that incorporation of radioactivity into hot trichloroacetic acid-insoluble material continues for at least 15 min and then levels off.[6]

The most difficult step in this procedure is the isolation of highly purified mRNA. The lipoprotein mRNA is the only mRNA of *E. coli* that has been purified to homogeneity.[39] When the unique features of an mRNA cannot be used to facilitate its isolation from whole cells, perhaps total cellular RNA or RNA synthesized *in vitro* from a DNA template can be used (for an example, see Queen and Rosenberg[40]).

[39] K. Takeishi, M. Yasumura, R. Pirtle, and M. Inouye, *J. Biol. Chem.* **251,** 6259 (1976).
[40] C. Queen and M. Rosenberg, *Cell* **25,** 241 (1981).

[6] Preparation of Microsomal Membranes for Cotranslational Protein Translocation

By PETER WALTER and GÜNTER BLOBEL

Secretory, lysosomal, and many integral membrane proteins are translocated *across* or asymmetrically integrated *into* the membrane of the rough endoplasmic reticulum.[1,1a] The events of this translocation or integration process can be faithfully reproduced *in vitro*.[2-4] For this purpose, an *in vitro* protein translation system programmed with a suitable mRNA is supplemented with microsomal membranes, a fraction of closed vesicles derived from the rough endoplasmic reticulum.[2]

In all cases investigated, translocation is a *co*translational process; i.e., the nascent chain is vectorially translocated across the membrane as it emerges from the ribosome. Consequently, the microsomal membrane fraction has to be present *during* protein synthesis. This also implies that

[1] Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*'tetraacetic acid; IAA, iodoacetamide; NEM, *N*-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; RM, rough microsomes; SDS, sodium dodecyl sulfate; SRP, signal recognition particle; TCA, trichloroacetic acid; TEA, triethanolamine; TPCK, L-1-tosylamide-2-phenylethyl chloromethyl ketone.
[1a] G. Palade, *Science* **189,** 347 (1975).
[2] G. Blobel and B. Dobberstein, *J. Cell Biol.* **67,** 852 (1975).
[3] F. N. Katz, J. E. Rothman, V. R. Lingappa, G. Blobel, and H. F. Lodish, *Proc. Natl. Acad. Sci. U.S.A.* **74,** 3278 (1977).
[4] A. H. Erickson, G. Conner, and G. Blobel, *J. Biol. Chem.* **256,** 11224 (1981).

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all the components added to study the translocation process have to be compatible with *in vitro* protein synthesis.[2] If the microsomal vesicles are added after protein synthesis is completed, a *post*translational translocation is not observed.

In most cases translocation is accompanied by the proteolytic removal of the signal peptide by signal peptidase, an endoprotease located on the luminal face or inside the lipid bilayer of the endoplasmic reticulum.[2,5,6] The conversion of the preprotein synthesized in the absence of microsomes to the processed form can therefore be taken as an assay for successful translocation. However, cleavage of the signal peptide (1500–3000 daltons) may be difficult to detect by mobility differences in SDS–PAGE in cases where the protein is larger than 60,000 daltons. Moreover, it is essential to determine the partial NH_2-terminal amino acid sequences of the processed chain to ascertain that cleavage did in fact occur at the signal peptidase site, not at some other nearby site by a protease other than signal peptidase. In addition to the signal peptide removal by signal peptidase, the translocated protein ends up on the inside of the microsomal vesicles and is therefore protected from degradation by exogenously added proteases.[2] This provides additional evidence that actual chain translocation occurred. In the case of proteins with uncleaved signal sequences, cosedimentation of the translocated protein with the vesicles under stringent conditions (high salt, high pH, urea, etc.) or the appearance of a coreglycosylated form of the translocated protein have been used as assays for translocation.[7,8] Occasionally, however, the translocation-coupled loss of the signal peptide and the gain of a single oligosaccharide compensate for each other so that the molecular weight difference between primary translation product and translocated chain is nil.[9]

The Translocation Machinery

We like to view the translocation machinery in the membrane of the endoplasmic reticulum as an assembly of proteins, representing the enzymic activities required for the specific polysome recognition and attachment, chain translocation, and the cotranslational modification of the nascent chain. A few of the activities of such a putative multienzyme

[5] R. C. Jackson and G. Blobel, *Proc. Natl. Acad. Sci. U.S.A.* **74,** 5598 (1977).

