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Import of frog prepropeptide GLa into microsomes requires ATP but does not involve docking protein or ribosomes

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Frog prepropeptide GLa, a precursor to a secretory protein containing 64 amino acids, was processed and imported by dog pancreas microsomes. These events did not depend on either docking protein or on the presence of ribosomes. A hybrid protein between the first 60 amino acids of prepropeptide GLa and an unrelated peptide of 49 amino acids fused to the carboxy terminus, however, behaved like a typical secretory protein precursor with regard to docking protein dependence. This suggests that independence of the need for docking protein, in the case of prepropeptide GLa, can be attributed to the size of the precursor protein. Processing and import of prepropeptide GLa by microsomes were ATP dependent. Therefore, import of proteins into the endoplasmic reticulum (ER) includes an ATP-requiring step not involving a ribosome/ribosome receptor or signal recognition particle (SRP)/docking protein interaction.

Key words: docking protein/endoplasmic reticulum/ATP/ribosomes/signal recognition particle

Introduction

The import pathway of secretory proteins into the endoplasmic reticulum (ER) can be resolved into a sequence of the following events: (i) specific association of a precursor polypeptide with the membrane; (ii) insertion of the precursor into the membrane and removal of the signal sequence by signal peptidase; and (iii) complete translocation of the mature protein across the membrane into the ER lumen. The first step has been examined in much detail (Hortsch and Meyer, 1984; Walter *et al.*, 1984). Recently it became apparent that this step involves the signal sequence, typically a transient peptide at the amino terminus of a secretory protein precursor, a soluble cytoplasmic ribonucleoprotein, the signal recognition particle (SRP) and a proteinaceous component of the microsmal membrane, docking protein, the receptor for SRP. We recently reported, however, that one precursor protein is able to bypass this elaborate system, namely honeybee prepromelittin: a 70 amino acid-long precursor polypeptide having a typical transient signal sequence (Zimmermann and Mollay, 1986). From this and some new data on hybrid proteins comprising prepromelittin and dihydrofolate reductase (Müller and Zimmermann, in preparation) we concluded that the SRP/docking protein system may function in keeping large precursor proteins competent for membrane association and insertion while small precursor proteins, such as M13 procoat protein (Watts *et al.*, 1983) and prepromelittin (Zimmermann and Mollay, 1986), do not depend on this system because of intrinsic properties. Furthermore, recently published data from several laboratories point to a role of both the ribosomes (Caulfield *et al.*, 1986; Mueckler and Lodish, 1986; Perara *et al.*, 1986) and the hydrolysis of ATP (Hansen *et al.*, 1986; Mueckler and Lodish, 1986; Perara *et al.*, 1986; Rothblatt and Meyer, 1986; Waters and Blobel, 1986) in post-translational membrane association and/or insertion.

We are interested in defining these different stages of membrane association, insertion and translocation of secretory proteins into microsomes, and to investigate their requirements. For prac-

