rough microsomes from dog pancreas, removal of ribosomes, protease/high salt treatment, in vitro translation, assays of translocation, and proc-

essing of immunoglobulin light chain, polyacrylamide gel electrophoresis, and autoradiography were all carried out as described in the companion paper (18).

Detergent Extraction of Stripped Rough Microsomes

Stripped rough microsomal membranes were suspended to a concentration of A_{280} = 50/ml (measured in 2% SDS) in the following buffer (referred to as buffer A): 0.25 M sucrose, 20 mM HEPES, pH 7.5, 0–500 mM KCl (see Results), 3 mM magnesium acetate, 1 mM dithiothreitol (DTT). 20% (wt/vol) Triton X-100 was added to a final concentration of 2%. After incubation for 20 min at 0°C, the solution was layered over a cushion of 20 ml of buffer A having a final sucrose concentration of 1.0 M, and centrifuged for 90 min at 105,000 g_{av} in a Beckman Ti 45 rotor (Beckman Instruments, Inc., Fullerton, Calif.). To remove residual detergent, the pelleted material was suspended in buffer A in a Dounce homogenizer (Kontes Co., Vineland, N. J.) and centrifuged for 1 h at 105,000 g_{av}.

Protease Digestion

The washed, detergent-extracted microsomal material was suspended to a concentration of A_{280} = 50/ml in buffer A with [KCl] = 0.5 M. The nonelastolytic activity of a 1% solution of elastase (EC 3.4.21.11, Merck Co., Inc., Darmstadt, Federal Republic of Germany) was inhibited by the addition of an equal volume of 1% trasylol (Boehringer Mannheim GmbH, Federal Republic of Germany). The protease was diluted and added to the suspended material to give a final elastase concentration of 1.0 µg/ml. The suspension was incubated at 0°C for 1 h. The reaction was terminated by the addition of phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 40 µg/ml. Membranes were separated from solubilized material (referred to as SE) by centrifugation of the incubation mixture for 2 h at 105,000 g_{av}.

Ion Exchange Chromatography

Cation exchange chromatography was performed with carboxymethyl (CM)-Sephadex (Pharmacia, Inc., Uppsala, Sweden). In all instances, the adsorbent was equilibrated with 0–500 mM KCl (see Results), 20 mM HEPES, pH 7.5, 1 mM DTT. Adjustment of [KCl] in SE was always performed by dialysis. Column effluents were monitored continuously at 280 nm. Fractions were analyzed for their ability to functionally reconstitute inactive rough microsomes (RM$_i$).

Gel Filtration

Chromatography was performed with Sephadex G-150 superfine (Pharmacia, Inc., Uppsala, Sweden) in a column 1.0 × 25 cm. The column was equilibrated and proteins eluted in 0.5 M KCl, 20 mM HEPES, pH 7.5, 1 mM DTT (buffer B) at a flow rate of 1.5 ml/h. Fractions were assayed for their ability to functionally reconstitute RM$_i$.

N-[ethyl-1-^{14}C]maleimide (NEM) Labeling

A purified fraction of SE (A_{280} = 0.3/ml) was dialyzed against buffer B with 40 µg/ml PMSF but without DTT for several hours to remove DTT. NEM (New England Nuclear, Boston, Mass.) was divided into portions of 12.5 µCi. Organic solvent was evaporated with a gentle stream of N_2. 0.8 ml SE was added and incubated for 30 min at 25°C. The reaction was terminated by the addition of DTT to a final concentration of 5 mM. The reaction products were analyzed by electrophoresis on SDS polyacrylamide gels and autoradiography.

Molecular Weight Determination

Molecular weight estimations were based on polyacrylamide gel electrophoresis of the following standards: bovine serum albumin, 68,000 mol wt; ovalbumin, 45,000 mol wt; chymotrypsinogen, 25,000 mol wt; and cytochrome c, 12,500 mol wt.