[6] V. R. Lingappa, A. Devillers-Thiery, and G. Blobel, *Proc. Natl. Acad. Sci. U.S.A.* **74,** 2059 (1977).

[7] V. R. Lingappa, D. Shields, S. L. C. Woo, and G. Blobel, *J. Cell. Biol.* **79,** 567 (1978).

[8] B. Goldman and G. Blobel, *J. Cell. Biol.* **90,** 236 (1981).

[9] D. J. Anderson and G. Blobel, *Proc. Natl. Acad. Sci. U.S.A.* **78,** 5598 (1981).

assembly have been isolated and characterized. Both specific polysome recognition[10] and binding[11] to the membrane are catalyzed by the signal recognition particle (SRP), a ribonucleoprotein consisting of six different polypeptide chains and one molecule of 7 S RNA of 300 nucleotides.[12,34] SRP appears to shuttle between a free, a ribosome/polysome-associated, and a membrane-bound state, and thus functions as adaptor between the cytoplasmic translation and the membrane-bound translocation machinery. It can be efficiently extracted from the membrane with solutions of high ionic strength.[13] On the membrane, SRP interacts with a SRP-receptor protein,[31] which was also termed docking protein.[32] SRP receptor is an integral membrane protein (72,000 daltons)[14] containing a 60,000-dalton cytoplasmic domain which can be removed by mild proteolytic digestion of the membrane. The 60,000-dalton fragment can be added back to proteolyzed membranes to reconstitute the translocation activity.[15–17,30,33]

Two integral membrane proteins (ribophorins)[18,19] have been identified on the basis of their apparent physical association with attached polysomes, but so far no evidence for their functional involvement in protein translocation has been presented.

The enzymic activities involved in cotranslational modifications of the nascent chain have not yet been fully characterized. Signal peptidase is an integral membrane protein that (or at least its active side) faces the luminal site of the endoplasmic reticulum membrane,[5,15] and, so far, there is no known inhibitor of its activity. If the microsomes are rendered translocation-inactive (e.g., by salt extraction, proteolysis, or *N*-ethylmaleimide treatment), the peptidase activity remains in a latent form in the membrane fraction and can be exposed by disrupting the lipid bilayer with detergents (this volume [62]). In detergent solutions it requires phospholipid for its activity.[20] The oligosaccharide transferase(s) that transfers the core oligosaccharide from the dolichol phosphate carrier to certain aspar-

[10] P. Walter, I. Ibrahimi, and G. Blobel, *J. Cell Biol.* **91**, 545 (1981).

[11] P. Walter and G. Blobel, *J. Cell Biol.* **91**, 551 (1981).

[12] P. Walter and G. Blobel, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 7112 (1980).

[13] G. Warren and B. Dobberstein, *Nature (London)* **273**, 569 (1978).

[14] D. I. Meyer, D. Louvard, and B. Dobberstein, *J. Cell Biol.* **92**, 579 (1982).

[15] P. Walter, R. C. Jackson, M. M. Marcus, V. R. Lingappa, and G. Blobel, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1795 (1979).

[16] D. I. Meyer and B. Dobberstein, *J. Cell Biol.* **87**, 498 (1980).

[17] D. I. Meyer and B. Dobberstein, *J. Cell Biol.* **87**, 503 (1980).

[18] G. Kreibich, C. M. Freienstein, P. N. Pereyra, B. C. Ulrich, and D. D. Sabatini, *J. Cell Biol.* **77**, 464 (1978).

[19] G. Kreibich, B. C. Ulrich, and D. D. Sabatini, *J. Cell Biol.* **77**, 488 (1978).

[20] R. C. Jackson and W. R. White, *J. Biol. Chem.* **256**, 2545 (1981).

agine residues in the nascent polypeptide chain has been considerably enriched from detergent extracts of chicken oviduct rough microsomes.[21]

Some General Remarks on the Preparation of Microsomal Membranes

At present, canine pancreas is our main source of actively translocating microsomal membranes. Because the pancreas actively secretes digestive enzymes, it is essential to take a number of precautions. We work as fast as possible. It usually takes us less than 90 min from sacrificing the dog to the start of the final centrifugation step to pellet the microsomes. All steps are carried out at 0–4° to minimize degradation. We also add PMSF (a covalent serine protease inhibitor) and EDTA (to inhibit metalloproteases) to the homogenization buffer. Under these conditions, we obtain a rough microsomal fraction which is essentially unproteolyzed (as judged by the intactness of the three higher-molecular-weight polypeptides of SRP by immunological criteria).

