Characterization of Molecules Involved in Protein Translocation Using a Specific Antibody

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ABSTRACT The vectorial translocation of nascent proteins through the membrane of the rough endoplasmic reticulum has been shown to require a specific membrane-bound protein whose cytoplasmic domain can be proteolytically cleaved and isolated as an active peptide of mol wt 60,000 (Meyer and Dobberstein, 1980, *J. Cell Biol.* 87:503-508). Rabbit antibodies raised against this peptide were used to further characterize the membrane-bound molecule. Immunoprecipitation of solubilized, radiolabeled rough microsomal proteins yielded a single polypeptide of mol wt 72,000, representing the membrane-bound protein from which the 60,000-mol wt peptide was proteolytically derived. The antibody could also be used to remove exclusively the 60,000-mol wt peptide, and thus the translocation activity, from elastase digests tested in a reconstituted system. Moreover, immunoprecipitation of elastase extracts alkylated with [^{14}C] *N*-ethylmaleimide selected a single species of mol wt 60,000.

Immunoprecipitation of in vivo radiolabeled proteins from the appropriate cell type yielded the 72,000-mol wt membrane protein irrespective of the duration of labeling, or if followed by a chase. Subsequent treatment with protease generated the 60,000-mol wt fragment. In addition, the antibody could be used to visualize reticular structures in intact cells which correspond to endoplasmic reticulum at the ultrastructural level. It is thus clear that one membrane component required in the vectorial translocation of nascent secretory (and membrane) proteins is a peptide of mol wt 72,000.

Isolated rough microsomes have been shown to be capable of procesing, translocating, and glycosylating a number of secretory and membrane protein precursors in vitro (1, 2; for reviews, 3 and 4). In an attempt to characterize the membrane components which mediate the vectorial translocation of such proteins, microsomal membranes have been fractionated in various ways (5–9). Recently, we have shown that mild proteolysis in conjunction with high-salt treatment solubilized the cytoplasmic domain of a microsomal membrane protein required for the vectorial translocation of nascent secretory proteins (7, 8). The cytoplasmic domain is a basic polypeptide of mol wt 60,000.

Aside from its being required for activity, little is known about this molecule's structure or the role it plays in vectorial translocation. To conclusively demonstrate the involvement of the 60,000-mol wt peptide in translocation, as well as to identify and characterize its membrane-bound form, antibodies were raised against this fragment in rabbits. Reported here is the characterization of the antibody (antifragment) raised against this proteolytically generated peptide and the results of its use in further probing the components involved in vectorial translocation.

MATERIALS AND METHODS

Assays for translocation and processing of nascent proteins, as well as polyacrylamide gel electrophoresis, were carried out as described (7).

Preparation of Antigen and Antibody

A crude preparation of the 60,000-mol wt protein fragment was purified from rough microsomes as described (7). After the ion-exchange step the purified elastase extract was fractionated on preparative (2-mm) slab gels, the relevant area of the gel (indicated by fluorescine-labeled standard proteins) was cut out, and chromatographically pure fragment was electrophoretically eluted from the gel slices (10). After dialysis (against 500 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 20 mM HEPES, and 50% glycerol) to remove SDS and "renature" the protein, 100 μg of fragment was injected into a rabbit as described (11), with one boost of 20 μg 4 wk later. 2 wk later and four times thereafter blood was collected at 10-d intervals. IgG was purified from the antiserum by anion-exchange chromatography (12).

Pulse Labeling of MDCK Cells

Madin-Darby Canine Kidney (MDCK) cells were grown to 90% confluence in 60-mm dishes in modified Eagle's medium (MEM) containing 10% fetal calf serum. Each dish received a pulse of 0.5 μCi of ^{35}Methionine (New England Nuclear, Boston, Mass.; sp act: 1,200 Ci/mmol) in methionine-free medium according to the scheme presented in the legend to Fig. 3. Cells were washed,

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scraped from the dish, and lysed in hypotonic buffer (10 mM HEPES, pH 7.5). Nuclei and cell debris were removed by centrifugation at 1000 *g* for 10 min. A crude membrane fraction was prepared from the postnuclear supernate by centrifugation at 100,000 *g* for 45 min. The pellet was dissolved in the following buffer: 50 mM Tris HCl, pH 7.5, 250 mM KCl, 0.5% (wt/wt) Triton X-100, 0.5% (wt/wt) Sodium Desoxycholate, 40 μg/ml Phenylmethylsulfonylfluoride (PMSF), 5 mM iodoacetamide, 10 μg/ml trasylol, 0.1 mg/ml benzamidine, 10 μg/ml pepstatin, 10 μg/ml antipain, 0.1% gelatin, and 2 mM EDTA.

