A Membrane Component Essential for Vectorial Translocation of Nascent Proteins across the Endoplasmic Reticulum: Requirements for Its Extraction and Reassociation with the Membrane

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ABSTRACT Previous reports have shown that rough microsomes treated with high salt (Warren and Dobberstein, 1978, *Nature*, 273:569–571) or proteases (Walter et al., 1979, *Proc. Natl. Acad. Sci. U. S. A.*, 76:1,795) are unable to vectorially translocate nascent proteins. Readdition of the high salt or protease extracts restored activity to such inactive rough microsomes.

A detailed study was carried out to determine how this factor interacts with the rough microsomal membrane. Proteolytic cleavage was found to be necessary but not sufficient to remove this factor from the membrane. A subsequent treatment with high salt had to be carried out. Endogenous (pancreatic) protease could effect the required cleavage, but low levels of trypsin, clostripain, or elastase were far more efficient. Several proteases were not effective. The minimum level of salt (after proteolysis) required to solubilize the active factor was ~200 mM KCl. Salt extracts prepared by treatment with one of the effective proteases were capable of restoring activity to inactive microsomes produced by treatment with one of the others.

During synthesis on ribosomes bound to the cytoplasmic face of the rough endoplasmic reticulum, secretory proteins pass across the membrane into the lumen (1, 2). In most cases, this is accompanied by the cleavage of an N-terminal extension of amino acids referred to as the signal sequence (references 3 and 4 and for review see references 5 and 6). This process can be reconstructed in a cell-free system where isolated rough microsomal vesicles translocate, process, glycosylate, and sequester nascent secretory (7–9) and insert nascent membrane (10–15) proteins. The same structures within the membrane may be required for the vectorial translocation of secretory and certain membrane proteins. A thorough understanding of this transfer process demands the separation of the components involved and their reassembly into functional complexes. Only then can we begin to understand this process at the molecular level.

The first attempt to characterize molecules involved in vectorial translocation made use of high salt extraction to remove a protein component(s) from isolated rough microsomes. These membranes were then incapable of vectorial translocation in vitro, and their function could only be restored by the readdition of the salt-removed material (16). A similar approach, taken by Walter et al. (17), used protease instead of salt to cleave a factor from the membrane that was essential for proper translocation. Such conflicting reports can be interpreted in two ways. Either the effectiveness of the high salt was based on an endogenous proteolysis that occurred previously or the two procedures led to the liberation of two distinct species. It remains difficult, however, to envision the manner in which a molecule, liberated by proteolysis alone, could become reassociated with the membrane during reconstitution.

Because functional reconstitution is of such crucial importance in understanding the process of translocation, we have reinvestigated, in detail, the conditions necessary to liberate the membrane-derived component. This has enabled us to optimize

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release of this component from the membrane, a step which has led to the identification and characterization of the active protein (18).

MATERIALS AND METHODS

Rough microsomes were prepared as described previously (7, 19) with the following exception: phenylmethylsulfonyl fluoride (PMSF) at a concentration of 40 μg/ml was present in the homogenization medium (if not stated otherwise). The aforementioned published procedure was followed precisely through to the first discontinuous sucrose gradient step.

To rough microsomes collected from the 1.75–2.1 M sucrose interface were added 3 vol of 25 mM HEPES, pH 7.5, 0.7 M KCl, 15 mM EDTA. After a 20-min incubation at 0°C, 50 ml of this suspension was layered over 15 ml of 0.75 M sucrose, 500 mM KCl, 20 mM HEPES, pH 7.5, and centrifuged for 90 min at 105,000 g_{av} in a Beckman Ti 45 rotor (Beckman Instruments, Inc., Fullerton, Calif.). Under these conditions, EDTA/KCl-stripped microsomes do not form a pellet, but instead remain suspended in the 0.75 M sucrose cushion. After aspirating off the clear, upper, EDTA-containing phase, the cloudy, lower, microsome-containing layer was decanted from above a tight ribosome-rich pellet. This suspension was diluted threefold with 20 mM HEPES, pH 7.5, 5 mM dithiothreitol, and centrifuged at 105,000 g_{av} for 2 h to sediment the membranes and remove any residual EDTA. Membranes prepared in this manner represent the starting material for all experiments carried out in this study. This method of preparing microsomes saves time and avoids the need to pellet them before or during the stripping procedure. It also allows the stripping agents greater access to the membrane with the result that ribosomes are more effectively removed. This is shown by the fact that the A_{260}/A_{280} ratio was 10–20% lower in membranes prepared according to this protocol.