The translocation activity of the microsomal membranes is dependent on free SH groups.[12,17,22] We therefore include 1 m*M* DTT in all buffers to keep the membranes under reducing conditions. Although there is no absolute requirement for the presence of DTT, we seem to obtain a better reproducibility as far as their translocation activity is concerned between different preparations if it is included. It should also be noted that, owing to the sulfhydryl requirement of the translocation activity, no reagents modifying SH groups (e.g., NEM, IAA, TPCK) can be added at any stage of the preparation.

Microsomal membranes can (and should) be rapidly frozen in small aliquots (<5 ml) in liquid N_2. We include 250 m*M* sucrose as a cryoprotectant. They can be stored at −80° for at least a year without a loss of activity. When stored at −20° (frozen or in 50% glycerol), they seem to lose activity more rapidly. For thawing, the tube is warmed up fast in a water bath at room temperature with rapid agitation; only after the contents are completely thawed is the tube placed on ice. We observe no detectable loss of activity upon at least three thawing and freezing cycles.

Solutions

A stock solution of 1.0 *M* triethanolamine was adjusted to pH 7.5 at room temperature with acetic acid and, as such, is referred to as TEA. A stock solution of 4.0 *M* KOAc was adjusted to pH 7.5 at room tempera-

[21] R. C. Das and E. C. Heath, *Proc. Natl. Acad. Sci. U.S.A.* **77,** 3811 (1980).
[22] R. C. Jackson, P. Walter, and G. Blobel, *Nature (London)* **284,** 174 (1980).

ture with acetic acid. A stock solution of 0.2 *M* EDTA was adjusted to pH 7.5 at room temperature with NaOH. Stock solutions of 2.5 *M* sucrose and 1.0 *M* $Mg(OAc)_2$ were not further adjusted. All stock solutions mentioned above were filtered through a 0.45-μm Millipore filter, except the sucrose solution, which was filtered through a 1.2-μm Millipore filter. Phenylmethylsulfonyl fluoride (PMSF) was freshly dissolved in ethanol or dimethyl sulfoxide to a concentration of 100 m*M* and diluted into the buffers immediately before use. Dithiothreitol was kept in small aliquots as a 1 *M* stock solution at −20° and diluted into the buffers immediately before use.

Buffer A: 250 m*M* sucrose, 50 m*M* TEA, 50 m*M* KOAc, 6 m*M* $Mg(OAc)_2$, 1 m*M* EDTA, 1 m*M* DTT, 0.5 m*M* PMSF

Buffer B: 250 m*M* sucrose, 50 m*M* TEA, 1 m*M* DTT

Buffer C: 50 m*M* TEA, 1.5 m*M* $Mg(OAc)_2$, 1 m*M* EDTA, 1 m*M* DTT, 0.5 m*M* PMSF

Preparation of Crude Rough Microsomes

Dogs weighing 10–25 kg, of either sex, were used. Although it appears to be an important parameter to check, the effect of fasting or feeding the animal prior to sacrifice has not been systematically investigated. Most (although not all) of our dogs were fed about 2 hr before sacrifice. Acepromacine maleate {[10-(3-methylaminopropylphenothiazin-2-yl)methyl ketone] maleate; Ayerst}, 1 ml of a 10 mg/ml solution, was injected intramuscularly as a sedative. After about 30–60 min the animals were anesthetized with an intraveneous injection of Nembutal (pentobarbital sodium salt; Abbott) (approximately 5 ml of a 50 mg/ml solution, the extract amount depending on the size of the dog). The dogs were bled by severance of the great vessels at the base of the heart or excision of the heart, and the pancreas was removed with scissors. The gland was immediately rinsed with 50 ml of ice-cold buffer A and immersed in another 50-ml aliquot of buffer A on ice. According to the size of the dog, the excised pancreas weighed 15–60 g. All subsequent steps were carried out at 0–4°.

