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The ATP requiring step in assembly of M13 procoat protein into microsomes is related to preservation of transport competence of the precursor protein

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M13 procoat protein is processed to transmembrane coat protein by dog pancreas microsomes after completion of synthesis and in the absence of the signal recognition particle (SRP)/docking protein system. ATP is required for fast and efficient processing of procoat protein by microsomes in a reticulocyte lysate. Requirement for ATP is also observed in the absence of ribosomes or docking protein. This indicates the existence of a unique assembly pathway for procoat protein into microsomes which depends on ATP but does not depend on the SRP/docking protein and ribosome/ribosome receptor systems. We suggest that the ATP requirement is linked to a so far unknown component of the reticulocyte lysate, acting on transport competence of precursor proteins.
Key words: endoplasmic reticulum/ATP/reticulocyte lysate/M13 procoat protein/protein import

Introduction

Import of proteins into mitochondria and chloroplasts, and export of proteins in *Escherichia coli* are not mechanistically coupled to protein synthesis (Wickner and Lodish, 1985). In all these systems the hydrolysis of ATP appears to be a prerequisite for membrane insertion of precursor proteins (Geller *et al.*, 1986; Flügge and Hinz, 1986; Pfanner and Neupert, 1986). In contrast to this, the import of proteins into the endoplasmic reticulum has been viewed in a completely different way (Blobel and Dobberstein, 1975). Import was presumed to be obligatorily coupled to protein synthesis, and chain elongation was seen as providing the driving force for the movement of the nascent precursor polypeptide across the microsomal membrane.

Recently, however, this concept has been seriously questioned by a number of groups (for review see Zimmermann and Meyer, 1986). We have shown that M13 procoat protein can assemble into dog pancreas microsomes in the absence of protein synthesis. This process is also independent of the signal recognition particle (SRP)/docking protein system (Watts *et al.*, 1983). Since this observation, a number of eucaryotic secretory and plasma membrane proteins have been reported to be able to enter microsomes post-translationally (Zimmermann and Meyer, 1986). Most, but not all, of these proteins depend on the SRP/docking protein and the ribosome/ribosome receptor systems. Furthermore, recent reports have indicated that there is an ATP requiring step also in this system.

Here we demonstrate that the assembly of M13 procoat protein into dog pancreas microsomes in a reticulocyte lysate is dependent on a component of the lysate and on ATP but does not depend on the presence of ribosomes. Furthermore, we show that processing of procoat protein by leader peptidase in liposomes as well as in the presence of detergent depends on ATP. The general mechanistic implications of these data are discussed.

Results

Import of M13 procoat protein into microsomes does not depend on the presence of ribosomes

We have established conditions for the post-translational import of small precursor proteins, synthesized in rabbit reticulocyte lysates, into dog pancreas derived microsomes. These include synthesis of the precursor proteins for 10 min followed by termination of protein synthesis by incubation in the presence of cycloheximide and RNase A for 5 min, and then protein transport by incubation in the presence of microsomes for 15 min. A control experiment to prove the post-translational nature of transport under these conditions is shown in Figure 1. Neither elongation (lane 1 versus 2) nor initiation (lane 4) took place under these conditions. Notably, cycloheximide alone was sufficient to block initiation (lane 5) and elongation on free ribosomes (dihydrofolate