Cell-Free Protein Synthesis

In vitro translation of purified immunoglobulin light chain mRNA was carried out, as described previously, with rabbit reticulocyte lysate (15). Unless otherwise stated, extracts derived from the protease and/or high salt treatment of rough microsomes were incubated for 20 min at 0°C with inactive membrane vesicles or the appropriate control before their inclusion in the cell-free system. The concentration of KCl in the system was adjusted when necessary to compensate for KCl added with the salt extracts. A typical translation, carried out for 1 h at 37°C, was composed of 1 μl mRNA (A_{260} = 3/ml), 9 μl translation cocktail containing 25–50 μCi [^{35}S]methionine, 10 μl reticulocyte lysate, and 5 μl of a membrane or inactive membrane/extract suspension or the appropriate control.

Determination of Translocation across Microsomal Membranes

An aliquot of the cell-free translation mixture (2.5 μl) was added to 25 μl of polyacrylamide gel electrophoresis (PAGE) sample buffer (0.1 M Tris, pH 8.8, 0.5 M sucrose, 0.01% bromphenol blue, 5 mM EDTA, 4% SDS, 1% methionine, 5 mM dithiothreitol), and electrophoresis was carried out on polyacrylamide gels (10–15% polyacrylamide gradient), as described previously (4). After fixation for 1 h in TCA, the gel was immersed in 3 vol of Enhance (New England Nuclear, Boston, Mass.) for 45 min, washed, dried, and placed in contact with Kodak X-Omat film for 16–24 h at −80°C. After the development of the fluorogram, the extent of processing could be approximated by scanning the appropriate bands on a Joyce-Lobel microdensitometer (Joyce, Lobel and Co., Ltd., Gateshead-on-Tyne, England).

As a test for translocation of IgG light chain across the microsomal membrane, post-translational proteolysis was performed as follows: 5 μl of the reticulocyte lysate system was incubated for 90 min at 0°C with 5 μl of 0.2 mg/ml proteinase K in 0.25 M sucrose. The reaction was terminated by the addition of 1 μl of 40 mg/ml PMSF in isopropanol. PAGE and fluorography were then carried out on 5-μl aliquots, as described above.

Protease Treatment of Stripped Microsomes

Membranes were suspended in 0.25 M sucrose, 50 mM KCl, 20 mM HEPES, pH 7.5 (buffer A), to a concentration of A_{280} = 50/ml (all absorbance measurements were performed in the presence of 2% SDS). Proteases were activated as required and the appropriate ions or chelators were added from stock solutions (for details, see reference 20). Incubations were carried out as described in Results, and reactions were terminated by the addition of PMSF and/or the appropriate chelating agent (20). Typically, 0.5 ml digests were layered over 0.5-ml cushions of 0.5 M sucrose in 1.5 ml Eppendorf tubes and centrifuged at 40,000 rpm for 90 min in a Beckman Ti 75 rotor (Beckman Instruments, Inc.) fitted with special adapters. The pellets were then washed with 0.5 M KCl, 20 mM HEPES, pH 7.5, and resedimented in Eppendorf tubes, as described above. Aliquots of the protease digest supernate, the membrane pellet (suspended in 0.5 ml buffer A), the salt wash, and the salt-washed membrane pellet (suspended in 0.5 ml buffer A) were tested for their ability to cotranslationally process light chain precursor (in the case of the membranes) or restore activity to proteolytically inactivated (5 μg trypsin/ml, 0°C, 60 min) rough microsomes (RM_i).