The gland was freed of connective tissue and large vessels, repetitively rinsed with buffer A, and finally extensively minced with a razor blade. No tissue press was required if the tissue was well minced. Four milliliters of buffer A were then added per gram of tissue. The tissue was extensively homogenized with 5 strokes (10 sec down, 10 sec up) in a motor-driven Potter–Elvehjem homogenizer, avoiding foam formation and heating. Milder homogenization (1 stroke) yielded a microsome fraction with identical activity, but at a reduced yield. The homogenate was centrifuged for 10 min at 1000 g_{av}. Floating fatty material was removed by

aspiration, and the supernatant was recentrifuged for 10 min at 10,000 g_{av}. The supernatant was immediately decanted from the pellet, taking care not to include the loose top layer of the pellet. Crude rough microsomes (RM) were collected by centrifugation of the 10,000 g_{av} supernatant for 2.5 hr at 140,000 g_{av} (Beckman Ti50.2 rotor at 40,000 rpm) through a cushion of 1.3 *M* sucrose in buffer A. The ratio of load to cushion was approximately 3:1. The supernatant, including the cushion and the membranous material at the interface, were removed by aspiration. The pellets were resuspended by manual homogenization in a Dounce homogenizer (A pestle; 2–3 strokes) in buffer B to a concentration of 50 A_{280} units/ml (determined in a 1% SDS solution). In a typical preparation 50 A_{280} units of RM were obtained from 1 g of tissue. The $A_{260}:A_{280}$ ratio was usually 1.84–1.92. The RM preparation obtained in this way can be used directly in an *in vitro* translation system.

Column Washing of RM

To remove adsorbed ribosomes and proteins we employed a column washing procedure.[23] Washed RM retained all their translocation activity and were consistently less inhibitory to protein synthesis; at most 20% inhibition of protein synthesis was observed at 3 Eq of washed RM per 25 μl of translation mix (see below for definition of Eq). The column washing buffer was of low ionic strength to avoid loss of membrane-bound SRP. The buffer also contained a low concentration of magnesium ions, enough to prevent unfolding of the membrane-bound ribosomes.

For a typical washing procedure a 20-ml portion of RM was loaded on a 200-ml Sepharose Cl-2B column (2.5 cm × 40 cm, flow rate 15 ml/hr) that was developed in upward flow in buffer C. The turbid fractions corresponding to the void volume of the column were pooled (about 40 ml), and the membranes were collected by centrifugation for 15 min at 50,000 g_{av}. The resulting washed RM were resuspended in 20 ml of buffer B.

EDTA Stripping of RM

The bulk of the membrane-bound ribosomes (all of the small subunits and at least half of the large subunits) and mRNA as well as many adsorbed proteins or peripheral membrane proteins can be removed from RM by an EDTA-extraction. The resulting membrane fraction has an approximately twofold decreased optical density and a considerably reduced endogenous mRNA activity. It should be noted, however, that the

[23] H. C. Hawkins and R. B. Freedman, *Biochim. Biophys. Acta* **558**, 85 (1979).

translocation activity of EDTA-stripped RM is not increased over that of the starting RM preparation; i.e., in spite of the fact that ribosomes have been unfolded and extracted, no new "translocation-active sites" are generated. SRP is not extracted by the EDTA treatment.

For a typical EDTA-stripping procedure, a 20-ml portion of RM was added to 20 ml of a solution of 50 m*M* EDTA (sodium salt) in buffer B. The mixture was incubated at 0–4° for 15 min. EDTA-stripped RM were collected by centrifugation of the mixture for 1 hr at 140,000 g_{av} through a cushion of 0.5 *M* sucrose in buffer B (without EDTA). The ratio of load to cushion was about 3 : 1. The resulting pellet was resuspended in 20 ml of buffer B.

Nuclease Treatment of RM

Rough microsomes can be treated with staphylococcal nuclease (EC 3.1.31.1)[24] to deplete them of endogenous mRNA activity, which, depending on the *in vitro* translation system used, might contribute more or less to the background.