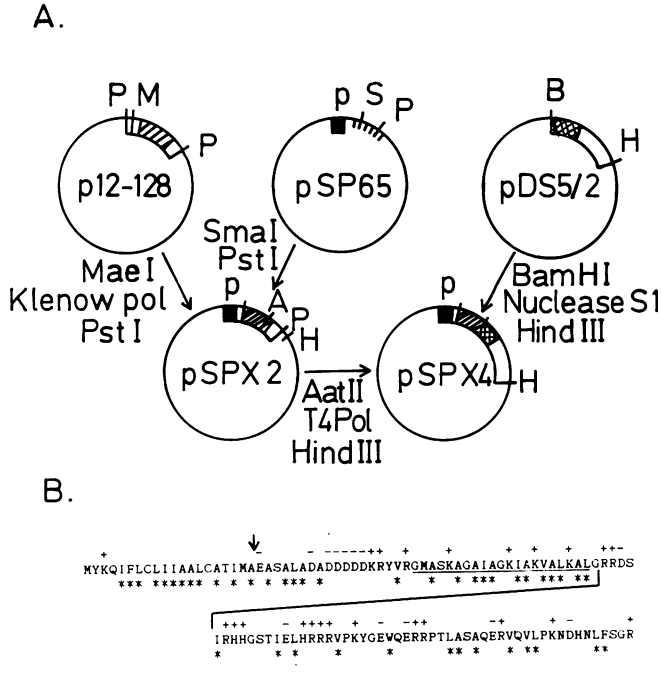

Fig. 1. Construction of plasmids and sequences of proteins. **(A)** Plasmid p12-128, containing the prepropeptide GLa cDNA, was treated with restriction endonuclease *Mae*I which cleaves between the G/C tail and the start codon of the cDNA. After treatment with Klenow fragment of DNA polymerase I and cleavage with *Pst*I, a DNA fragment was isolated and ligated into plasmid pSP 65 (Melton *et al.*, 1984), which was treated with *Pst*I, *Sma*I and calf intestine phosphatase. The resulting plasmid pSPX2 contains the bacteriophage SP6 promoter in front of the prepropeptide GLa cDNA. Ligation of a DNA fragment derived from plasmid pDS5/2 (Stueber *et al.*, 1984) by cleavage with *Bam*HI, treatment with exonuclease S1 and cleavage with *Hin*dIII into plasmid pSPX2 treated with *Aat*II, T4 polymerase, *Hin*dIII and calf intestine phosphatase gave rise to plasmid pSPX4. This plasmid contains the SP6 promoter in front of an open reading frame and encodes a hybrid protein between prepropeptide GLa and an out-of-frame sequence of mouse dihydrofolate reductase cDNA. Restriction endonuclease sites are indicated in single letter code. The solid squares indicate the SP6 promoter. Hatched squares indicate the coding region of prepropeptide GLa cDNA. Cross-hatched squares indicate the coding region used in pSPX4, and open squares the non-coding regions. **(B)** The primary structure of prepropeptide GLa is taken from Hoffmann *et al.* (1983) and Richter *et al.* (1985) and is shown in the upper line. The putative primary structure of the hybrid protein is deduced from the apparent mol. wt of the transcription/translation product of the corresponding plasmid and published sequences (Nunberg *et al.*, 1980; Hoffmann *et al.*, 1983; Richter *et al.*, 1985) and is shown in two lines connected by the solid bar. The predicted cleavage site for signal peptidase is indicated by an arrow. Positively (+) and negatively (−) charged amino acids are indicated as well as hydrophobic (*) amino acids. The underlined sequence represents the mature peptide which is amidated at the C terminus.

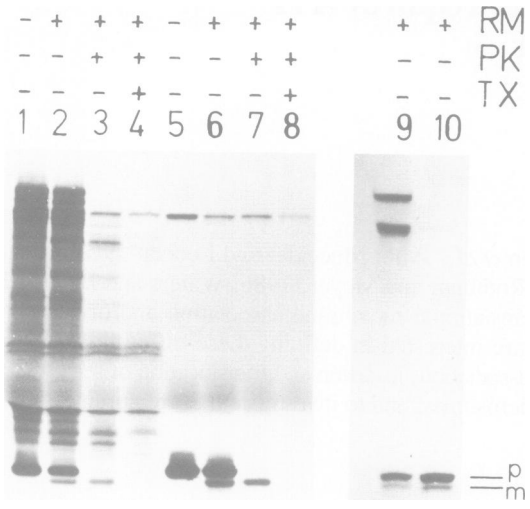

Fig. 2. Processing and sequestration of frog prepropeptide GLa by dog pancreas microsomes. Prepropeptide GLa was synthesized in rabbit reticulocyte lysates programmed with frog skin poly(A)$^+$ RNA **(lanes 1–4** and **lane 9)** or a transcript of plasmid pSPX2 **(lanes 5–8** and **lane 10)** in the absence of membranes **(lanes 1** and **5)** or the presence of dog pancreas rough microsomes (RM) **(lanes 2–4, 6–8, 9** and **10)**. After incubation for 30 min at 37°C, the samples containing membranes were divided into three aliquots. One aliquot was analyzed without proteinase K treatment, one with proteinase K treatment (PK) and one with protease treatment in the presence of Triton X-100 (TX) by gel electrophoresis (22% acrylamide) and fluorography. **Lanes 9** and **10** represent immunoprecipitates corresponding to **lanes 2** and **6**, respectively. Immunoprecipitation was carried out starting with ten times the amount of translation products as compared with **lanes 2** and **6** after denaturation in sample buffer and subsequent renaturation with antibodies directed against peptide GLa and Protein A Sepharose. p, prepropeptide GLa; m, propeptide GLa.