Materials

Dog pancreas was obtained from either the University of Heidelberg or Boehringer Mannheim GmbH, Federal Republic of Germany. mRNA from MOPC 41 cells was prepared as described previously (4). [^{35}S]Methionine and a cell-free translation system (rabbit reticulocyte) were purchased from New England Nuclear, Boston, Mass. Proteases were obtained from the following companies: trypsin (EC 3.4.21.4), Sigma Chemical Co., St. Louis, Mo.; subtilisin (EC 3.4.21.14), thermolysin (EC 3.4.24.4), and papain (EC 3.4.22.2), Boehringer Mannheim GmbH, Federal Republic of Germany; elastase (EC 3.4.21.11) and proteinase K (EC 3.4.21.14), Merck & Co., Inc., Darmstadt, Federal Republic of Germany; clostripain (EC 3.4.22.8), Worthington Biochemical Co., Freehold, N. J.; and *Staphylococcus aureus* V-8 (EC 3.4.21.19), Miles Laboratories, Elkart, Ind.

RESULTS

Translocation of nascent peptides can be determined by demonstrating the protection of newly synthesized proteins from proteolytic attack. Because it was necessary in this study to express reconstitution quantitatively, the percent of light chain processed of the total synthesized is given in the figures and tables. Protection assays, although omitted from the Results, were routinely performed to verify the correlation between translocation and processing.

In previously reported experiments (16), high salt alone was used to remove an active component from rough microsomes. No steps were taken to eliminate the effects of endogenous proteolysis, which is quite likely to occur during the fractionation of a protease-rich tissue, such as pancreas. It is, therefore, conceivable that the effectiveness of high salt treatment in liberating this component was in part caused by previous proteolytic activity. To verify this, rough microsomes were prepared in the presence or absence of the protease inhibitor PMSF. We determined that when PMSF was present throughout the isolation procedure, high salt alone was incapable of removing the active component from the membrane (see Fig. 1*A*). When PMSF was omitted from such preparations, high salt removed varying amounts of active material (data not shown). It would appear then that proteolysis is required before

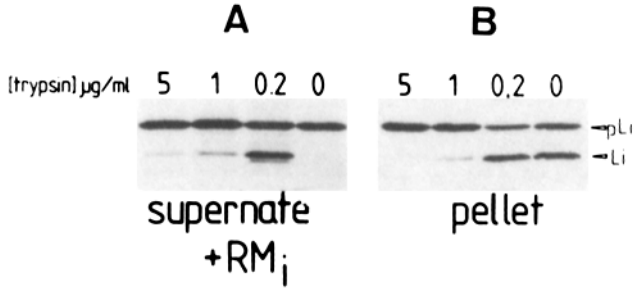

FIGURE 1 Protein translocation activity of microsomes as a function of trypsin treatment. Microsomes were treated with the concentrations of trypsin shown, washed in 0.5 M KCl, and pelleted. (*A*) The ability of the resulting supernate to reconstitute translocation/processing in inactive rough microsomes (RM_i). (*B*) Level of translocation/processing activity remaining in rough microsomes following protease treatment. The fluorogram depicts the conversion of IgG light chain precursor (*pLi*) to authentic light chain (*Li*) upon translocation.

active components can be removed reversibly. Accordingly, subsequent preparations of rough microsomes included PMSF in the homogenization medium. Microsomes prepared in this way could be washed in high salt, in addition to EDTA, to remove peripheral membrane proteins and ribosomes without affecting the active component involved in protein translocation.

The conditions necessary for the quantitative removal of the active component from the membrane and the nature of its interaction with the membrane were investigated using a variety of proteases and varying salt concentrations.

The effect of trypsin/0.5 M KCl on the liberation of an active factor (referred to henceforth as salt extract or SE) capable of restoring the translocating capacity to RM_i is shown in Fig. 1 *A*. Of the three concentrations used (0.2, 1.0, and 5.0 μg/ml), only the lowest (0.2 μg/ml) yielded an active SE. At this trypsin concentration, the microsomes retained a substantial amount of activity (Fig. 1 *B*). To render rough microsomes totally inactive, it is necessary to raise trypsin concentrations to ~5 μg/ml. Thus, RM_i in all experiments represents membranes treated with trypsin at 5 μg/ml (see Materials and Methods for details).