To a 2-ml fraction of RM, washed RM, or EDTA-stripped RM, 20 μl of a 100 m*M* $CaCl_2$ solution were added. Staphylococcal nuclease (Boehringer, Catalog No. 107921) was added to a final concentration of 20 units/ml (here we added 8 μl of a stock solution of 5000 units/ml—which is stable for at least 2 years when stored in aliquots at −80°). Digestion was carried out for 10 min at 23°. It was stopped by the addition of 40 μl of a 100 m*M* EGTA solution (adjusted to pH 7.5 with NaOH). Membranes were pelleted at 100,000 g_{av} for 30 min and resuspended in 2 ml of buffer B.

No endogenous mRNA activity was detectable when the nuclease-treated RM were assayed in the reticulocyte lysate translation system. The translocation activity of the microsomes and the activity of membrane-associated SRP were not affected by the small concentrations of nuclease used.

Microsomes in Different Translation Systems

We have described here a rapid isolation procedure that reproducibly yields highly active microsomal membranes. We also described procedures for refining this crude RM fraction by column washing, EDTA stripping, or nuclease treatment. None of these procedures affects the translocation activity of the vesicles. The choice of procedure(s) will depend on the specific application—in particular, on which *in vitro* trans-

[24] H. R. B. Pelham and R. J. Jackson, *Eur. J. Biochem.* **67**, 247 (1976).

lation system will be used. For example, if one works in a wheat germ translation system, the readout of the RM membrane-bound polysomes is generally negligible, and therefore neither EDTA nor nuclease treatment will be required. A column wash, however, is advantageous, since otherwise microsomes tend to inhibit considerably.[25] In the reticulocyte lysate system readout of membrane-bound polysomes is very effective,[15] and therefore EDTA stripping and/or nuclease treatment will be required.

Rough microsomes can also be extracted with high-ionic-strength buffers and still retain their translocation activity in the reticulocyte lysate system. The same salt-extracted membranes, however, are completely inactive in the wheat germ translation system, indicating that a factor required for translocation (now identified as SRP) is removed from the membranes with the salt extract, but is present in the reticulocyte lysate.[32] Therefore, translocation in the wheat germ translation system is SRP dependent (and sensitive to salt extraction of the microsomes), whereas in the reticulocyte lysate it is independent of SRP added directly or with the microsomes (E. Evans and P. Walter, unpublished observation).

In summary: column-washed RM have in our hands almost no inhibitory effects on protein synthesis; EDTA-stripped RM have a high "specific activity" (translocation activity per milligram of protein), because of removal of ribosomes and proteins; nuclease-treated RM have essentially no endogenous mRNA activity. All these microsome preparations still contain SRP. They are therefore translocation-active in wheat germ as well as reticulocyte lysate.

Use of Microsomes in Translation Systems

We relate all concentrations of microsomes back to the original crude RM preparation, which has been adjusted to a concentration of 50 A_{280} units/ml. We refer to 1 μl of this suspension as 1 equivalent (1 Eq). Upon column washing, nuclease treatment, or EDTA extraction, UV-absorbing material is removed, but in theory the number of recovered vesicles should remain constant. We therefore resuspend these treated RM to the original volume to obtain the same (equivalent) vesicle concentration, rather than readjusting the concentration to the same optical density. As a result, all microsome preparations described here are at 1 Eq/μl.

Using bovine pituitary RNA translated in a wheat germ system, we obtain approximately 50% of the synthesized chains of preprolactin translocated (and processed to prolactin) with a microsome concentration of 1 Eq per 25 μl of translation mix. The optimal microsome concentration for different mRNAs ranges from 0.5–3 Eq per 25 μl of translation mix.

[25] D. Shields and G. Blobel, *J. Biol. Chem.* **253**, 3753 (1978).

When using the wheat germ system, translocation activity can be boosted to 100% at a fixed microsome concentration of 1 Eq/μl if an excess of purified SRP is added.

To add microsomes to a translation system, we first mix all other components except the microsomes and the mRNA. We than add the microsomes, mix, and add the mRNA. All our translations contain human placental RNase inhibitor at a concentration of 0.16 A_{280} units/ml.

It is not necessary to compensate for the buffer, sucrose, or DTT added with the membrane fraction. None of these components effects protein synthesis noticeably (unless the DTT is badly oxidized). The membrane fraction therefore simply displaces water.

