Characterization of Molecules Involved in Protein Translocation Using a Specific Antibody

DAVID I. MEYER, DANIEL LOUVARD, and BERNHARD DOBBERSTEIN
Division of Cell Biology, European Molecular Biology Laboratory, Heidelberg, Federal Republic of Germany

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.

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 MgCl₂, 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 \[^{35}S\]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.
Scrapped 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 Phenylmethylsulfonyl fluoride (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 [14C]N-ethylmaleimide ([14C]NEM) as described previously (8). Bolton-Hunter Reagent and [14C]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 [14C]NEM. Fig. 1, lanes 7 and 8 indicates 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 immunoadsorbed from an elastase extract, the remainder of the extract would no longer restore function to inactivated rough microsomes (RM). 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, 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 (RM).

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 [35S]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 radiiodinated proteins derived from detergent-solubilized rat liver (smooth or rough) microsomes.
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 ba, 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 RMi 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 reconstituting 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 vectorial translocation should, however, serve to elucidate this phenomenon in greater detail.

We would like to thank Elke Krause for expert technical assistance, John Stanger for photography, and Ines Benner for typing the manuscript.

This work was supported in part by a grant from the Deutsche Forschungsgemeinschaft (No. Do 199/3).

Received for publication 14 September 1981.

REFERENCES

1. Blobel, G., and B. Dobberstein. 1975. Transfer of proteins across membranes. II. Reconstruction of functional rough microsomes from heterologous components. J. Cell Biol. 67: 652–662.
2. Schiebel, G., R. Jacoby, and T. Carne. 1980. Mechanism of compartmentation of secretory proteins: transport of exocrine pancreatic proteins across the microsomal membrane. J. Cell Biol. 87:611–625.
3. Davis, B. D., and P.-C. Tai. 1980. The mechanism of protein secretion across membranes. Nature (Lond.). 283:433–438.
4. Wickner, W. 1980. Assembly of proteins into membranes. Science (Wash. D. C.). 210:861–868.
5. Warren, G. B., and B. Dobberstein. 1978. Protein transfer across microsomal membranes reassembled from separated membrane components. Proc. Natl. Acad. Sci. U. S. A. 75:1795–1799.
6. Walter, P. R., C. Jackson, M. M. Marcus, V. R. Lingappa, and G. Blobel. 1979. Tryptic digestion and reconstitution of translocation activity for nascent presecretory proteins across microsomal membranes. Proc. Natl. Acad. Sci. U. S. A. 76:1975–1979.
7. Meyer, D. I., and B. Dobberstein. 1980. A membrane component essential for vectorial translocation of nascent proteins across the endoplasmic reticulum: requirements for its extraction and reassociation with the membrane. J. Cell Biol. 87:498–502.
8. Meyer, D. I., and B. Dobberstein. 1980. Identification and characterization of a membrane component essential for the translocation of nascent proteins across the membrane of the endoplasmic reticulum. J. Cell Biol. 87:503–506.
9. Walter, P., and G. Blobel. 1980. Purification of a membrane-associated protein complex required for protein translocation across the endoplasmic reticulum. Proc. Natl. Acad. Sci. U. S. A. 77:112–1116.
10. Matlin, K. S. 1979. Ph.D. Thesis. Studies on chloroplast protein synthesis in vivo and in vitro. The Rockefeller University, New York.
11. Gooden, R. B., C. W. Herne, and P. A. Wilkinson. 1966. A simple method for producing antibody specific to a single diffusible antigen. Lancet. 11:1224.
12. Fahey, J. L., and E. W. Terry. 1973. Jr. Handbook of Experimental Immunology. Vol. I. Immunocytochemistry. (Ch. 7), D. M. Weir, editor. Blackwell Scientific Publications, Oxford. 1–16.
13. Bolton, A. E., and W. M. Hunter. 1973. The labeling of proteins to high specific radioactivities by conjugation to a '1'1'-containing acylating agent. Biochem. J. 133:529–533.
14. Dobberstein, B., H. Garoff, G. Warren, and P. J. Robison. 1979. Cell-free synthesis and membrane insertion of mouse H-2Dk histocompatibility antigen and β2-microglobulin. Cell 17:759–769.
15. Ash, J. F., D. Loward, and S. J. Singer. 1977. Antibody-induced linkages of plasma membrane proteins to intracellular cytosol-containing filaments in cultured fibroblasts. Proc. Natl. Acad. Sci. U. S. A. 74:5584–5588.
16. Ternynck, T., and S. Avrameas. 1976. Polymerization and immobilization of proteins using edchyloliprase and diethylpyrocarbonate. Scand. J. Immunol. Supp. 3:29–35.
17. Brandtzaeg, P. 1973. Conjugates of Immunoglobulin G with different fluorochromes. I. Characterization by anionic exchange chromatography. Scand. J. Immunol. 2:273–280.
18. Jackson, R. C., P. Walter, and G. Blobel. 1980. Secretion requires a cytoplasmically-distributed sulphhydril of the RER membrane. Nature (Lond.). 286:174–176.
19. Loward, D., H. Reggio, and G. Warren. 1982. Antibodies to the Golgi Complex and the rough endoplasmic reticulum. J. Cell Biol. 92:92–197.
20. Loward, D. 1980. Apical membrane aminopeptidase appears at site of cell-cell contact in cultured kidney epithelial cells. Proc. Natl. Acad. Sci. U. S. A. 77:4312–4316.
21. Neubrenner, S. G., and S. E. Culler. 1974. Biochemical properties and immunoochemical- genetic relationship of mouse H-2 alloantigens. Biochim. Biophys. Acta. 344:1–25.
22. Medusin, J., G. Corte, G. Pietrini, and N. Borgese. 1980. Localization and biosynthesis of NADP-dependent cytochrome b, reductase, an integral membrane protein, in rat liver cells. Il. Evidence that a single enzyme accounts for the activity in its various subcellular locations. J. Cell Biol. 85:516–526.
23. Loward, D., S. Marou, C. Vanner, and P. Desautel. 1975. Topological studies on the hydrolases bound to the intestinal brush border membrane. I. Solubilization by papain. J. Cell Biol. 27:351–352.
24. Spatz, L., and P. Strittmatter. 1971. A form of cytochrome b, that contains an additional hydrolase bound to the intestinal brush border membrane. I. Solubilization by papain. J. Cell Biol. 27:351–352.
25. Tartakoff, A. M. 1980. The golgi complex: crossroads for vesicular traffic. Int. Rev. Exp. Pathol. 22:226–251.
26. Jia, A., and G. Palade. 1978. Presence of NAD P-dependent cytochrome P-450 reductase in rat liver Golgi membranes. Evidence obtained by immunosorbent method. J. Cell Biol. 76:590–597.