Membrane Biogenesis

Edited by

Jos A. F. Op den Kamp

Laboratory of Biochemistry
State University of Utrecht
Padualaan 8, P.O. Box 80054
3508 TB Utrecht, The Netherlands

Springer-Verlag
Berlin Heidelberg New York London Paris Tokyo
Published in cooperation with NATO Scientific Affairs Division

CONTENTS

IMPORT OF SMALL SECRETORY AND PLASMA MEMBRANE PROTEINS INTO THE ENDOPLASMIC RETICULUM

Richard Zimmermann, Maria Sagstetter, Gabriel Schlenstedt, Hans Wiech, Birgitta Kaßeckert and Günter Müller
Institut für Physiologische Chemie, Physikalische Biochemie und Zellbiologie
Universität München
Goethestr. 33
D-8000 München 2
Federal Republic of Germany

SUMMARY

We are employing precursors of small secretory and plasma membrane proteins as tools for defining the different stages in the import of proteins into the endoplasmic reticulum (ER). The precursor proteins that we selected are M13 procoat protein, the precursor of a bacterial plasma membrane protein, and the precursors of two eucaryotic secretory proteins, honeybee prepromelittin and frog prepropeptide GLa. Our experimental systems involves in vitro systems, suitable for translation as well as import of the translation products into microsomes. The following stages in import can be resolved: i) Specific association of a precursor protein with the ER-membrane; ii) Insertion of a precursor protein into the ER-membrane; and iii) Assembly of a protein into the ER-membrane and transport of a protein across the ER-membrane, respectively. We present a working model for the import of small precursor proteins into ER.

NATO ASI Series, Vol. H16
Membrane Biogenesis. Edited by J. A. F. Op den Kamp

INTRODUCTION

Every polypeptide has a specific function as well as a unique functional location, i.e. an intra- or extracellular location where it fulfils its function (Zimmermann, 1986; Zimmermann and Meyer, 1986). There are two facts which turned the latter notion into a central problem in cell biology: i) There is essentially only one site of protein synthesis, the cytoplasm, but there are many different potential functional locations, the cell organelles and the extracellular space; ii) The site of synthesis is separated from these locations by phospholipid bilayers. Therefore, there must exist mechanisms which guarantee the specific transport of proteins across membranes. Membrane assembly of proteins can be visualized as a similar problem.

About fifteen years ago a first hypothesis was put forward which tried to explain specific import of proteins into the endoplasmic reticulum (ER), the "signal hypothesis" (for review see Zimmermann and Meyer, 1986). According to this hypothesis there are specific signals present in the proteins to be imported and the signals, typically aminoterminal and transient, guide the nascent polypeptides and the translating ribosomes to pores in the ER membrane. The tight interaction between ribosomes and pores and the resulting coupling of translation and import were suggested to prevent folding of a particular protein before it had reached its functional location. Therefore, the mechanism was termed cotranslational. On first sight the identification of a system which apparently decodes the signals, the signal recognition particle (SRP)/docking protein-system, seemed to provide support for this hypothesis.

However, during the last year the whole concept has been seriously questioned by data published by several laboratories. The novel themes seem to be that there does not have to be a coupling between translation and import and that folding of precursor proteins in the cytoplasm is prevented by the action of different cytoplasmic systems, at least one of which depends on ATP.

We are employing precursors of small secretory and membrane proteins as tools for defining the different stages in the import of proteins into the endoplasmic reticulum. The logic behind this approach is: i) that these precursor proteins have typical signal sequences, are processed by signal peptidase and thus presumably share some steps of the import pathway with

larger precursor proteins, and ii) that these proteins may be able to bypass certain steps, obligatory for larger precursor proteins, possibly because of a lower tendency for folding into structures, incompatible with membrane insertion or transport. The precursor proteins, we selected, are M13 procoat protein (73 amino acid residues), the precursor of a bacterial plasma membrane protein, and the precursors of two eucaryotic secretory proteins, honeybee prepromelittin (70 amino acid residues) and frog prepropeptide GLa (64 amino acid residues) (Figure 1). An additional reason for choosing these precursor proteins is that they could be expected to be imported posttranslationally just because of their size and, therefore, to give a technical advantage: Approximately 40 amino acid residues of a nascent polypeptide are shielded within a protease-inaccessible domain of the ribosome. This does not leave much time between the point where a 20 to 23 residues long signal peptide emerges from the ribosome and becomes free to interact with a receptor or a membrane and the point of termination of protein synthesis.