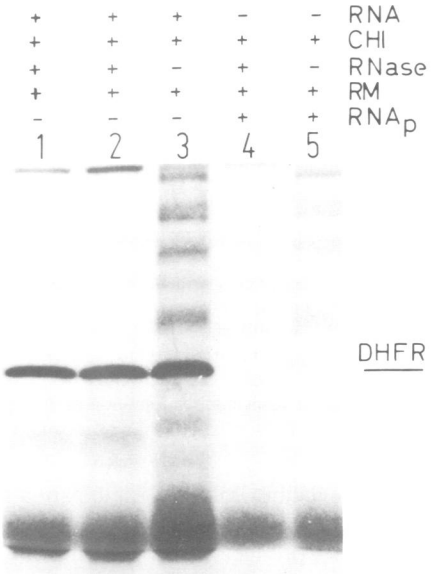

Fig. 1. Conditions for post-translational import of proteins into microsomes. Translation was carried out for 5 min at 37°C in a rabbit reticulocyte lysate in the presence (**lanes 1-3**) or absence (**lanes 4** and **5**) of an *in vitro* transcript coding for mouse dihydrofolate reductase. The translation reaction was divided into five aliquots and the different aliquots were supplemented with cycloheximide (100 μg/ml) (**lanes 3** and **5**) or a combination of cycloheximide and RNase A (80 μg/ml) (**lanes 1**, **2** and **4**). Following further incubation for 5 min at 37°C, dog pancreas microsomes (RM) were added. Aliquots represented by lanes 4 and 5 were supplemented with the transcript coding for dihydrofolate reductase. After incubation at 0°C (**lane 1**) or 37°C (**lanes 2-5**) for 15 min further, the samples were analyzed by gel electrophoresis and fluorography. RNA, DHFR transcript present during translation; CHI, cycloheximide; RNase, RNase A; RNAp, DHFR transcript added post-translationally; DHFR, mouse dihydrofolate reductase.

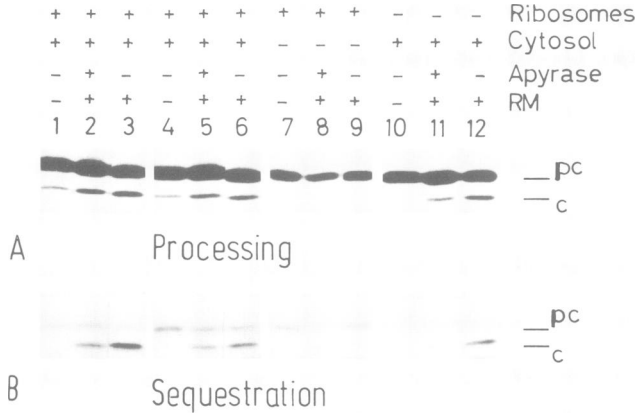

Fig. 2. Processing of procoat protein and sequestration of coat protein by microsomes in the presence and absence of ribosomes. Procoat protein was synthesized in a rabbit reticulocyte lysate for 10 min at 37°C. The translation reaction was split into two samples. One sample was supplemented with water (**lanes 1, 3, 4, 6, 7, 9, 10** and **12**), the other one with potato apyrase (4 units/ml) (**lanes 2, 5, 8** and **11**). Both samples were supplemented with a combination of cycloheximide and RNase A and were then divided into three aliquots. The first aliquot was kept on ice during the centrifugation of the other two aliquots for 15 min at 430 000 *g* in a Beckman TL 100 centrifuge (rotor TLA 100.2). After centrifugation the pellet from the second aliquot was resuspended in its own supernatant (cytosol), the pellet from the third aliquot was resuspended in a supernatant from an aliquot without any procoat protein, which had been prepared in parallel. These three samples and the supernatant from aliquot three were incubated for 5 min at 37°C. Prior to the following incubation for 15 min at 37°C, the different samples were split into two reactions; one was supplemented with water, the other one with dog pancreas microsomes. All reactions were then divided into two parts; one was incubated in the absence (processing), the other one in the presence of proteinase K (sequestration) for 60 min at 0°C. All samples were analyzed by gel electrophoresis and fluorography. A control experiment showed that the supernatant was free of ribosomes: when an aliquot without transcript, cycloheximide or RNase A was subjected to centrifugation and the pellet was resuspended in its own supernatant and supplemented with transcript, translation products were produced during an incubation for 15 min at 37°C; when the supernatant was incubated with transcript in the absence of the pellet no translation products could be detected (data not shown). Translation and the subsequent post-translational incubation gave rise to a small amount of a protein (pseudo coat protein) which corresponded to coat protein according to electrophoretic mobility (**A, lane 1**). This background was subtracted when processing efficiencies were determined from densitometric analyses of X-ray films in the following experiments. The pseudo coat protein was not protected, however, from degradation by externally added protease, our assay for sequestration of coat protein (**B, lane 1**). RM, dog pancreas microsomes; pc, procoat protein; c, coat protein.

reductase) but only cycloheximide in combination with RNase A was able to block elongation on membrane-bound ribosomes (microsome-dependent products) (lane 2 versus 3).