RESULTS

In the preceding paper (18), we showed that rough endoplasmic reticulum membranes treated with elastase and high salt gave rise to a soluble component (SE) that can confer protein translocating activity on Rm$_i$ vesicles. Because microsomes treated in this manner (elastase/high salt) gave optimal recov-

ery of SE, this procedure was used to identify the active molecule(s) involved.

Two approaches to the purification of the active factor can be envisioned. The SE could be subjected directly to further fractionation with standard techniques. It is possible, however, that the extract may consist of a complex mixture of proteins that does not lend itself to such treatment. Alternatively, a preliminary fractionation may be used to produce a less complex starting material that would lead to a simpler SE after protease/high salt. Our results indicate that this latter approach was advantageous in ultimately purifying the activity-restoring material.

Determination of Molecular Weight

When the SE was fractionated on sucrose density gradients, activity was associated with proteins in the 40–80,000 mol wt range (data not shown). More defined molecular weight measurements could be made using gel filtration on Sephadex G-150. Fig. 1 shows that the activity of the SE correlates with a protein of 60,000 mol wt. Further experiments were designated to demonstrate that this 60,000-mol wt peptide possesses the ability to restore activity to RM$_i$.

Triton X-100 Extraction of Membranes

Ion exchange chromatography revealed that the band at 60,000 mol wt was not composed of one species of peptide. When SE was prepared from whole membrane vesicles, a component was liberated by high salt treatment alone, irrespective of the presence or absence of protease (data not shown). This contaminant hampered isolation efforts because it was so similar to the putative active factor. For this reason, and to reduce the number of proteins in the starting material, a detergent extraction step was introduced to selectively remove this contaminant before the extraction of active material by protease/high salt.
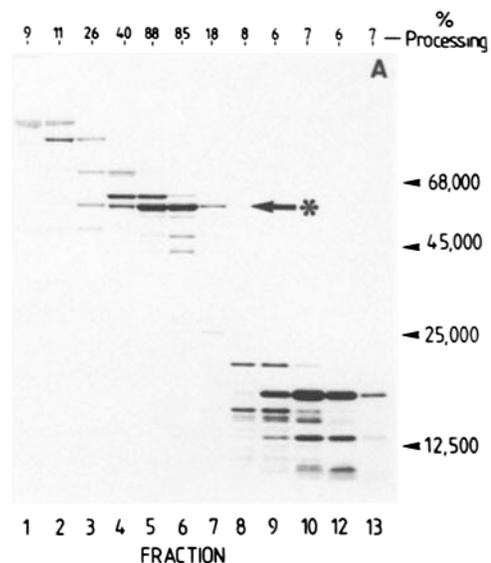

FIGURE 1 Gel filtration of elastase/high salt-solubilized microsomal proteins. Microsomal membranes were treated with elastase/high salt (see Materials and Methods). The solubilized proteins were fractionated on Sephadex G-150. Fractions were tested for their ability to restore the translocation and processing activity to RM$_i$. These activity measurements are indicated as percent processing of precursor IgG light chain. The asterisk/arrow indicates the protein whose distribution exhibits the best correlation with activity.

In the presence of detergents, the amount of membrane material solubilized is proportional to the salt concentration (21, 22). We, therefore, determined the KCl concentration below which translocation restoring activity remains associated with the insoluble, and, thus, sedimentable material. Microsomes were treated with 2% Triton X-100 and increasing concentrations of KCl. Insoluble material was pelleted, washed, resuspended, and treated with 1 μg/ml elastase in 0.5 M KCl. After centrifugation to sediment the insoluble material, the solubilized extract was tested for its ability to restore translocation activity to RM$_i$. Fig. 2 shows that the activity can be obtained from the sedimentable fraction after microsomes were treated with Triton at a KCl concentration <100 mM. Thus, the membrane proteins from which active component is derived are not solubilized under these conditions. Furthermore, the 60,000-mol wt component is retained only in extracts derived from microsomes extracted with Triton at KCl concentrations of 100 mM and below. Ion exchange chromatography revealed that the aforementioned contaminant was extracted under low ionic strength into the Triton-soluble material (data not shown). Thus, all subsequent purification steps were carried out on material extracted with 2% Triton X-100/50 mM KCl before protease treatment.