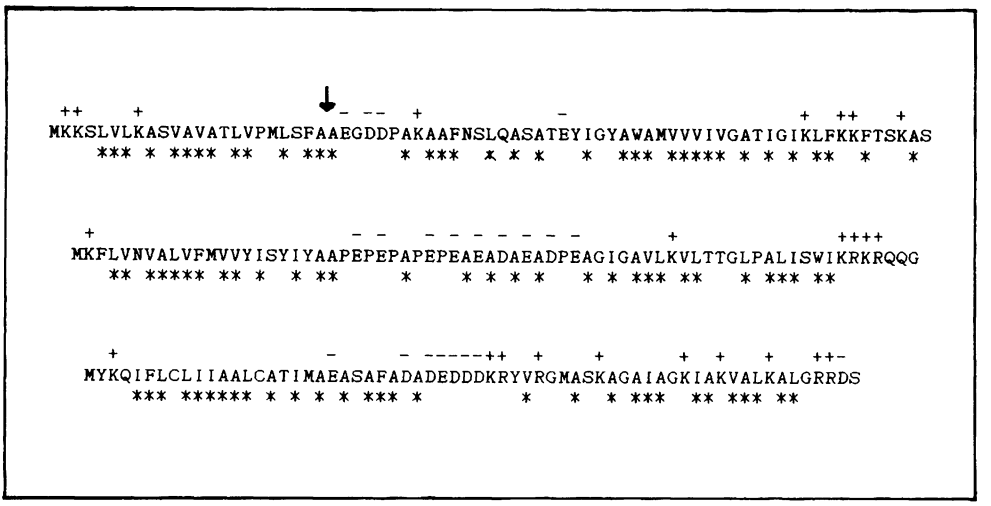

Figure 1. Primary Structure of M13 Procoat Protein, Honeybee Prepromelittin and Frog Prepropeptide GLa.
The cleavage site for signal peptidase is indicated by an arrow. Positively (+) and negatively (-) charged amino acid residues are indicated as well as hydrophobic ones (*).

RESULTS

Our experimental system involves in vitro systems, isolated from E.coli, wheat germs or rabbit reticulocytes, for translation of native mRNAs or in vitro-transcripts and ER-derived vesicles, isolated from dog pancreas. We assay association of small precursor proteins with microsomes in binding experiments where precursors are incubated with microsomes at low temperatures and microsomes are reisolated by gradient centrifugation or gel filtration. Membrane insertion is typically assayed as removal of the signal peptide by signal peptidase on the luminal side of the membrane. The standard assays for assembly or transport are testing sequestration, i.e. protection against externally added protease in the absence of detergent but sensitivity in the presence of detergent, and subfractionation of microsomes at neutral and alkaline pH-values.

Specific Association of a Precursor Protein with the ER-Membrane

The prediction for a specific association of a precursor with its target membrane is that the precusor is soluble and is able to expose its signal peptide. The classic model for specific association of precursors with microsomes involves SRP and docking protein. The small precursors, however, do not use the SRP/docking protein-system (Watts et al, 1983; Zimmermann and Mollay, 1986; Schlenstedt and Zimmermann, 1987). Therefore, we concluded that the SRP/docking protein-system has a role in keeping the signal peptide exposed while the small precursors can do so because of intrinsic properties (Müller and Zimmermann, 1987a).

A set of typical experiments on the docking protein-independence of prepromelittin processing is shown in Figures 2 to 4: While even the lowest concentration of trypsin used for pretreatment of microsomes is sufficient to completely destroy docking protein and, accordingly, to inactivate the microsomes with respect to their ability to process pre-kappa light chain, there is no effect on the ability of such pretreated microsomes to process prepromelittin (for further details refer to Zimmermann and Mollay, 1986 and Schlenstedt and Zimmermann, 1987).