Immunoprecipitation

Stripped rough microsomes (7) were solubilized in 200 mM KCl, 1.3% (wt/wt) Triton X-100, 20 mM HEPES, pH 7.5, and 40 μg/ml PMSF. Insoluble material was removed by centrifugation at 100,000 *g* av for 1 h. Elastase extracts were prepared as described (8). Samples were radioiodinated according to the method of Bolton and Hunter (13) or alkylated with [^{14}C]*N*-ethylmaleimide ([^{14}C]NEM) as described previously (8). Bolton-Hunter Reagent and [^{14}C]NEM were purchased from New England Nuclear. Protein samples (50 μl) were immunoprecipitated according to the procedure published by Dobberstein et al. (14).

Immunofluorescence Microscopy

Cells were fixed and labeled with antifragment using procedures described by Ash et al. (15). The second antibody was a goat antirabbit IgG that had been affinity purified on a column containing bound rabbit IgG (16) and then conjugated to rhodamine (17). Cells were observed using epifluorescence on a Zeiss photomicroscope III equipped with a 63 times' oil immersion objective.

RESULTS

Immunoprecipitation of Radioiodinated Membrane Proteins

The antibody was first used to ascertain from which membrane protein the fragment was proteolytically derived. Stripped rough microsomes were either solubilized in detergent or used to prepare an elastase/high-salt extract containing the 60,000-mol wt fragment. In this way the antigens of nonproteolyzed membranes could be compared with those derived from proteolytic digestion of the cytoplasmic surface. Fig. 1 displays the results of immune precipitations of detergent-solubilized microsomes, of a crude elastase/high-salt extract, and of a purified elastase/high-salt extract.

The species precipitated from solubilized membranes has an apparent mol wt of 72,000 (Fig. 1, lane *2*). As endogenous proteolysis was avoided through the use of PMSF, no significant amount of the fragment was obtained. As would be expected, the antibody precipitated a peptide of mol wt 60,000 from elastase/high-salt extracts (Fig. 1, lane *4*). When elastase extracts were further purified (with respect to the 60,000-mol wt species) by ion-exchange chromatography (8), and then used for immunoprecipitations, the relative amount of 60,000-mol wt peptide precipitated was correspondingly higher (Fig. 1, lane *6*). Interestingly, the material precipitated from extracts thus purified contains a limited amount (<2% of the material precipitated) which has an apparent mol wt of 72,000, corresponding to the nonproteolyzed species.

Immunoprecipitation of NEM-labeled Extracts

It is known that a free thiol group is required for vectorial translocation to occur (18). It has been demonstrated that a species of 60,000 mol wt, present in relatively pure elastase extracts, can be alkylated by NEM (8). To demonstrate that this molecule is antigenically the same as that which can be precipitated by anti-60,000 antibody, immunoprecipitation was performed on an elastase extract inactivated and radiolabeled by alkylation with [^{14}C]NEM. Fig. 1, lanes *7* and *8* indicates