The data shown in Fig. 1 demonstrate that precisely defined protease concentrations are needed to obtain an active SE. Accordingly, a series of trypsin concentrations within the determined effective range (Fig. 1) was tested as a function of the requirement for high salt. Membranes were sedimented after proteolysis and resuspended in 0.5 M KCl or 0.05 M KCl. After centrifugation, the two series of supernates were tested for their ability to reconstitute functional microsomes. The results are shown in Fig. 2. The optimal protease concentration used in conjunction with 0.5 M KCl was found to be between 0.2 and 0.6 μg trypsin/ml. Under low salt conditions, it was difficult to determine an optimum because values were barely above background levels.

The need for high salt to remove the active component from the microsomal membrane implies an electrostatic interaction. To examine this more precisely, membranes treated with an optimal level of trypsin (0.4 μg/ml) were extracted with different concentrations of KCl. It was found that concentrations in excess of 200 mM were required to liberate the majority of the activity (Fig. 3).

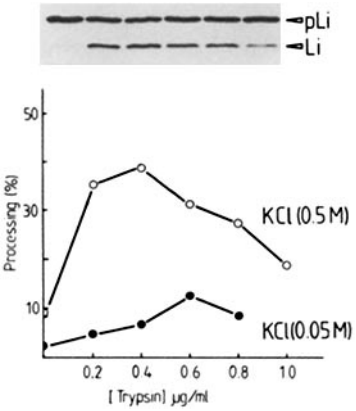

FIGURE 2 Solubilization of translocation/processing activity from rough microsomes as a function of protease and salt concentrations. Microsomes were treated with the trypsin concentrations shown. SE was prepared by washing with 0.5 or 0.05 M KCl. The ability of such extracts to restore translocation/processing activity to RM_i was tested. The curves describe a quantitation of reconstitution. The fluorogram depicts the conversion of IgG light chain precursor (*pLi*) to authentic light chain (*Li*) in a reconstituted system composed of RM_i and SE. ○, Translocation/processing activity released with 0.5 M KCl after proteolysis. ●, Translocation/processing activity released with 0.05 M KCl after proteolysis.

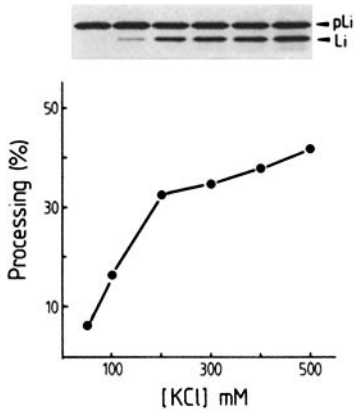

FIGURE 3 Solubilization of translocation/processing activity as a function of salt concentration. Microsomes were treated with trypsin (0.4 μg/ml) and washed with KCl at the concentrations shown. The resulting SEs were tested for their ability to restore the translocation/processing activity of RM_i. Translocation/processing activity is displayed as described in Fig. 2.

Under conditions leading to the optimal recovery of active SE, microsomes retain a considerable capacity for translocation. Thus, there appears to be an equilibrium between cleavage of material at a site that leaves the molecule active and further cleavage that inactivates the molecule. This inactivation could be seen when trypsin concentrations in excess of 0.5 μg/ml were used (Fig. 2). Consistent with this notion is the fact that SE prepared from membranes that had been trypsinized for 30, 60, or 120 min showed the same level of processing in a reconstituted system (data not shown). Furthermore, membranes trypsinized once for 1 h could be resubjected to trypsinization after salt washing to yield a second active SE. In both SEs, the level of processing obtained upon reconstitution with RM_i was nearly identical (data not shown). These data indicate that at any given time there is a relatively constant amount of active component in a cleaved state that can be removed by treating the membrane with salt. To effect a complete recovery of activity, it would, thus, seem that numerous cycles of proteolysis high salt treatment are necessary.