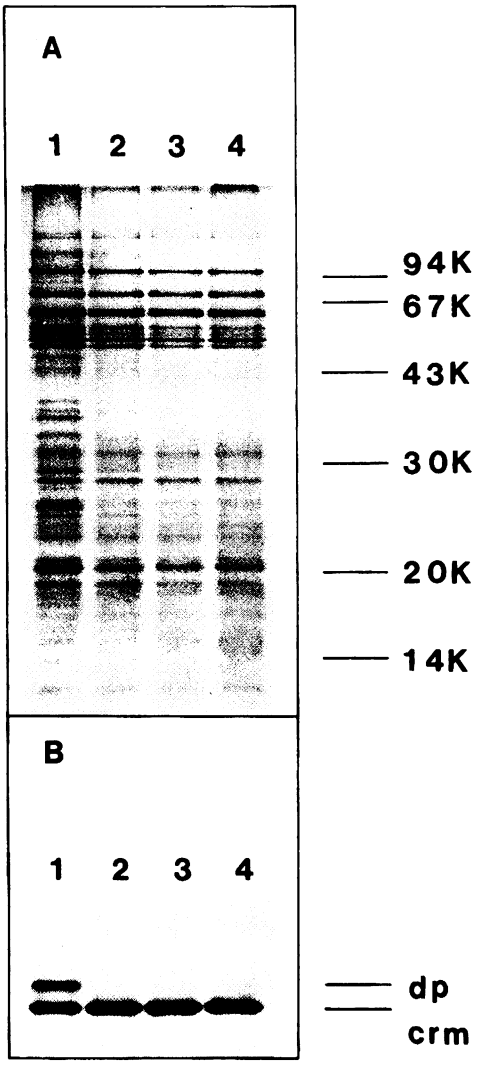

Figure 2. Effect of Trypsin Treatment of Microsomes on the Total Protein Pattern and on Docking Protein.
Four aliquots of dog pancres microsomes were incubated without trypsin (mock-treatment) (lane 1) or in the presence of trypsin at final concentrations of 10 (lane 2), 20 (lane 3) or 50 µg/ml (lane 4). After incubation for 60 min at 0 °C soybean trypsin inhibitor (twofold molar excess) and PMSF (1 mM) were added and the incubation was continued for 5 min at 0 °C. For analysis of the total protein pattern (A) and the docking protein content (B) two aliquots of each sample were run on SDS polyacrylamide gels (17.5 %). One set of samples was analysed by staining with Coomassie Brilliant Blue (A). The other set was electrophoretically transferred onto nitrocellulose and then probed with a rabbit antiserum directed against docking protein (dp) and with 14C-protein A (B). The relevant portion of an autoradiograph of this blot is shown. The antiserum crossreacts with a luminal protein (crm).

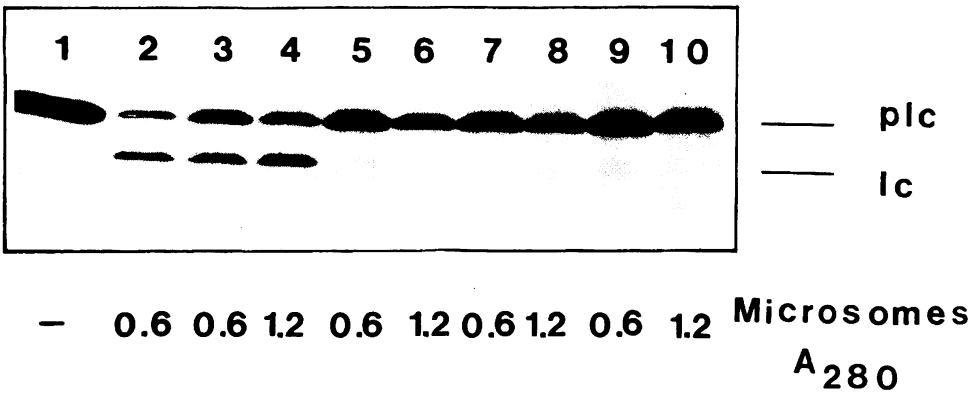

Figure 3. Effect of Trypsin Treatment of Microsomes on Processing of Pre-kappa-light Chain.
Pre- kappa-light chain (plc) was synthesized in rabbit reticulocyte lysates for 60 min at 37 °C in the absence of membranes (lane 1) or in the presence of dog pancreas microsomes, which were either not treated prior to their inclusion in the translation reactions (lane 2), mock-treated (lanes 3 and 4) or treated with trypsin and trypsin inhibitors as described in the legend to Figure 2. The trypsin concentrations during the treatment were 10 µg/ml (lanes 5 and 6), 20 µg/ml (lanes 7 and 8) and 50 µg/ml (lanes 9 and 10). The relevant portion of a fluorograph after electrophoretic analysis is shown.