We studied the precursor of a bacterial plasma membrane protein, M13 procoat protein, in this post-translational assay. To achieve efficient translation of this *E. coli* phage protein in the eucaryotic translation system, the DNA coding for procoat was inserted into plasmid pSP 65. Transcription with SP6 polymerase and subsequent translation in a rabbit reticulocyte lysate yielded procoat protein (Figure 2A, lane 1). When microsomes were present during the post-translational incubation, procoat protein was processed to coat protein (Figure 2A, lane 3) and coat protein was sequestered into microsomes (Figure 2B, lane 3). From densitometric analysis of X-ray films, and taking into account the distribution of methionines in procoat protein (three) and coat protein (one), processing efficiencies between 30 and 50% were calculated.

We first addressed the question as to whether procoat requires the presence of ribosomes for sequestration into microsomes. Procoat was synthesized and the translation mixture was subjected to high-speed centrifugation after termination of protein synthesis. When the pellet fraction of this centrifugation (containing ribosomes and some procoat protein) was resuspended in the corresponding supernatant fraction (containing cytosol and most of the procoat protein) and supplemented with microsomes, processing of procoat protein and sequestration of coat protein took place (Figure 2A and B, lane 4 versus 6). Furthermore, when the supernatant fraction was supplemented with microsomes in the absence of the pellet fraction there was also processing and sequestration (Figure 2A and B, lane 10 versus 12). The same result was obtained when the pellet fraction was resuspended in a supernatant fraction which did not contain any procoat protein and was supplemented with microsomes (Figure 2A and B, lane 7 versus 9). These data demonstrate that the procoat protein present in both fractions was competent for membrane insertion. The presence of ribosomes is not a prerequisite for insertion of procoat protein into microsomal membranes.

ATP is required for efficient import of procoat protein into microsomes

To test whether the presence of ATP is a prerequisite for insertion of procoat protein into microsomes, the various fractions containing procoat protein were depleted of ATP and other nucleoside triphosphates by incubation with potato apyrase (Waters and Blobel, 1986; Schlenstedt and Zimmermann, 1987). This ATP depletion led to complete inhibition of polypeptide synthesis, our assay for a nucleoside triphosphate requiring process (data not shown). When microsomes were added to the various fractions, depleted of ATP, a reduction in the formation and sequestration of coat protein was observed (Figure 2A and B, lanes 2, 5, 8 and 11). ATP depletion by treatment with hexokinase plus glucose, instead of apyrase, had the same effect (data not shown). This indicates that ATP is involved in processing of procoat protein present in ribosomal and cytoplasmic fractions. Apparently, procoat protein is imported in a pathway which does not involve the ribosome/ribosome receptor system but involves ATP. However, the ATP requirement in the case of procoat protein was not as stringent as, for example, in the case of prepropeptide gla (Schlenstedt and Zimmermann, 1987) (see below).

The amount of microsomes used in the post-translational incubation and the incubation time of the import reaction was varied. Figure 3A shows the result of the titration with microsomes with respect to processing and sequestration in the presence and absence of ATP. The efficiencies of both processing and sequestration decreased after treatment with apyrase. The effect of apyrase treatment was due to the depletion of ATP (or other nucleoside triphosphates) since (i) denatured apyrase had no effect (Figure 3A), and (ii) the apyrase effect was partially reversed when the apyrase concentration was reduced and the transport reaction was supplemented with ATP prior to the addition of microsomes (Figure 3A). Taken together, ATP (or another nucleoside triphosphate) enhances the efficiency of processing of procoat protein by microsomes. The same observation was made when microsomes were used which had been pretreated with trypsin (Figure 3B). This confirms our earlier conclusion that the SRP/docking protein system is not involved in the assembly of procoat protein into microsomes. Apparently, there is a unique pathway for procoat protein which does not involve the SRP/docking protein system but involves ATP. ATP appears also to affect the rates of processing and sequestration since both reactions were slowed down after apyrase treatment (Figure 4).