tical reasons we are studying SRP/docking protein-independent precursor polypeptides. Here we describe another such precursor, the prepropeptide GLa which occurs in skin cells of frogs. According to cDNA sequence analysis (Hoffmann *et al.*, 1983; Richter *et al.*, 1985), the prepropeptide is 64 amino acids long and contains a typical signal sequence. We present evidence that the import of prepropeptide GLa into the ER is docking protein independent, probably due to the size of this precursor. The presence of ribosomes is apparently not a prerequisite for translocation, either. Furthermore, we show that ATP is required for the import of this protein into microsomes. The general mechanistic implications of these findings are discussed.

Results

Dog pancreas microsomes process frog prepropeptide GLa and sequester propeptide GLa

Plasmid pSPX2 (Figure 1A), containing the prepropeptide GLa cDNA behind the bacteriophage SP6 promoter, was transcribed *in vitro*. After *in vitro* translation of the plasmid-derived transcripts in rabbit reticulocyte lysates a protein with an approximate mol. wt of 7000 was observed as the predominant translation product (Figure 2, lane 5). This protein corresponded in electrophoretic mobility to the major product that was obtained after translation of *Xenopus laevis* skin poly(A)$^+$ RNA (Figure 2, lane 1); both proteins were recognized by antibodies directed against peptide GLa (Figure 2, lanes 9 and 10, upper band). This confirmed the identity of the plasmid-directed transcription/translation product.

In the presence of dog pancreas microsomes, prepropeptide GLa was processed to a protein with a lower mol. wt (Figure

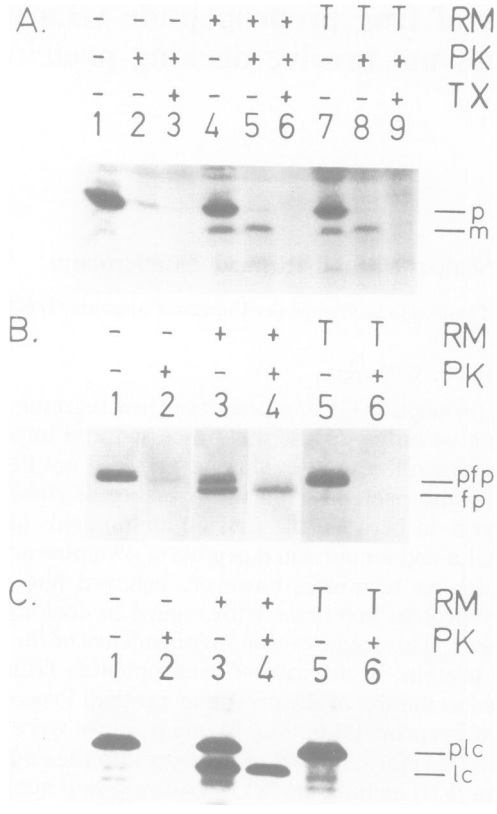

Fig. 3. Effect of pretreatment of microsomes with trypsin on processing and sequestration of propropeptide GLa and a related hybrid protein. Prepropeptide GLa **(A)**, the related fusion protein **(B)** and preimmunoglobulin light chain **(C)**, respectively, were synthesized in rabbit reticulocyte lysates in the absence of rough microsomes (RM) (reaction one) or the presence of microsomes which were either not treated prior to their inclusion in the translation reactions (reaction two) or treated with trypsin (T) (reaction three). Following incubation for 30 min at 37°C, the translation reactions were divided into two **(B** and **C)** or three aliquots **(A)**. On aliquot was analyzed without proteinase K (PK) treatment, one with proteinase K treatment and one with protease treatment in the presence of Triton X-100 (TX). The samples were analyzed directly **(A** and **C)** or after precipitation with $(NH_4)_2SO_4$ **(B)**, according to Hoober *et al.* (1982) by gel electrophoresis and fluorography; the gels employed were urea/SDS gels (25% acrylamide in **A**, 19% acrylamide in **B)** and a 17.5% acrylamide SDS gel in **C**. p, prepropeptide GLa; m, propeptide GLa; pfp, fusion protein precursor; fp, processed fusion protein; plc, preimmunoglobulin light chain; lc, immunoglobulin light chain.