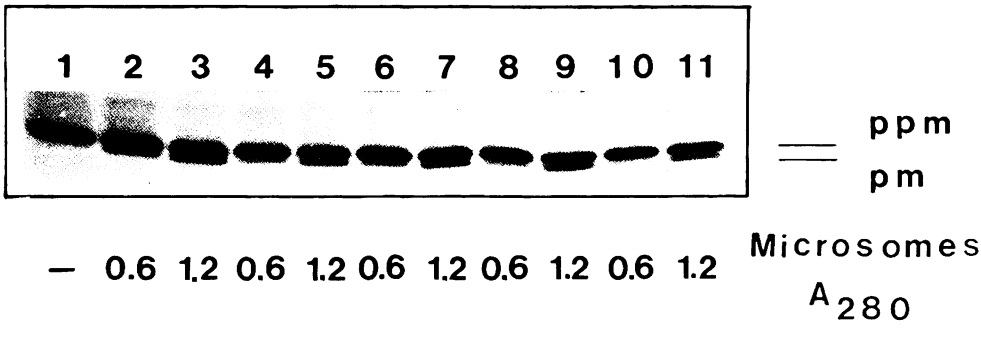

Figure 4. Effect of Trypsin Treatment of Microsomes on Processing of Prepromelittin.
Prepromelittin (ppm) was synthesized in rabbit reticulocyte lysates for 60 min at 37 °C in the absence of membranes (lane 1) or in the presence of dog pancreas microsomes, which were either not treated prior to their inclusion in the translation reactions (lanes 2 and 3), mock-treated (lanes 4 and 5) or treated with trypsin and trypsin inhibitors as described in the legend to Figure 2. The trypsin concentrations during the treatment were 10 µg/ml (lanes 6 and 7), 20 µg/ml (lanes 8 and 9) and 50 µg/ml (lane 10 and 11). The relevant portion of a fluorograph after butanol extraction and electrophoretic analysis is shown.

Insertion of a Precursor Protein into the ER-Membrane

The insertion of a precursor into the hydrophobic core of its target bilayer can be expected to be the second step in the import pathway and to precede membrane assembly as well as completion of translocation, i.e. transport. For the classic examples of proteins to be imported into mirosomes the ribosome in collaboration with a ribosome receptor at the surface of the microsomal membrane seem to be involved at this step. Again, however, the small precursors do not show such a requirement (Schlenstedt and Zimmermann, 1987; Wiech et al., 1987; Müller and Zimmermann, 1987b). On the other hand, we observed a requirement for the hydrolysis of ATP and a so far unknown cytosolic component in these cases. We concluded from these data that the ribosome in the case of large precursor proteins and the ATP-dependent component in general have a role in keeping precursor proteins competent for membrane insertion, i.e. preventing an unfavourable conformation. This notion is supported by our observation that the processing of small precursor proteins are inhibited at this step by formation of intramolecular disulfide bridges (Müller and Zimmermann, 1987b).

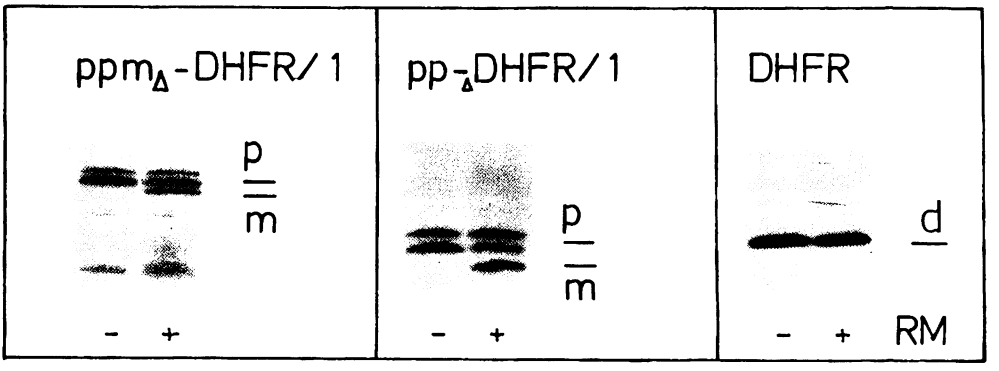

Figure 5. Processing of Hybrid Proteins between Prepromelittin and Dihydrofolate Reductase by Microsomes.
Hybrid Proteins between prepromelittin and dihydrofolate reductase, one containing prepromelittin with a minor deletion at the carboxy terminus and full length dihydrofolate reductase (ppmΔ-DHFR/1) and the other one containing prepromelittin without the melittin domain and dihydrofolate reductase with a large amino terminal deletion (ppΔDHFR/1), and dihydrofolate reductase (DHFR, d), respectively, were synthesized in rabbit reticulocyte lysates for 30 min at 37 °C in the absence or presence of microsomes (RM). The relevant portion of a fluorograph after electrophoretic analysis is shown.