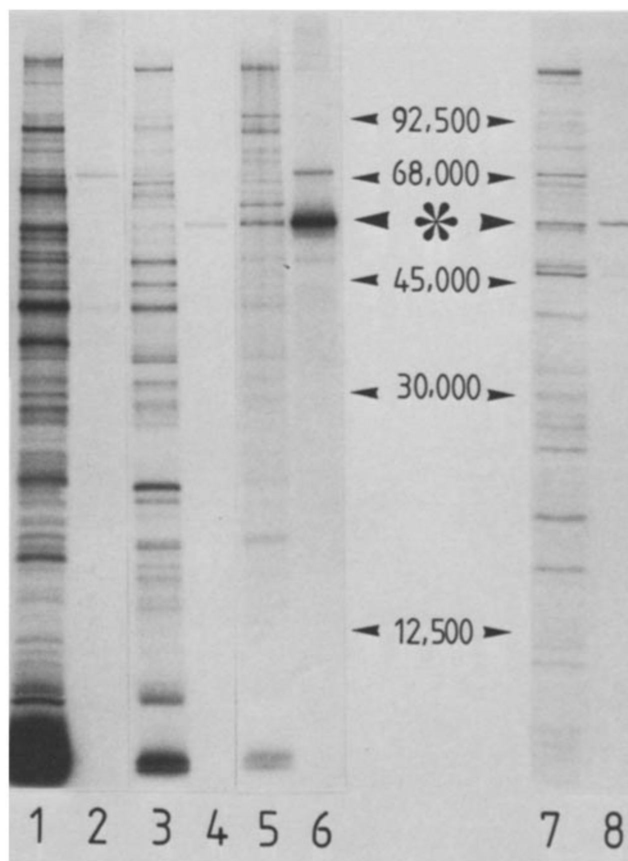

FIGURE 1 Immunoprecipitation of membrane and elastase extract (*EE*) proteins by antifragment IgG. Precipitations were carried out as described by Dobberstein et al. (14). Lanes *1–6* depict proteins radioiodinated with Bolton-Hunter reagent. Lanes *7* and *8* are labeled with [^{14}C]NEM. Shown in this figure are antifragment immunoprecipitations (lanes *2, 4, 6,* and *8*) of detergent-solubilized membrane proteins (lane *1*), crude elastase/high-salt extracts (lane *3* and *7*), or purified elastase/high-salt extracts (lane *5*). Asterisk indicates fragment against which the antibody was raised.

that one of the major labeled species has a 60,000 mol wt and is precipitated by the antibody, thus indicating that this species is antigenically identical to the one identified previously by this technique.

Functional Inhibition of Translocation by Antibody in a Reconstituted System

The aforementioned data verify the fact that a specific peptide can be precipitated from elastase extracts, and that this molecule would appear to be the active factor which restores translocation activity to inactivated membranes. A conclusive proof of this would be the demonstration that once the 60,000-mol wt peptide was immunoabsorbed from an elastase extract, the remainder of the extract would no longer restore function to inactivated rough microsomes (RM_i). Such an experiment was carried out by first adsorbing antifragment IgG (or the appropriate control) to protein A-Sepharose. After washing to remove unbound IgG, an aliquot of elastase extract was added to the protein A-IgG complex. After a short incubation, the protein A-IgG was removed by centrifugation and the supernate was added to RM_i which were then assayed for translocation activity.

As can be seen in Fig. 2, the absorption of elastase extract on protein A Sepharose or protein A Sepharose-preimmune

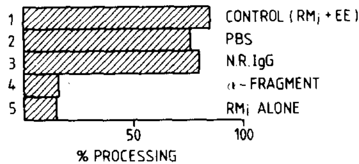

FIGURE 2 Inhibition by antibody of the restoration of translocation/processing activity to inactivated rough microsomes (*RMi*). Immunoabsorption to Protein A Sepharose (PrA-S) IgG was carried out as described in Materials and Methods. Lane *1*—positive control for reconstitution using *RMi* and elastase extract (*EE*) that had not been absorbed. Lane *2*, as in lane *1*, only absorbed on PrA-S incubated in PBS. Lane *3*—PrA-S preincubated with Normal Rabbit (*N.R.*) *IgG*. Lane *4*—PrA-S preincubated with antifragment IgG. Lane *5*—*RMi* to which no *EE* was added.

IgG had no effect on the ability of the extract to restore translocation activity to RM_i. In the case of antifragment IgG bound to protein A Sepharose, virtually no activity remained in the supernate. These data confirm the fact that translocation of nascent peptides has an absolute requirement for the molecule represented in elastase extracts by the 60,000-mol wt species, and that this species is the only one required in such extracts.