2, lanes 2 and 6). The processing product apparently represents prepropeptide GLa, i.e. the form which lacks the predicted signal sequence (Hoffmann *et al.*, 1983; Richter *et al.*, 1985). The identity of this processing product was confirmed by immunoprecipitation (Figure 2, lanes 9 and 10, lower band). The propeptide GLa was also sequestered into the lumen of the microsomes. This was demonstrated by the resistance of the processed protein to externally added protease in the absence of detergent (Figure 2, lanes 3 and 7) and its sensitivity in the presence of detergent (Figure 2, lanes 4 and 8). The notion that protease resistance of propeptide GLa reflects sequestration was supported by our observation that propeptide GLa was extracted from microsomes in 0.1 M sodium carbonate at pH 11.5 (data not shown).

The intensity of the bands corresponding to prepropeptide GLa and propeptide GLa (Figure 2, lanes 2 and 6) are not proportional to the efficiency of processing since [^{35}S]methionine was used for radiolabelling. There are three methionines present in prepropeptide GLa but only one methionine is present in propeptide GLa (Figure 1B) (Hoffmann *et al.*, 1983; Richter *et al.*, 1985).

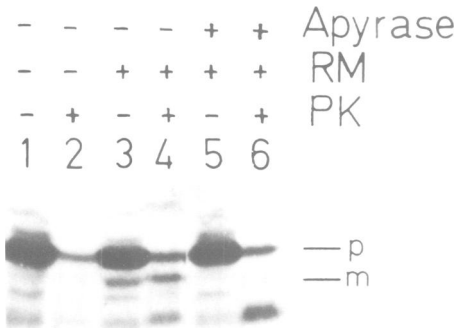

Fig. 4. Effect of ATP depletion on post-translational processing and sequestration of prepropeptide GLa by microsomes. Prepropeptide GLa was synthesized in a rabbit reticulocyte lysate, programmed by transcripts of plasmid pSPX2, at 37°C for 5 min. Protein synthesis was terminated by the addition of cycloheximide (100 μg/ml) and RNase A (80 μg/ml). The translation reaction was divided into three reactions which were further incubated at 37°C for 5 min. In the third reaction, potato apyrase (70 U/ml) was present during this incubation. Following this second incubation, water **(lanes 1** and **2)** or dog pancreas rough microsomes (RM) **(lanes 3–6)** were added. After a third incubation at 37°C for 15 min, the samples were divided into two aliquots each. One aliquot was incubated at 0°C for 60 min in the absence of proteinase K (PK) **(lanes 1, 3** and **5)**, the other one in the presence of proteinase K **(lanes 2, 4** and **6)**. The samples were analyzed by gel electrophoresis (25% acrylamide) and fluorography. A control experiment showed that the effect of apyrase treatment was due to the enzymatic activity and not the incubation conditions: when the apyrase solution was boiled for 2 min prior to addition to the translation reaction the result of the subsequent incubation in the presence of microsomes was identical to aliquot two **(lanes 3** and **4)** (data not shown). p, prepropeptide GLa; m, propeptide GLa.