The following set of experiments points to a role of the ribosome in the case of large, SRP and docking protein-dependent, precursor proteins (Figures 5 to 8): While methotrexate is able to bind to a hybrid protein consisting of prepromelittin and dihydrofolate reductase in the absence of microsomes and to induce a native conformation in the dihydrofolate reductase domain (dd), it cannot do so in the presence of microsomes and, accordingly, does not interfere with import (for further details refer to Müller and Zimmermann, 1987a and b).

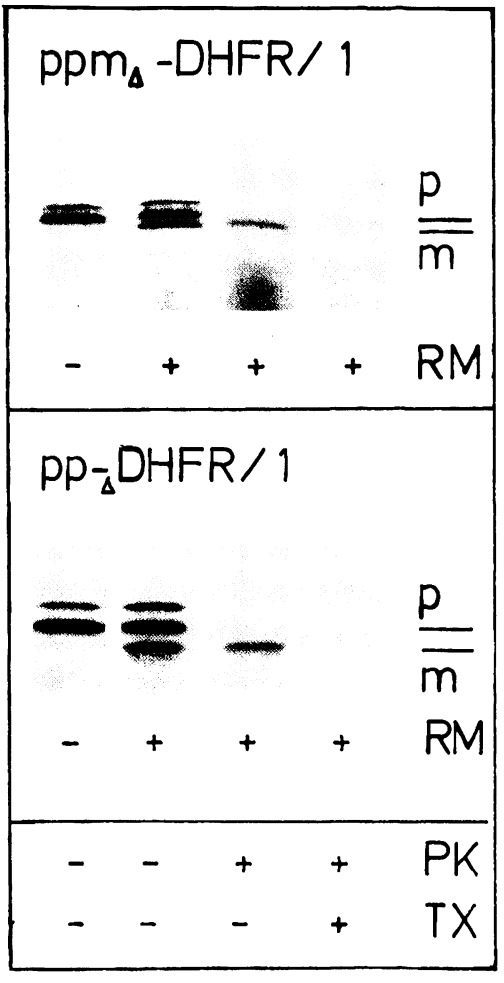

Figure 6. Import of Hybrid Proteins between Prepromelittin and Dihydrofolate Reductase into Microsomes. Hybrid proteins between prepromelittin and dihydrofolate reductase were synthesized in rabbit reticulocyte lysates for 30 min at 37 °C in the absence or presence of microsomes (RM). Afterwards each sample was divided into three aliquots. One aliquot was incubated in the absence of proteinase K (PK), the second one in the presence of proteinase K (50 µg/ml) and the third one in the presence of both, Triton X-100 (TX) and proteinase K, for 60 min at 0 °C. The relevant portion of a fluorograph after electrophoretic analysis is shown.

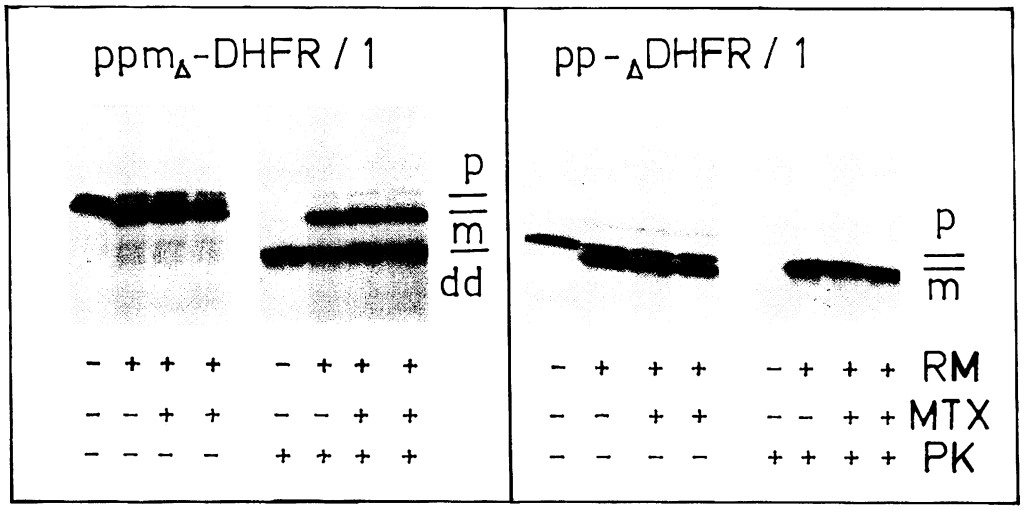

Figure 7. Effect of Methotrexate on the Import of Hybrid Proteins between Prepromelittin and Dihydrofolate Reductase into Microsomes.
Hybrid proteins between prepromelittin and dihydrofolate reductase were synthesized in rabbit reticulocyte lysates for 30 min at 37 °C in the absence of methotrexate (MTX) or in the presence of methotrexate (10 µM or 100µM) in the absence or presence of microsomes (RM). Afterwards each sample was divided into two aliquots. One aliquot was incubated in the absence of proteinase K (PK), the other one in the presence of proteinase K (50 µg/ml) for 60 min at 0 °C. The relevant portion of a fluorograph after electrophoretic analysis is shown.