Immunoprecipitation of Metabolically Labeled MDCK Cell Proteins

To verify the conclusion that the membrane-associated antigen of 72,000 mol wt represented the native component of endoplasmic reticulum, metabolically labeled MDCK cell proteins were immunoprecipitated. Cells were pulsed and pulse-chased with [^{35}S]methionine for various periods (see legend to Fig. 3). A crude membrane fraction was isolated and subsequently solubilized in detergent-containing buffer (see Materials and Methods). In addition, one aliquot was prepared without protease inhibitors and treated with exogenously added elastase. In Fig. 3 it can be seen that, regardless of the duration of the pulse or the chase, only the 72,000-mol wt species was precipitated by antifragment (lanes *1–4*). When protease inhibitors were omitted and elastase was added (lane *5*), the 60,000-mol wt fragment was generated and precipitated as well. This latter result was obtained regardless of whether the elastase treatment preceded or followed immunoprecipitation.

Indirect Immunofluorescence in MDCK Cells

To establish that the serum obtained had the ability to recognize intact, native cellular structures, and not merely solubilized forms, indirect immunofluorescence microscopy was used. MDCK cells were chosen because the antigen was derived from canine tissue. On the basis of the ultrastructural morphology of MDCK cells as well as previous experience with antibody labeling of rough endoplasmic reticulum (RER) (19), one would expect that an antiserum capable of combining with antigenic sites in the endoplasmic reticulum would yield a characteristic reticulated staining pattern. Such a pattern would be nonvesicular, randomly distributed throughout the cytoplasm, concentrated in the perinuclear region, and would include the nuclear envelope. Fig. 4 depicts the results of such an experiment. It is clear that the antibody recognizes those structures which correspond to the known distribution of endoplasmic reticulum as seen at the ultrastructural level and as confirmed by using other anti-ER sera in cultured cells (19). There does not appear to be any staining pattern characteristic of antigens in either the apical or basolateral plasmalemma (20), the Golgi apparatus (19), or vesicular structures (20). Furthermore, there was no labeling of the cell surface in experiments in which the cells were not permeabilized to the antibody.

This same technique was used to determine the species specificity of the antibody. Positive immunofluorescence was obtained with human fibroblasts (WI-38 cells), whereas staining was not observed in rat (NRK or hepatoma) or hamster (BHK) cell lines. Moreover, the antibody failed to precipitate any radioiodinated proteins derived from detergent-solubilized rat liver (smooth or rough) microsomes.