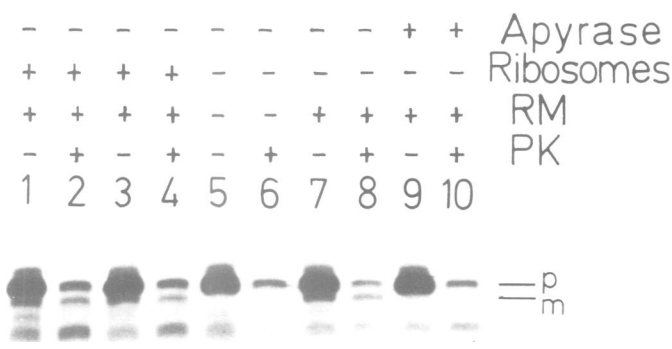

Fig. 5. Processing and sequestration of prepropeptide GLa do not involve ribosomes. Prepropeptide GLa was synthesized in a rabbit reticulocyte lysate at 37°C for 5 min. Translation was stopped by the addition of cycloheximide (100 μg/ml) and RNase A (80 μg/ml). The translation mixture was divided into five reactions. The first raction **(lanes 1** and **2)** was kept on ice during the centrifugation of the other samples. To the fifth reaction, apyrase (3.5 U/ml) was added. After further incubation at 37°C for 5 min reactions two to five were centrifuged at 4°C for 15 min in a Beckman TLA-100 rotor at 430 000 *g*. The ribosomal pellet of the second reaction **(lanes 3** and **4)** was resuspended in its own supernatant. The third to fifth reactions **(lanes 5–10)** represent the post-ribosomal supernatants. Prior to the following incubation at 37°C for 15 min, water **(lanes 5** and **6)** or microsomes (RM) **(lanes 1–4, 7–10)** were added. All samples were divided into two aliquots each, one incubated at 0°C for 60 min in the absence of proteinase K (PK) **(lanes 1, 3, 5, 7** and **9)**, the other in the presence of proteinase K **(lanes 2, 4, 6, 8** and **10)**. The samples were analyzed by gel electrophoresis (22% acrylamide) and fluorography. A control experiment showed that the supernatants were free of translation-active ribosomes: when a post-ribosomal supernatant of a lysate containing no transcript was incubated with plasmid-derived transcript after centrifugation no protein synthesis occurred (data not shown). p, prepropeptide GLa; m, propeptide GLa.

Taking this into account densitometric analysis of X-ray films indicates a processing efficiency of up to 30%. For comparison, ~70% of the precursor of immunoglobulin light chain was processed under identical conditions (Figure 3C).

Import of propeptide GLa into microsomes does not depend on docking protein

To determine whether prepropeptide GLa follows the SRP/docking protein-dependent pathway of protein entry into microsomes or not, microsomes were depleted of intact docking protein prior to their inclusion in translation reactions. Treatment of microsomes with trypsin leads to degradation of docking protein (Meyer and Dobberstein, 1980). As expected, this caused inactivation of these microsomes with respect to processing of the precursor of immunoglobulin light chain (Figure 3C). No inactivation was observed when these microsomes were assayed for processing of prepropeptide GLa (Figure 3A), as has been previously reported for the processing of prepromelittin (Zimmermann and Mollay, 1986). Owing to the similar behaviour of the two precursor proteins in this respect, we suggest that prepropeptide GLa is inserted into microsomal membranes without the involvement of SRP and docking protein. In contrast to prepromelittin, however, there was also sequestration of propeptide GLa into the microsomes which had been pretreated with trypsin.

Docking protein independence of prepropeptide GLa import is due to the nature of the mature part of the precursor

Figure 1A shows the construction of plasmid pSPX4 which codes for a hybrid protein (pfp) between prepropeptide GLa, missing four carboxy-terminal amino acids, and a random peptide containing 49 amino acids (Figure 1B). The transcription/translation system yielded a precursor protein with an approximate molecular mass of 12 000 (Figure 3B, lane 1). Upon addition of microsomes to the translation reaction programmed with the pSXP4 transcript, a processing product (fp) was obtained (Figure 3B, lane 3) which was sequestered into the lumen of the microsomes (Figure 3B, lane 4).

To understand the reason for the docking protein independence of prepropeptide GLa import into microsomes, processing of the precursor pfp was assayed in the presence of trypsin-pretreated microsomes. The precursor of the hybrid protein behaved like the precursor of immunoglobulin light chain (Figure 3B, lanes 5 and 6), i.e. it could be neither processed nor imported in the absence of active docking protein. Apparently, this prepropeptide GLa-related precursor strictly depends on docking protein, at least when synthesized in the presence of SRP. A related hybrid protein comprising prepropeptide GLa, missing four carboxy-terminal amino acids, and a peptide with the sequence Pro-Trp-Asp-Phe-Cys-Trp-Leu behaved like authentic prepropeptide GLa with respect to processing and sequestration (data not shown). We conclude that it is not the signal sequence of prepropeptide GLa but more likely the nature of the mature part of this precursor, i.e. primarily the size, which accounts for the SRP/docking protein independence. Similar experiments with prepromelittin-related hybrid proteins led to the identical conclusion for prepromelittin (Müller and Zimmermann, in preparation).