Assembly of a Protein into the ER-Membrane and Transport of a Protein across the ER-Membrane

Membrane assembly of a membrane protein and completion of translocation of a soluble protein, respectively, can be seen as the final step of an import pathway. In the case of our small precursor proteins we were able to suggest a component of the microsomal membrane to be involved in transport of promelittin (Mollay and Zimmermann, 1986). However, not all small precursor proteins depend on this component (Schlenstedt and Zimmermann, 1987; Müller and Zimmermann, 1987a).

A typical experiment on the involvement of a protease-sensitive component of the microsomal membrane on the sequestration of promelittin is shown in

Figure 9: While trypsin pretreatment of microsomes does not inactivate the microsomes with respect to prepromelittin processing (see above), it completely inhibits such pretreated microsomes with respect to promelittin sequestration (for further details refer to Zimmermann and Mollay, 1986 and Müller and Zimmermann, 1987a).

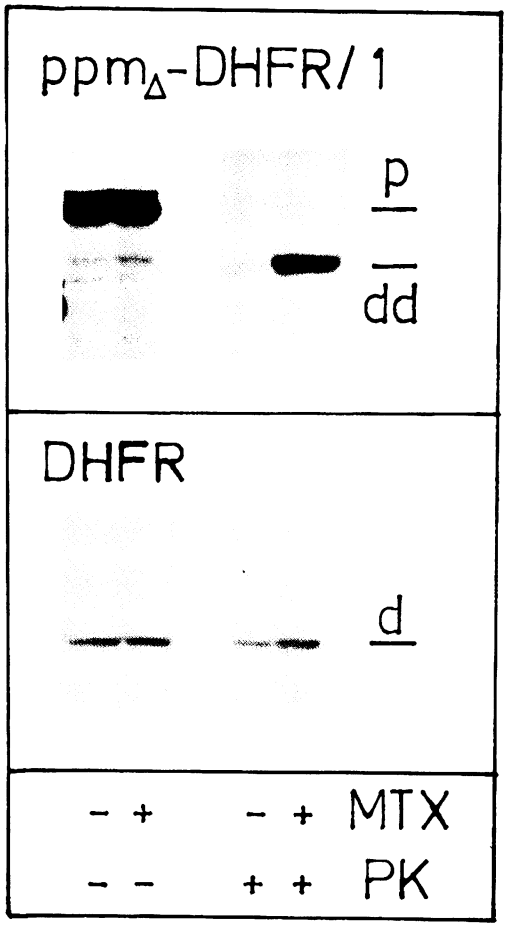

Figure 8. Effect of Methotrexate on the Folding of Hybrid Proteins between Prepromelittin and Dihydrofolate Reductase.
A hybrid protein between prepromelittin and dihydrofolate reductase (ppm$_\Delta$-DHFR/1) and dihydrofolate reductase (DHFR, d), respectively, were synthesized in rabbit reticulocyte lysates for 30 min at 37 °C in the absence of methotrexate (MTX) or in the presence of methotrexate (100 µM). Afterwards each sample was divided into two aliquots. One aliquot was incubated in the absence of proteinase K (PK), the other one in the presence of proteinase K (50 µg/ml) for 60 min at 0 °C. The portion of a fluorograph is shown.