Ion Exchange Chromatography

KCl concentrations in excess of 0.2 M are necessary to remove a significant amount of SE from membranes (18). It would, therefore, follow that the active factor possesses considerable charge and that ion exchange would be useful in its purification. Of the four exchangers tested (two anionic and two cationic), CM-Sephadex gave the best results. In a simple but conclusive binding study, it was shown that at pH 7.5, a salt concentration of ~0.3 M KCl was necessary to prevent adsorption of the active factor to the exchanger (data not shown). To better characterize the active component, the SE was bound to CM-Sephadex and gradually eluted with a gradient of increasing KCl concentration. As with gel filtration, an extremely good correlation exists between the location of the 60,000-mol wt band and the ability to reconstitute Rm$_i$ (Fig. 3). Based on these data, a batch purification step was devised in which SE was bound to CM-Sephadex at [KCl] = 200 mM, and active material eluted at 350 mM.

Gel Filtration

Material purified on CM-Sephadex was subjected to gel filtration such that an activity-containing fraction could be shown to be composed of virtually one protein species. The results of this purification are shown in Fig. 4. A depicts the protein composition of total elastase/high salt-extracted material. When this mixture is subjected to batch purification on CM-Sephadex, a considerably simpler pattern is obtained (B). After gel filtration on Sephadex G-150, the highest activity was
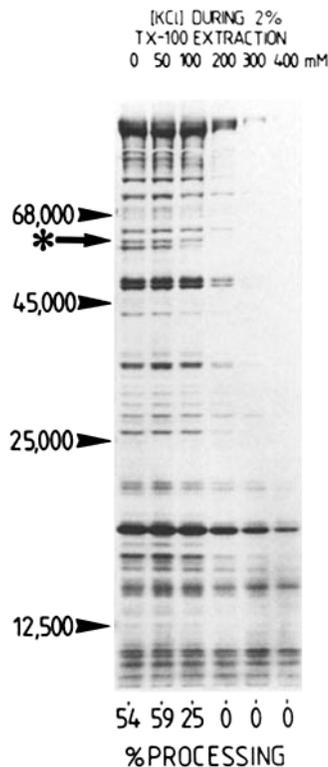

FIGURE 2 Solubilization of membrane protein from which the active component is derived. EDTA/high salt-stripped rough microsomes were treated with 2% Triton X-100 and increasing concentrations of KCl. Insoluble material was pelleted and extracted with elastase/0.5 M KCl. The protein compositions of these extracts were examined by gel electrophoresis (above) and tested for their ability to restore translocation/processing activity to RM$_i$ (shown along the bottom). Asterisk/arrow indicates the 60,000-mol wt protein.
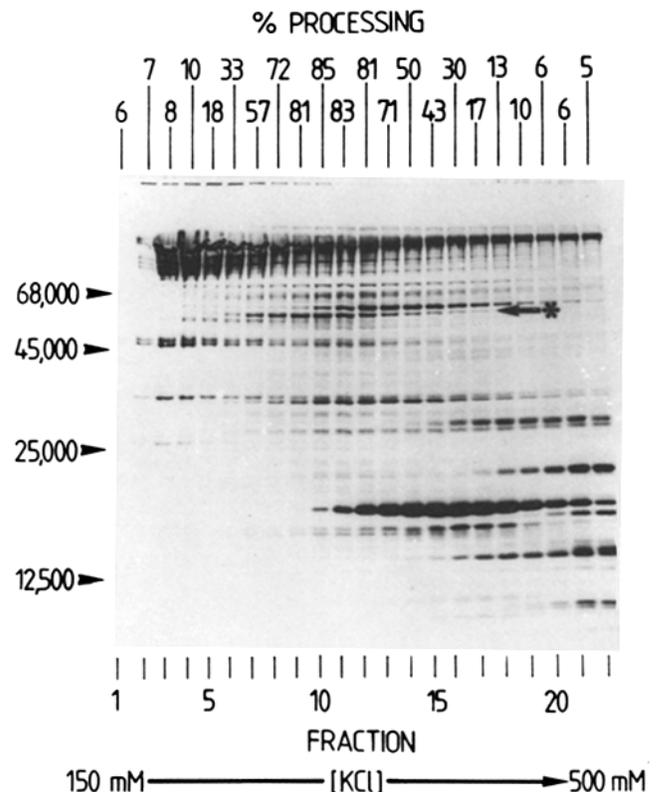

