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-
easing 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_{260} = 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% trypsol (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 0.5 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.

**Protease Digestion**

The washed, detergent-extracted microsomal material was suspended to a concentration of A_{260} = 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% trypsol (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.

**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 active rough microsomes (RM).

**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 elicited 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.

**N- [ethyl-1-14C]maleimide (NEM) Labeling**

A purified fraction of SE (A_{260} = 0.5/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 DT 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 vesicles. Because microsomes treated in this manner (elastase/high salt) gave optimal recov-
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. 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. (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](image-url) 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 (shown along the bottom). Asterisk/arrow indicates the 60,000-mol wt protein.

![Figure 3](image-url) 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. (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.

FIGURE 5 Titration of SE with a constant amount of RM,. Serial dilutions of SE (or fractions derived from SE during purification of the active factor) were added to RM, 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 A280-

Table 1

| Purification of Active Factor from Rough Microsomes |
|--------------------------------------------------|
| Elastase extract (+RM,) | 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 A280 x 10^-3) needed to convert 50% of IgG light chain precursor to authentic light chain in one 25-µl cell-free translation.
† A280.

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, 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 purification achieved are given in Table 1. 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, 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).
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