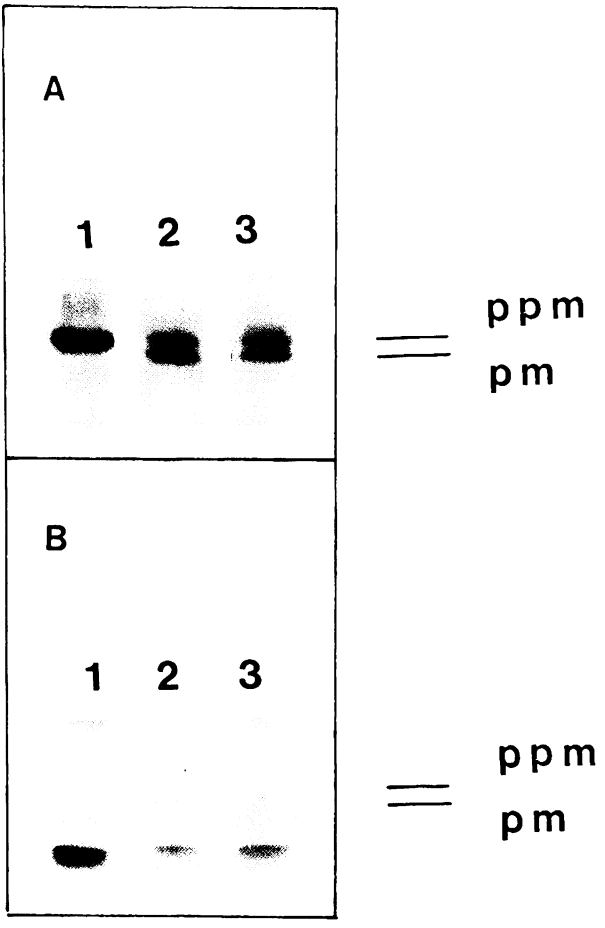

Figure 9. Effect of Trypsin Treatment of Microsomes on Processing of Prepromelittin and Sequestration of Promelittin.
Prepromelittin (ppm) was synthesized in rabbit reticulocyte lysates for 60 min at 37 °C in the absence of membranes (lanes 1) or in the presence of dog pancreas microsomes, which were either not treated prior to their inclusion in the translation reactions (lanes 2), or treated with trypsin and trypsin inhibitors as described in the legend to Figure 2 at a concentration of 10 µg/ml (lanes 3). Afterwards each sample was divided into two aliquots. One aliquot was incubated in the absence of proteinase K (A), the other one in the presence of proteinase K (B) for 60 min at 0 °C. The relevant portion of a fluorograph after electrophoretic analysis and butanol extraction is shown.

DISCUSSION

We propose the following mechanism for the import of small secretory and membrane proteins into the endoplasmic reticulum (Figure 10): Precursor proteins with a content of less than 80 amino acids are released from the ribosome before an interaction between either the signal peptide and signal recognition particle or the ribosome and the ribosome receptor can occur (Schlenstedt and Zimmermann, 1987; Müller and Zimmermann, 1987a; Wiech et al., 1987). Their primary structures, however, have evolved in a way which allows them to stay competent for membrane association and insertion without the aid of these components (Müller and Zimmermann, 1987a). Possibly, a loop is formed by the mature part, thereby bringing the amino- and carboxy-terminal parts in close proximity of each other. Ionic interactions may stabilize this structure, the presence of unbalanced charges in these positions may act destabilizing (Müller and Zimmermann, 1987a and b). Even disulfide bridges are allowed at this point, although, they probably never occur in vivo. This structure can associate with the microsomal membrane. It is presently unclear, whether a signal receptor is involved at this step or whether it is a pure protein-lipid interaction. We assume that an unfolding, i.e. destabilization of this structure, has to take place before membrane insertion can occur (Müller and Zimmermann, 1987b). Typically this is catalysed by a cytoplasmic protein which depends on the hydrolysis of ATP for its action (Wiech et al., 1987). Strikingly, the protein without any charged amino acid residues at the mature termini does not depend on this activity and disulfide bridges cannot be dissociated (Müller and Zimmermann, 1987b). Insertion may occur in form of a hairpin structure between the signal peptide and the mature amino terminus. This event leads to exposure of the cleavage site to the luminal side of the membrane and typically results in processing by leader peptidase. We assume that this takes place in a lipid environment because we observed the same characteristics for insertion into detergent micelles (Müller and Zimmermann, 1987b; Wiech et al, 1987). The following membrane assembly or transport of the mature proteins may occur in one or more steps and may or may not involve membrane components. We have defined one protease sensitive component which seems to be necessary in order to expell the hydrophobic melittin domain within promelittin out of the membrane (Zimmermann and Mollay, 1986; Müller and Zimmermann, 1987a). Accordingly,

proteins which do not contain a similar domain do not depend on this component. The alternatives for the action of such a component are still the ones proposed by either the signal hypothesis or the membrane triggered folding hypothesis or something in between. There may be transient proteinaceous pores involved or just the phospholipids, possibly in form of a transient bilayer distortion. Or there may be membrane proteins which do not form a pore but trigger or / and control the bilayer distortion.