FIGURE 3 Elution of SE from CM-Sephadex with a gradient of KCl. SE derived from Triton X-100/50 mM KCl-extracted microsomes was bound to CM-Sephadex at 150 mM KCl, pH 7.5, and eluted with a linear gradient of 150–500 mM KCl, pH 7.5. Each fraction was assayed for restoration of translocation/processing activity to RM$_i$ (shown across the top), and proteins characterized on Coomassie Blue-stained polyacrylamide gels (shown above). Asterisk/arrow indicates the species showing the best correlation with activity.
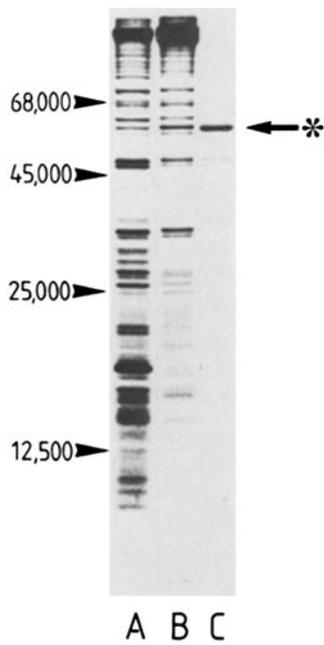
FIGURE 4 Purification of material bound to CM-Sephadex by gel filtration. SE derived from Triton X-100/50 mM KCl-extracted microsomes was bound to CM-Sephadex at 200 mM KCl and eluted at 350 mM KCl. The eluted material was concentrated and further fractionated on Sephadex G-150, as described in Materials and Methods. SE fractions containing translocation/processing activity were separated on polyacrylamide gels and stained with Coomassie blue. *A*, elastase/high salt extract of microsomes (SE); *B*, material eluted from CM-Sephadex at 350 mM KCl; *C*, translocation/processing activity–containing fraction obtained by gel filtration on Sephadex G-150. Asterisk/arrow indicates 60,000-mol wt protein.

purification achieved are given in Table I. Specific activity measurements indicate that the final product was purified some 70-fold compared with the elastase/KCl-extracted starting material. The data presented for the Sephadex-purified protein were derived from the most active of several active fractions. Hence, a high specific activity value was obtained with a correspondingly low recovery of total protein. Based on such measurements, it is possible to approximate that the active factor represents ~1% of the total protein present in the SE.

A crucial proof demonstrating that the ability to restore translocating activity to RM_i is associated with the 60,000-mol wt protein was undertaken. Protein patterns from active and inactive SE were compared on polyacrylamide gels. One would expect that the 60,000-mol wt protein would only be present in the pattern representing the active SE. High salt treatment alone and excessive protease/high salt treatment was used to produce the inactive mixtures. The presence of elastase at the right concentration gave rise to the active SE. Although the patterns show a great similarity, the 60,000-mol wt band is located solely in the pattern of the active SE (Fig. 6).