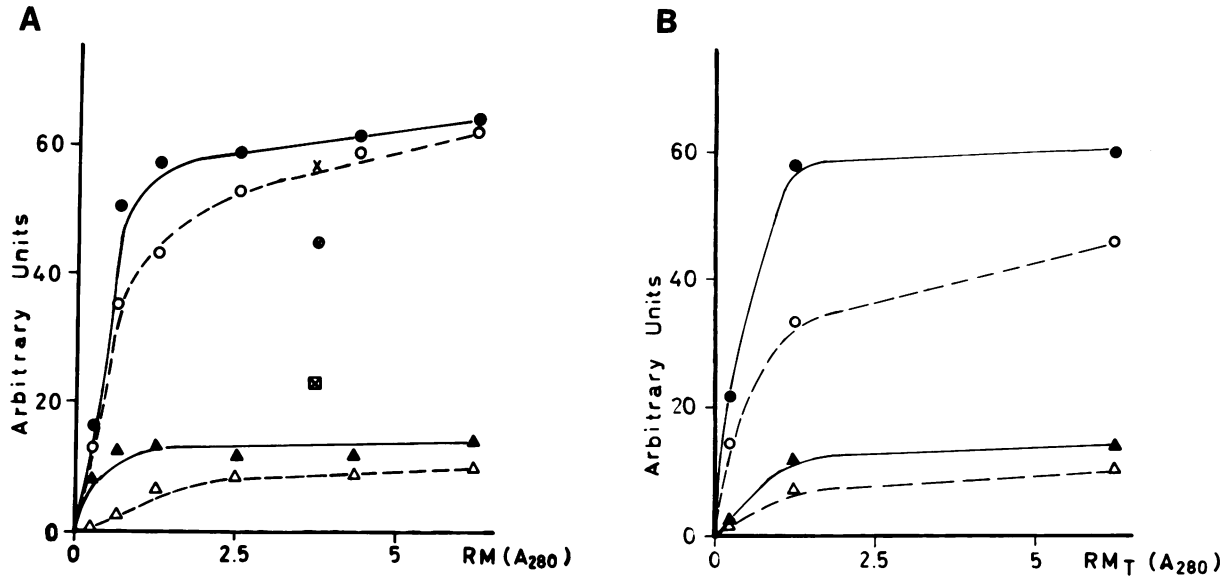

Fig. 3. Efficiences of processing of procoat protein and sequestration of coat protein by microsomes in the presence and absence of ATP. Translation of procoat protein was carried out in a rabbit reticulocyte lysate for 10 min at 37°C. After the addition of cycloheximide and RNase A the sample was divided in two aliquots; one aliquot (▲,△) was supplemented with apyrase (80 units/ml), the other aliquot (●,○) with water, and both aliquots were incubated for 5 min at 37°C. The samples were then incubated for 15 min at 37°C with increasing amounts of untreated microsomes (**A**) or trypsin-treated microsomes (**B**) present. Each reaction was divided into two parts; one was incubated at 0°C for 60 min in the absence of proteinase K (●,▲), the other in the presence of proteinase K (○,△). After immunoprecipitation the samples were analyzed by gel electrophoresis and fluorography. Densitometric analysis of the X-ray film with a Hirschmann densitometer yielded arbitrary units, corrected, in the case of processing, for the background (pseudo coat protein). The trypsin-treated microsomes, used here, had been shown to be incapable of translocating a precursor of immunoglobulin light chain (Schlenstedt and Zimmermann, 1987). (●) total amount of coat protein; (○) protected coat protein (▲) total amount of coat protein after apyrase treatment; (△) protected coat protein after apyrase treatment; (×) protected coat protein after treatment with apyrase which had been boiled for 15 min at 95°C; (⊠) protected coat protein after treatment with diluted apyrase (4 units/ml); (⊗) protected coat protein after treatment with diluted apyrase and supplementation with 4 mM ATP.

ATP increases the efficiency of processing of procoat protein by leader peptidase liposomes and by leader peptidase in detergent

We investigated whether the ATP exerts its action at the level of the reticulocyte lysate or of the membrane. Procoat protein was synthesized in the reticulocyte lysate and subjected to post-translational processing by leader peptidase reconstituted into liposomes or in detergent in the presence and absence of ATP. The efficiency of processing of procoat protein by leader peptidase liposomes (Figure 5, lane 2 versus 6), by Triton X-100 solubilized leader peptidase liposomes (Figure 5, lane 3 versus 7), and by isolated leader peptidase in Triton X-100 (Figure 5, lane 4 versus 8) was reduced by ATP depletion. Since leader peptidase does not show an ATP requirement *in vivo* (Date *et al.*, 1980), our data indicate that the ATP effect, shown here, is due to the reticulocyte lysate. At present, however, it cannot be excluded that there is an additional ATP requiring step at the level of the membranes in import into microsomes. Procoat protein has to be presented to leader peptidase by a membrane or a detergent micelle in order to be a substrate for processing (Ohno-Iwashita and Wickner, 1983). Our observations support this view and suggest that the efficiency of insertion of procoat protein into phospholipid bilayers and into Triton X-100 micelles is increased by a component of the reticulocyte lysate which depends on ATP. An equilibrium may exist between competent and incompetent procoat protein in aqueous solution, and the ATP-dependent component may influence this equilibrium. The differences between various precursor proteins and their requirement for ATP (e.g. procoat protein versus prepropeptide gla) may reflect differences in the equilibrium distribution of the particular precursor protein between competent and incompetent state.