To avoid such a time-consuming, cumbersome purification scheme, the ability of other proteases to produce SE was investigated. It was hoped that an enzyme could be found that would be able to produce effective cleavage without significant inactivation.

Of seven proteases tested (Table I), four (thermolysin, subtilisin, *S. aureus* V8, and papain) were unable to produce an active SE, whereas trypsin, clostripain, and elastase could liberate the active component. To compare levels of activity released by the various proteases, SEs were titrated to determine the amount needed to restore 50% of the translocation/processing activity to RM_i. Clostripain, with a more defined substrate specificity than trypsin, released a comparable amount of activity at the same optimal concentration. At higher enzyme levels (as with trypsin), SE was inactivated. In the case of elastase, expressing a specificity for uncharged nonaromatic amino acids, a concentration of 1–2 μg/ml was optimal in releasing SE. Strikingly, this enzyme yielded about a fivefold higher amount of activity when compared with trypsin or

TABLE I

Protease Treatment of Rough Microsomes and Reconstitution of Protein Translocation

Protease	Specificity	Concentrations used	Reconstitution*	Concentrations for		Optimal condition
				RM_i	SE	
		μg/ml		μg/ml	μg/ml	
Trypsin	Arg, Lys	0.2–5.0	+	1.0	0.2–0.4	0°C, 30′
Clostripain	Arg	0.05–5.0	+	1.0	0.2	0°C, 30′
Elastase	Uncharged nonaromatic amino acids	0.1–25.0	+	5.0	1.0–2.0	0°C, 30′
Papain	Arg, Lys	2–2,000	–	–	–	–
S. aureus V-8	Glu, Asp	0.2–200	–	–	–	–
Subtilisin	Aromatic amino acids	0.2–200	–	–	–	–
Thermolysin	Hydrophobic amino acids	1.0–200	–	–	–	–

* Reconstitution is defined as the ability of the SE derived from protease-treated membranes to restore translocation/processing activity (of immunoglobulin light chain) to RM_i.

TABLE II

*Heterologous Reconstitution of Translocation/ Processing Activity with SE and RM_i Derived from Microsomes Digested with Various Proteases**

	SE			
	None	Trypsin	Clostripain	Elastase
RM_i				
Trypsin	–	+	+	+
Clostripain	–	+	+	ND‡
Elastase	–	+	ND	+

* Indicated is the ability (+) or inability (–) to functionally reconstitute translocation/processing. SE and RM_i were produced by treating rough microsomes with the proteases at their optimal concentrations (see Table I).
‡ ND, not determined.

clostripain. Inactivation of SE was nonetheless observed when a full removal of active component was attempted with higher elastase concentrations. Removal of active SE was always incumbent upon the presence of high salt (0.5 M KCl) after digestion, regardless of which of the three proteases was used.

As three different proteases were capable of producing SE, it was instructive to examine the ability of SE from one protease to successfully reconstitute RM_i produced by another. Table II shows that SE and RM_i produced by either trypsin or clostripain are interchangeable in reconstitution experiments. The same type of recombination experiment with trypsin and elastase was equally successful, with either SE being able to effectively reconstitute either RM_i. This latter finding is noteworthy in light of the substantially different substrate specificities of trypsin and elastase.

DISCUSSION

This study characterizes the interaction of a protein with the cytoplasmic face of rough microsomes. This component is required for vectorial translocation of nascent peptides. Low concentrations of trypsin, clostripain, or elastase in conjunction with at least 200 mM KCl are required to liberate this activity-restoring material. Lowering the salt concentration to physiological levels in the presence of inactive microsomal membrane vesicles enables the reconstitution of a functionally active translocating system.