TABLE I

Purification of Active Factor from Rough Microsomes

Elastase extract (+RM_i)	Specific activity*	Total protein‡
Crude	4.4	27
After CM-Sephadex	13.3	2.0
After Sephadex G-150	320	0.05

* Specific activity is expressed as the reciprocal of the amount of material (in A_{280}) $\times 10^{-3}$ needed to convert 50% of IgG light chain precursor to authentic light chain in one 25-μl cell-free translation.
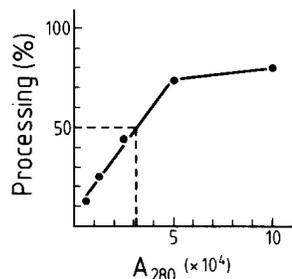‡ A_{280}.

FIGURE 5 Titration of SE with a constant amount of RM_i. Serial dilutions of SE (or fractions derived from SE during purification of the active factor) were added to RM_i and assayed for the ability to cotranslationally convert IgG light chain precursor to authentic light chain (see reference 18 for method). SE protein is expressed as A_{280}. Above, a titration of crude SE. The dashed line indicates that 50% processing, the level used for specific activity measurements (see Table I), is within the linear range of this type of assay.

found to be localized in a fraction containing primarily the 60,000-mol wt species. Densitometric quantitation of Coomassie Blue–stained gels showed that this protein accounted for 85% of the total absorbance of the fraction.

The degree of purification achieved with the procedures described was measured as follows: the amount of protein was determined (in a given fraction of SE) which was required to convert 50% of precursor to authentic light chain when added to a standard aliquot of RM_i in an in vitro translation. This level of conversion was selected because it falls within the linear portion of the titration curve (Fig. 5). The levels of
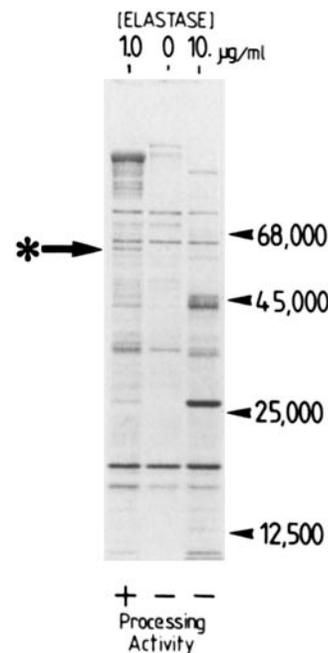

FIGURE 6 Comparison of proteins of active and inactive SE. SE derived from Triton X-100/50 mM KCl-extracted microsomes was prepared using the protease concentrations shown across the top. Proteins were separated on polyacrylamide gels and stained with Coomassie Blue. On the bottom, the ability to restore translocation/processing activity to RM_i is indicated. The asterisk/arrow points out the 60,000-mol wt species.
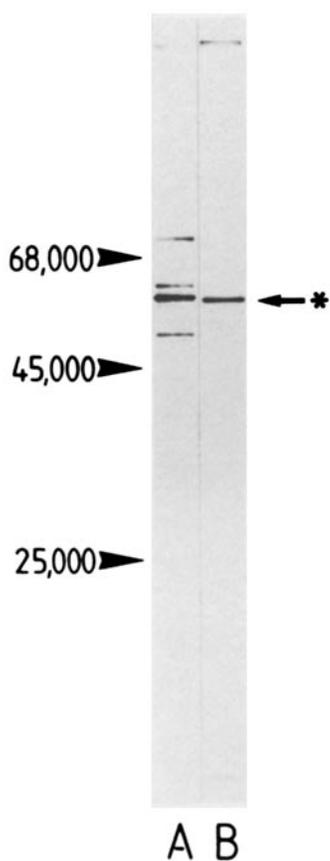
68,000 ►

45,000 ►

25,000 ►

A B

FIGURE 7 Alkylation of sulfhydryl groups of purified SE. A Sephadex-purified fraction of SE was alkylated with NEM (for details see Materials and Methods). Samples were characterized by gel electrophoresis. (A) NEM-labeled sample stained with Coomassie Blue. (B) An aliquot of the NEM-labeled sample detected by autofluorography. Asterisk/arrow indicates the 60,000-mol wt species.