Import of prepropeptide GLa into microsomes requires ATP

When prepropeptide GLa was synthesized in the absence of microsomes and the translation mixture was treated with cycloheximide and RNase A, there was also processing to propeptide GLa following the addition of microsomes (Figure 4, lane 3). A control experiment showed that there was no protein synthesis taking place after addition of cycloheximide and RNase A (Wiech, Sagstetter, Müller and Zimmermann, in preparation). The pro-

cessing product of the post-translational incubation was also sequestered within the microsomes (Figure 4, lane 4). An independent experiment demonstrated that prepropeptide GLa was present as completed polypeptide and not as peptidyl-tRNA in this post-translational assay; when puromycin (0.5 mM) and RNase A were used to block protein synthesis, post-translational processing and sequestration took place (data not shown). However, when the translation reaction was depleted of ATP prior to the addition of microsomes by treatment with potato apyrase (Waters and Blobel, 1986), neither processing nor sequestration occurred (Figure 4, lanes 5 and 6). From this we conclude that membrane association and/or insertion of prepropeptide GLa requires ATP or another high energy phosphate compound. Various ionophores [including calcium ionophore A23187 (calimycin), carbonyl cyanide *m*-chlorophenyl hydrazone, nigericin, valinomycin or a combination of nigericin plus valinomycin] did not affect the processing or sequestration (data not shown).

Import of prepropeptide GLa into microsomes can occur in the absence of ribosomes

To investigate whether or not ribosomes are required for translocation (i.e. beyond their role in translation), the translation mixture containing cycloheximide, RNase A and prepropeptide GLa was fractionated into a ribosomal pellet and a post-ribosomal supernatant. Most of the precursor protein was found in the supernatant under the centrifugation conditions employed (data not shown). Processing and sequestration occurred when the post-ribosomal supernatant was incubated in the presence of microsomes (Figure 5, lanes 7 and 8). Post-translational import was as efficient as in the presence of ribosomes (Figure 5, compare lanes 1–4 and lanes 7 and 8). Addition of apyrase to the lysate prior to centrifugation abolished processing and sequestration of prepropeptide GLa, directly demonstrating that the ATP-requiring step does not involve the ribosomes (Figure 5, lanes 9 and 10).

Discussion

The general mechanism for the entry of proteins into the ER seems to commence as soon as the signal sequence of a nascent secretory protein precursor emerges from the ribosome (Blobel and Dobberstein, 1975). At this point, a complex system, involving SRP and docking protein, comes into action (Hortsch and Meyer, 1984; Walter *et al.*, 1984). One function of this system may be to prevent folding of the precursor protein into structures which are incompatible with membrane insertion (Müller and Zimmermann, in preparation). Following membrane association of the nascent chains, membrane association of the translating ribosomes appears to occur; this reaction seems to involve a ribosome receptor on the microsomal surface (Blobel and Dobberstein, 1975; Hortsch *et al.*, 1986). The precise mechanisms of microsome insertion and translocation are poorly understood. Membrane insertion somehow involves the signal sequence and is usually accompanied by removal of the signal peptide (Blobel and Dobberstein, 1975). In many cases, additional covalent modifications take place at this point. However, there is no indication for a direct involvement of any of these processing events in membrane insertion and translocation. Completion of translocation, at least in certain cases, seems to require membrane proteins of the ER (Gilmore and Blobel, 1985; Zimmermann and Mollay, 1986). Recent work shows that hydrolysis of high energy phosphate bonds is involved at some point of the pathway (Hansen *et al.*, 1986; Mueckler and Lodish, 1986; Perara *et al.*, 1986; Rothblatt and Meyer, 1986; Waters and Blobel, 1986).