An indication that the loss of competence is due to a conformational change came from an experiment where protease sensitivity of procoat protein in the presence and absence of ATP was assayed. It was observed that procoat protein was considerably more resistant to both trypsin and elastase in the absence of ATP than in its presence (Figure 6). However, the differential protease sensitivity could also be due to aggregation of pro-

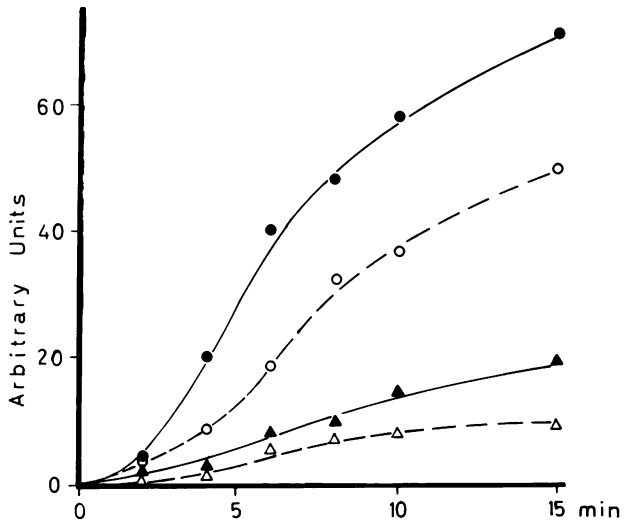

Fig. 4. Rates of processing of procoat protein and sequestration of coat protein by microsomes in the presence and absence of ATP. The experiment was carried out and analyzed as described in the legend to Figure 3, except that the samples were incubated for different times at 37°C. (●) total amount of coat protein; (○) protected coat protein; (▲) total amount of coat protein after apyrase treatment; (△) protected coat protein after apyrase treatment.

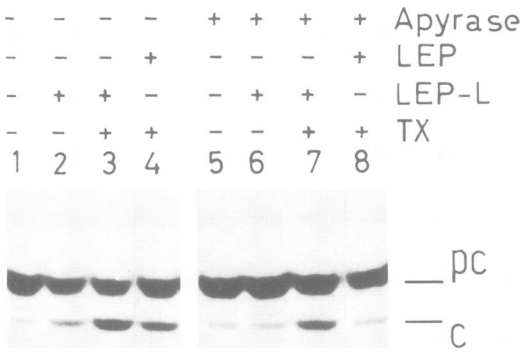

Fig.5. Processing of procoat protein by leader peptidase in the presence and absence of ATP. Translation of procoat protein was carried out in a reticulocyte lysate for 5 min at 37°C. After addition of cycloheximide and RNase A the sample was divided into two aliquots; one aliquot (**lanes 5–8**) was incubated in the presence of apyrase (80 units/ml), the other one in the presence of water (**lanes 1–4**) for 5 min at 37°C. Water (**lanes 1** and **5**), leader peptidase liposomes (Ohno-Iwashita and Wickner, 1983) (**lanes 2** and **6**), leader peptidase liposomes in Triton X-100 (final concentration 0.33%) (**lanes 3** and **7**), or leader peptidase (400 μg/ml) in Triton X-100 (final concentration 0.33%) (**lanes 4** and **8**) were added. All samples were incubated for 30 min at 37°C and then subjected to immunoprecipitation. After gel electrophoresis and fluorography, densitometric analysis was performed as described in the legend to Figure 3. The following values were determined for the percentage of processing of procoat protein due to the action of leader peptidase under the different conditions: 26% (lane 2), 52% (lane 3), 32% (lane 4), 1% (lane 6), 29% (lane 7), 7% (lane 8). LEP, leader peptidase; LEP-L, leader peptidase liposomes; TX, Triton X-100; pc, procoat protein; c, coat protein.