NEM Labeling

A recent report by Jackson et al. (23) has shown that the active component in SE has a sulfhydryl group that is essential for activity. To demonstrate that this fact is consistent with the notion that the 60,000-mol wt peptide is the active factor, a sample of Sephadex-purified SE was labeled with NEM. Of the four proteins present in the sample, only the major band at 60,000 mol wt was labeled by the alkylating agent (Fig. 7). Moreover, such an alkylated sample was no longer able to restore translocation activity to RM$_i$.

DISCUSSION

The results of this study characterize a protein species that is essential for the translocation of nascent peptides across rough microsomal membranes. This is the first example in which the isolation of such a molecule has been accomplished based on a functional requirement for translocation. The purification of this molecule was greatly facilitated by its water-solubility. This was accomplished by proteolytic cleavage and high salt extraction of a cytoplasmically exposed domain from a larger membrane protein. The advantage of a soluble fragment for further purification has previously been demonstrated in the isolation of histocompatibility antigens (24, 25), cytochrome b_5 (26), and enzymes located in the intestinal brush border (27). The component isolated in this study is unique, as it is able to recombine with the membrane and restore a function.

The overall characteristics of the molecule required for translocation have been determined in this and the previous study (18). The peptide is the cytoplasmically disposed domain of a larger membrane protein. It is rendered water-soluble through cleavage with elastase in conjunction with high salt. It is basic in character, contains at least one free sulfhydryl group, and has an apparent molecular weight of 60,000.

It was seen that the active fragment can be cleaved from either an intact membrane or after disruption of the membrane structure by detergents. Because the intact membrane form remains insoluble in the presence of detergent at low salt and becomes soluble in the presence of detergent at high salt concentrations, one can conclude that the molecule interacts electrostatically with other membrane proteins. In addition, the ability to cleave off the same functional portion regardless of the intactness of the membrane cannot be overlooked. In this way, one will then be able to perform translocation assays in a reconstituted system during the purification of the intact membrane protein.

The ability of the 60,000-mol wt peptide to bind to CM-Sephadex at pH 7.5 in the presence of 200 mM KCl suggests that it has a basic isoelectric point. This feature may, on the one hand, allow binding with other components in the membrane to take place, as was demonstrated in the previous paper to be necessary for translocation to occur (18). It may, on the other hand, be involved in an electrostatic interaction with components in the initiated ribosomal complex.

Membrane proteins that bind to ribosomes have been found in rough microsomes of rat liver. Referred to as ribophorins, this pair of glycoproteins has molecular weights of 63,000 and 65,000, respectively (12, 13). The question arose whether or not ribophorins represent the intact membrane proteins from which the 60,000-mol wt fragment is derived. Preliminary results have shown that the intact membrane protein is not a glycoprotein. This result was obtained by lectin affinity chromatography of Triton X-100–solubilized rough microsomal proteins (D. I. Meyer, unpublished observations). Thus, it is unlikely that the 60,000-mol wt species is derived from a ribophorin like protein.

The fact that the 60,000-mol wt fragment contains a free sulfhydryl group is consistent with the findings of Jackson et al. (23). This would also suggest, as pointed out previously (18), that the active components derived by high salt (19) and by protease treatment (20) are one and the same. Moreover, the presence of free thiol groups could allow thiol:disulfide interchange to play a role in the function of this molecule. Such interactions have been proposed to occur in the active sites of enzymes (9).

All of the fractionation and analytical procedures used in this study showed an excellent correlation between the presence of the 60,000-mol wt protein, as visualized on polyacrylamide gels, and the ability to restore translocation activity to RM$_i$. The data presented here do not yet allow definitive conclusions to be made concerning the role that this·molecule plays in the translocation of nascent peptides across the endoplasmic reticulum membrane. Its location on the cytoplasmic face of the rough microsome suggests that it is involved in one of the first stages of this multistep process. However, its identification and characterization provides a spring-board from which information concerning its precise function in protein translocation can be obtained.

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