The extreme sensitivity of the intact component to protease treatment at low temperature indicates that special precautions must be taken during the isolation of rough microsomes to avoid endogenous proteolysis. In the absence of suitable protease inhibitors, active SE can be prepared solely by the salt washing of microsomal membranes, as was observed previously (16). The experiments reported here demonstrate that when PMSF is included throughout the isolation procedure, the active component cannot be removed by high salt alone. Thus, limited proteolysis, whether endogenous or exogenous in origin, is a basic requirement in the preparation of SE. The protease requirement observed here is consistent with the findings of Walter et al. (17). However, their lack of a requirement for high salt and their use of a 10–25-fold higher trypsin concentration is not consistent with our data. Their ability to obtain active SE under such conditions may be caused in part by different enzyme sources and/or centrifugation conditions used (after proteolysis) to isolate solubilized material.

The findings presented here clearly indicate that after proteolysis, high salt treatment is a necessary second step in the liberation of active factor from the membrane. Furthermore, these data indicate that the same component was solubilized in previous investigations with high salt (16) or protease (17).

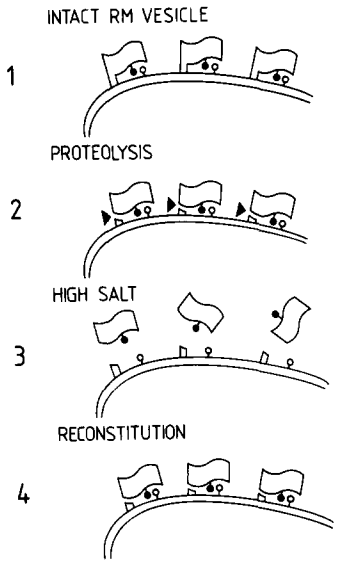

FIGURE 4 Model describing the interaction of the active component with the rough microsomal vesicle. (*1*) In the intact RM vesicle, the active component (⌒) is part of a larger membrane protein and is further bound via an electrostatic interaction (●♀). (*2*) Treatment with protease (▶) cleaves the active component. The molecule is retained on the membrane by the electrostatic bond. (*3*). Treatment with high salt liberates the component. (*4*) Functional reconstitution occurs when the salt concentration is reduced to physiological levels.

Based on our results, a model can be constructed which describes the interaction of the active component (see reference 18) with the membrane. In the intact microsomal vesicle, the active factor exists as the cytoplasmically disposed portion of an integral membrane protein (Fig. 4 *1*), i.e., it cannot be removed by high salt or EDTA. Limited proteolysis will result in cleavage at a site that neither removes the molecule from the membrane nor prevents its functioning in the translocation process (Fig. 4 *2*). Subsequently, the raising of the salt concentration reduces the electrostatic interaction of the active component with the membrane to the extent that it is released into the medium (Fig. 4 *3*). Upon lowering the salt concentration, the released molecule relocates on the membrane via the electrostatic interaction (Fig. 4 *4*). Microsomal vesicles reconstituted in this way are fully capable of translocating nascent peptides.

Although this model does not allow any conclusions to be drawn pertaining to the actual role of the active component in translocation, several functional implications are obvious. With protein synthesis and cotranslational transport occurring at a KCl concentration of 80 mM, the active factor would be located on the membrane in a reconstituted system. Thus, conformational changes need not be postulated to account for the functional relocation of the factor on to the membrane (in contrast to reference 17).

It is noteworthy that proteases with such different substrate specificities as trypsin and elastase gave rise to active SE. It must be recognized that both the site functioning in translocation and the electrostatic membrane binding site were preserved when these enzymes performed the cleavage necessary for the liberation of the factor from the membrane. This suggests that the active component has a domainlike structure. The specific location of cleavage may vary, as was indicated by the success of trypsin and elastase treatments and their respective heterologous reconstitutions. Accordingly, one would anticipate that the active factors derived from trypsin and elastase have different molecular weights. The fivefold higher yields of activity obtained with elastase could then be explained by a more limited range of cleavage sites.

The procedure of removing active factor from microsomes with elastase and high salt, as described here, gives rise to a relatively stable, highly active extract which can be used for further purification studies. The identification of the active fragment is the next essential step in the characterization of the structure and function of the membrane protein from which it is derived (18).

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