We are employing precursors of small eucaryotic secretory proteins as tools for defining the different stages in the import of proteins into the ER. The logic behind this approach is: (i) that these proteins have typical signal sequences, are processed by the signal peptidase and thus presumably share some steps of the import process with larger secretory proteins, and (ii) that these proteins may be able to bypass certain steps, obligatory for larger secretory proteins, possibly because of a lower tendency to lose import competence. This view is supported by the particular import requirements of the hybrid protein employed here. We recently demonstrated that honeybee prepromelittin can bypass the SRP/docking protein system (Zimmermann and Mollay, 1986). According to the data presented here frog prepropeptide GLa behaves similarly in this respect. Furthermore, removal of the signal peptide is not the only step which SRP/docking protein-independent and -dependent precursors have in common: prepropeptide GLa also depends on ATP, or another high energy phosphate compound, for import into microsomes. The presence of ribosomes, however, is not required for the import of prepropeptide GLa. This is in contrast to the import of human placental lactogen (Caulfield *et al.*, 1986) and of other larger precursor proteins with tRNAs still attached to the carboxy termini (Perara *et al.*, 1986; Mueckler and Lodish, 1986). Therefore, we assume that a ribosome/ribosome receptor interaction may be an additional way of minimizing the chances of unfavourable folding of large precursor proteins.

On the basis of these data we propose that the common features of the mechanisms involved in the import of large and small precursor proteins into the ER are the presence of a signal peptide, be it cleavable or non-cleavable, and the ATP requirement. None of the proteins so far described to be involved in protein import into the ER (SRP, docking protein, ribosome, ribosome receptor, signal peptidase) is obligatory for all precursor proteins. Therefore, we conclude that ATP is not consumed by, or used for a modification of any of these proteins. On the other hand, ATP in this system does not seem to be used for the generation of a membrane potential (Hansen *et al.*, 1986; Mueckler and Lodish, 1986; Rothblatt and Meyer, 1986; Waters and Blobel, 1986; and this study). Furthermore, we can exclude that ATP is required for glycosylation since prepropeptide GLa does not contain a potential Asn-X-Thr/Ser glycosylation site. We are left with the question of how and where the ATP is acting (cytoplasm or membrane). The answer to this problem seems to be equally important for our understanding of membrane transport of proteins in the bacterial system (Geller *et al.*, 1986), in mitochondria (Pfanner and Neupert, 1986) and in chloroplasts (Flügge and Hinz, 1986).

Materials and methods

Materials

Enzymes used for cloning were purchased from Boehringer Mannheim, except for S1 nuclease which was obtained from Pharmacia/P-L Biochemicals as was 7mGpppG and nucleotriphosphates. SP6 RNA polymerase, RNasin, RNase A, trypsin, soybean trypsin inhibitor and proteinase K were from Boehringer Mannheim. Potato apyrase (grade VIII), cycloheximide, calcium ionophore A23187, carbonyl cyanide *m*-chlorophenyl hydrazone, nigericin and valinomycin were from Sigma. [^{35}S]Methionine (1000 Ci/mmol) and Amplify were from Amersham Corp. X-ray films (Kodak X-Omat AR) were from Kodak.

Construction of plasmids and in vitro transcription

Construction of plasmids was carried out according to standard procedures (Maniatis *et al.*, 1982). *In vitro* transcription was performed following established procedures (Melton *et al.*, 1984; Krieg and Melton, 1984). Following transcription for 45 min at 40°C, samples were either immediately used for translation or were frozen in liquid nitrogen and stored at −80°C until they were used.

In vitro translation and protein transport

Cell-free translation was carried out as described previously (Zimmermann and Mollay, 1986) in the presence of [^{35}S]methionine and poly(A)$^+$ RNA from *X. laevis* skin, mineral oil-induced plasmacytoma 41 RNA, or the transcription system. Where indicated, dog pancreas microsomes which were isolated and pretreated as described previously (Watts *et al.*, 1983; Zimmermann and Mollay, 1986) were present. The absorbance at 280 nm (as measured in 2% SDS) of microsomes in a particular *in vitro* translation mixture was 1.2.

Analytical procedures

Protection assays were carried out as described previously (Zimmermann and Mollay, 1986) except for the proteinase K concentration which was 125 μg/ml. All samples were diluted with an equal volume of double strength sample buffer (Laemmli, 1970) and analyzed on SDS–polyacrylamide gels (17.5%) (Laemmli, 1970) or on urea-containing SDS–polyacrylamide gels (19, 22 or 25%) (Ito *et al.*, 1980). For fluorography, the gels were treated with sodium salicylate (Chamberlain, 1979) or Amplify (in the case of 25% gels), dried, and exposed to X-ray films at −80°C for 1 or 2 days.

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