Core Glycosylation Activity of RM

Glycoproteins containing asparagine-linked sugars are core-glycosylated by microsomal membranes *in vitro* cotranslationally. Most of our microsome preparations are glycosylation active, but there is a high variability from preparation to preparation in the extent of glycosylation obtained. Whereas essentially all translocated chains are cleaved by signal peptidase, usually only a fraction of them (ranging from 0 to 70%) will be glycosylated). There is evidence[26] that it is the dolichol-oligosaccharide intermediate that is limiting in the poorly glycosylating microsomal preparations, but no successful way to charge *in vitro* the microsomes with the corresponding core sugar precursors has been reported. In general, screening of a couple of different microsome batches with a suitable mRNA preparation (like rat mammary gland RNA, which codes for a number of major and readily detectable glycoproteins) is used to select a microsomal preparation which glycosylates satisfactorily.

After disruption of the lipid bilayer with detergents [e.g., 0.1% of the nonionic detergent Nikkol (Nikko Chem. Corp., Tokyo)], core glycosylation does not occur, but signal peptidase cleavage still takes place. This allows one to observe processing uncoupled from glycosylation.

Posttranslational Proteolysis Assay

Translocated proteins are protected from exogenously added proteases by the microsomal membrane. In practice, the choice of the proteolysis conditions will determine the successful outcome of these protection experiments. Too extensive protease digestion leads to degradation of even the segregated forms, presumably owing to breakdown of the

[26] D. D. Carson, B. J. Earles, and W. J. Lennarz, *J. Biol. Chem.* **256,** 11552 (1982).

membrane barrier. The reasons for this permeabilization are not clear and have not been systematically investigated.

We generally obtain good protection (80–100% of the translocated protein) by employing the following protocol. Immediately after translation the translation mix (25 μl, wheat germ or reticulocyte lysate) is cooled to 0° by placing the tube in an ice-water bath. A 2-μl aliquot of a 15-m*M* $CaCl_2$ solution is added. Calcium ions seem to stabilize the vesicles and improve recovery of the protected form. A 3-μl aliquot of a trypsin–chymotrypsin (Boehringer) solution (3 mg/ml each) is added. Digestion is allowed to proceed for 30–90 min on ice and is terminated by the sequential addition of 3 μl of 10 m*M* PMSF in DMSO and 5 μl of Trasylol (FBA Pharmaceuticals, New York), followed by PAGE sample buffer. The sample is placed in a boiling water bath immediately. The use of amphipathic molecules to further improve recovery of protected material is described elsewhere (this volume [7]). Controls should include a sample, where in addition to the proteases a detergent (Triton X-100 to 1% final) is added to destroy the lipid bilayer. For protection experiments we usually use a membrane aliquot that has not been more than once frozen and thawed.

Microsomal Membranes from Other Sources

Microsomal membranes from sources other than dog pancreas have also been employed in cotranslational studies. The most successfully used alternative systems are probably chicken oviduct RM[27] (which glycosylate very well) and adrenal microsomes.[28] Rat liver RM,[28] ascites RM,[28] bovine pituitary RM,[6] and an RM fraction of *Drosophila melanogaster* embryos[29] also have been reported to be translocation active *in vitro*.

[27] R. C. Das, S. A. Brinkley, and E. C. Heath, *J. Biol. Chem.* **255,** 7933 (1980).

[28] M. Bielinska, G. Rogers, T. Rucinsky, and I. Boime, *Proc. Natl. Acad. Sci. U.S.A.* **76,** 6152 (1979).

[29] M. D. Brennan, T. G. Warren, and A. P. Mahowald, *J. Cell Biol.* **87,** 516 (1980).

[30] R. Gilmore, G. Blobel, and P. Walter, *J. Cell Biol.* **95,** 463 (1982).

[31] R. Gilmore, P. Walter, and G. Blobel, *J. Cell Biol.* **95,** 470 (1982).

[32] D. I. Meyer, E. Krause, and B. Dobberstein, *Nature (London)* **297,** 647 (1982).

[33] D. I. Meyer and B. Dobberstein, this volume [54].

[34] P. Walter and G. Blobel, *Nature (London)* **299,** 691 (1982).