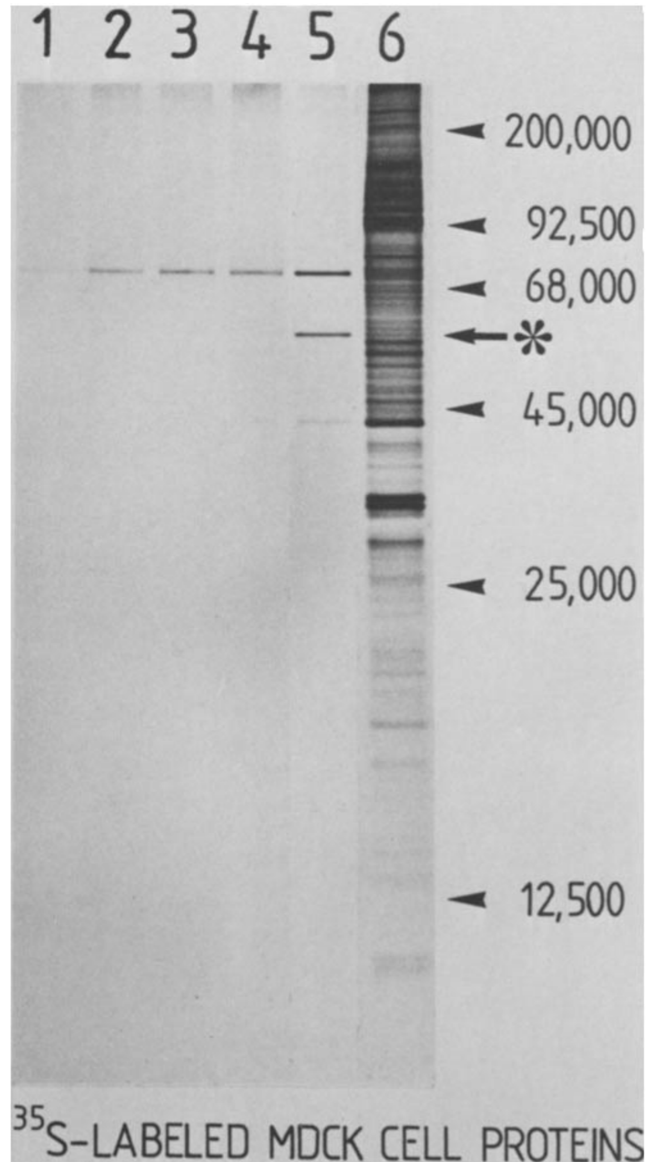

FIGURE 3 Immunoprecipitation by antifragment of pulsed and pulse-chased MDCK cells. Cells were pulsed for 40 min (lane *1*) and chased for either 60 min (lane *2*) or 3 h (lane *3*); or continuously labeled for 4 h (lane *4*). Lane *5* represents continuously labeled samples prepared in the presence of exogenously added elastase at 1 μg/ml. Lane *6* represents the protein composition of detergent-solubilized membranes before immunoprecipitation. Asterisk indicates the 60,000 mol wt fragment. Cell extracts were prepared as described in Materials and Methods.