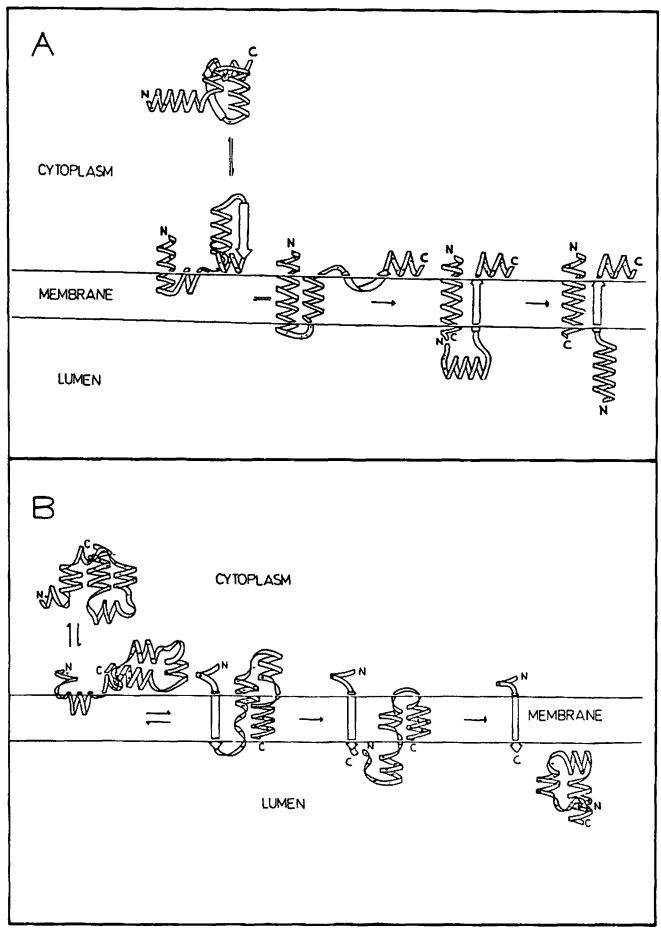

Figure 10. Working Model for the Import of Small Proteins into the Endoplasmic Reticulum.
Models for the assembly of M13 coat protein into microsomal membranes (A) and the transport of small secretory proteins, in this case promelittin into the ER-lumen (B), respectively, are shown. Charged amino acids are indicated.

ACKNOWLEDGEMENTS

We are thankful to Dr. Günther Kreil for mRNA and cDNA coding for prepromelittin and prepropeptide GLa, to Dr. William Wickner for DNA coding for procoat protein, and to Drs. Hermann Bujard and Dietrich Stueber for cDNA coding for dihydrofolate reductase. This work was supported by grants Zi234/2-1 and Zi234/2-2 from the 'Deutsche Forschungsgemeinschaft' and by grant B10 from the 'Sonderforschungsbereich 184: Molekulare Grundlagen der Biogenese von Zellorganellen'.

REFERENCES

Müller G, Zimmermann R (1987) Import of honeybee prepromelittin into the endoplasmic reticulum: structural basis for independence of SRP and docking protein. EMBO J 6: 2099-2107.

Müller G, Zimmermann R (1987) Import of SRP and docking protein independent precursor proteins into the endoplasmic reticulum: ATP requirement and effect of disulfide bond formation. Submitted for publication.

Schlenstedt G, Zimmermann R (1987) Import of frog prepropeptide GLa into microsomes requires ATP but does not involve docking protein or ribosomes. EMBO J 6: 699-703.

Watts C, Wickner W, Zimmermann R (1983) Mi3 procoat and pre-immunoglobulin share processing specificity but use different membrane receptor mechanisms. Proc Natl Acad Sci USA 80: 2809-2813.

Wiech H, Sagstetter M, Müller G, Zimmermann R (1987) The ATP requiring step in assembly of M13 procoat protein into microsomes is related to preservation of transport competence of the precursor protein. EMBO J 6: 1011-1016.

Zimmermann R (1986) Import of proteins into mitochondria. In: Strauss A W, Boime I, Kreil G (eds) Protein compartmentalization. Springer, Berlin Heidelberg New York, pp 119-136.

Zimmermann R, Meyer D I (1986) 1986: a year of new insights into how proteins cross membranes. Trends Biochem Sci 11: 512-515.

Zimmermann R, Mollay C (1986) Import of honeybee prepromelittin into the endoplasmic reticulum: requirements for membrane insertion, processing and sequestration. J Biol Chem 261: 12889-12895.