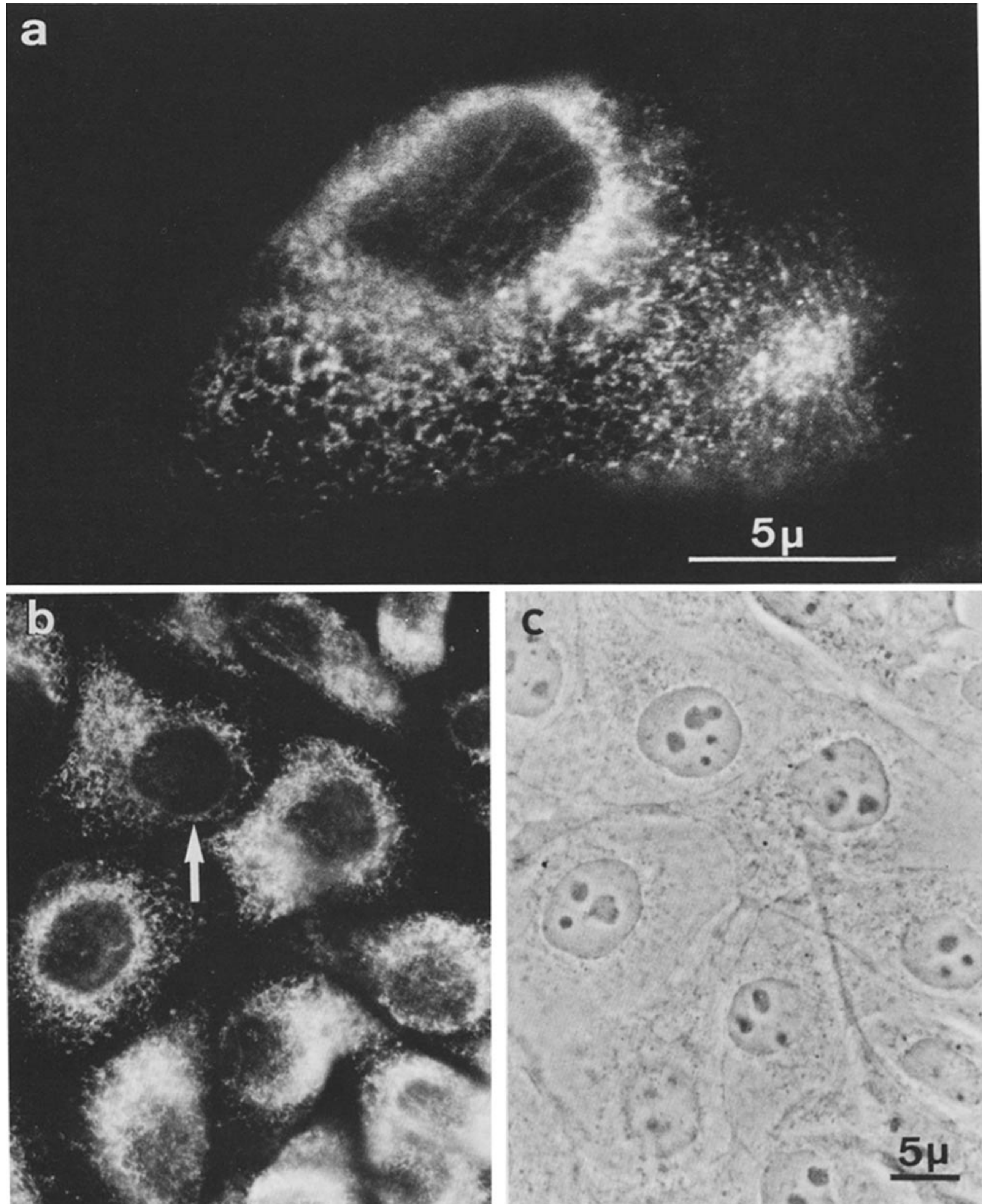

FIGURE 4 Immunofluorescence microscopy of MDCK cells using antifragment IgG. Cells were fixed and stained as described in Materials and Methods. Second antibody was goat antirabbit labeled with rhodamine. *a*) High magnification of a single spread cell. *b*) Typical field of labeled cells (arrow indicates staining of nuclear envelope. *c*) Same as *b*) in phase contrast. Bar, 5 μm.

DISCUSSION

The use of antibodies directed against water-soluble cytoplasmic domains of membrane proteins have previously yielded considerable information about the molecules from which they were derived (21–23). In much the same way, we have been able to generate a tool that will enable further characterization of the components involved in vectorial protein translocation.

On the basis of our model (7), we anticipated that the 60,000-mol wt fragment was part of a larger, membrane-associated

component. Through immunoprecipitation, a larger (mol wt 72,000) peptide was indeed isolated from detergent-solubilized dog pancreas microsomes as well as from cultured cells. The addition of elastase generated the 60,000-mol wt fragment. It is reasonable to conclude that the 72,000-mol wt protein represents the species from which the fragment is proteolytically derived. As it does not bind to Con A (data not shown), it is probably not glycosylated.

One can therefore only speculate as to this molecule's topological relationship to the membrane. The most reasonable assumption is that it interacts with the membrane in a manner similar to that of cytochrome b_5, i.e., a hydrophobic tail is anchored into but does not span the bilayer (24). We are, however, in a position to answer such questions concerning the insertion and assembly of this component now that an appropriate antibody is available.

Although the antigen was purified by preparative gel electrophoresis in the presence of SDS, the antibody recognized not only native molecules in solution but also antigenic sites in the intact rough microsomal membrane. This fact enabled us to demonstrate conclusively that the 60,000-mol wt fragment is the species that restores translocation activity to inactivated rough microsomes.

It should be pointed out that Fab' fragments of the antibody were not capable of blocking vectorial translocation when added to intact rough microsomes. Nor were they able to prevent the rebinding of the 60,000-mol wt fragment to RM_i in a reconstitution assay. This implies that the antigenic sites against which antifragment is directed belong probably neither to the region of the active site nor to the membrane recombining site. This is by no means a disadvantage, as the antibody's ability to recognize the native intact membrane-bound molecule is the sole requirement for its use in assessing the interaction of the 72,000-mol wt species with other RER components.

We have shown that this antibody can also be put to good use as a marker for RER at the light microscopic and ultrastructural levels. As was seen, a reticular pattern of staining was obtained which corresponds to the localization of RER within MDCK cells. Such an antibody can be used as a morphological marker for studies on intracellular protein transport (25) or even for immunologically based methods of cell fractionation (26).

Recently, Walter and Blobel have reported that a multimeric protein complex was involved in vectorial translocation (9). This complex could be isolated from rough microsomes by high-salt treatment alone. It was of interest to determine whether any of the six peptides reported to comprise this complex were antigenically related to the 60,000-mol wt fragment. Immunoprecipitations of radioiodinated, purified complex prepared as described (9) were all negative, i.e., antifragment did not specifically precipitate any of the six subunits under either native or denaturing conditions. This suggests that the two components are not related and that they are probably fulfilling different requirements in vectorial translocation.

It is clear that a large number of questions regarding the specific function of this molecule remain unanswered. The ability to selectively probe the RER by means of an antibody specific for a single component required for vectoral translocation should, however, serve to elucidate this phenomenon in greater detail.

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