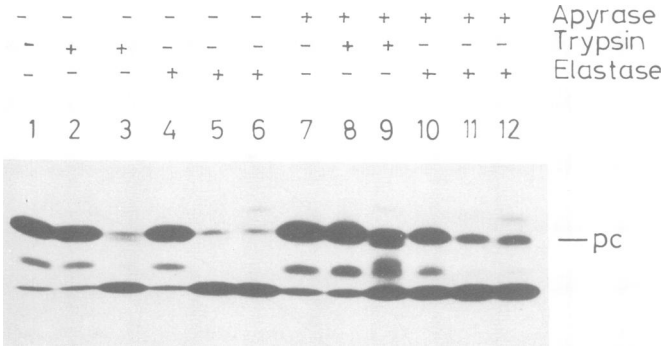

Fig. 6. Protease sensitivity of procoat protein in the presence and absence of ATP. Translation of procoat protein was carried out in a reticulocyte lysate for 10 min at 37°C. After addition of cycloheximide and RNase A the sample was divided into two aliquots; one aliquot (**lanes 7–12**) was incubated in the presence of apyrase (80 units/ml), the other one in the presence of water (**lanes 1–6**) for 5 min at 37°C. Then each aliquot was split into six reactions; one was incubated in the presence of water (**lanes 1** and **7**), the others in the presence of trypsin at 50 μg/ml (**lanes 2** and **8**) and 100 μg/ml (**lanes 3** and **9**) — or elastase at 50 μg/ml (**lanes 4** and **10**), 100 μg/ml (**lanes 5** and **11**) or 200 μg/ml (**lanes 6** and **12**), for 60 min at 0°C. After addition of phenylmethylsulfonyl fluoride (1 mM) all samples were analyzed by gel electrophoresis and fluorography. Differential protease sensitivities also were obtained with proteinase K; however, this was observed only at a concentration of 20 μg/ml but not at a concentration of 175 μg/ml (the conditions used in the sequestration assay) (data not shown). pc, procoat protein.

coat protein molecules with each other or with the putative cytoplasmic component.

An ATP-dependent component of the reticulocyte lysate is required for efficient import of procoat protein into microsomes

To test directly our suggestion that the ATP effect, described above, was due to a soluble component of the reticulocyte lysate, we tested whether the reticulocyte lysate can restore competence

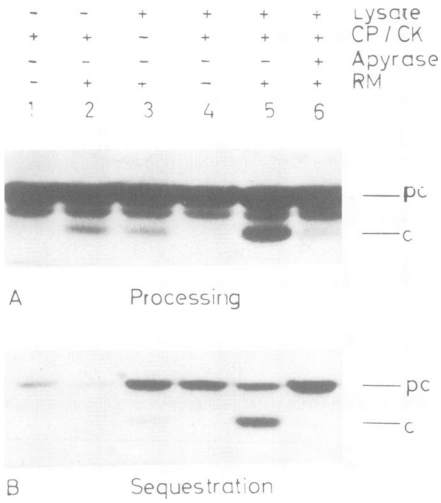

Fig. 7. Processing of procoat protein and sequestration of coat protein by microsomes in the presence and absence of a reticulocyte lysate. Procoat protein was synthesized in a bacterial extract for 30 min at 37°C as described previously (Watts *et al.*, 1983), then cycloheximide (100 μg/ml) and RNase A (80 μg/ml) were added and the incubation was continued for 5 min. The sample was divided into six aliquots; two aliquots were supplemented with 1.5 vol of water and with creatine phosphate (10 mM) and creatine kinase (50 μg/ml) (**lanes 1** and **2**); the other four aliquots were supplemented with 1.5 vol of nuclease-treated rabbit reticulocyte lysates (**lanes 3–6**) which had been incubated in the absence of any additions (**lane 3**) or in the presence of creatine phosphate (16.6 mM) and creatine kinase (83.3 μg/ml) (**lanes 4–6**), or in the presence of apyrase at a concentration of 80 units/ml (**lane 6**) for 5 min at 37°C. After addition of water and microsomes, respectively, all reactions were incubated for 30 min at 37°C. Then all reactions were divided into two parts; one was incubated in the absence (processing), the other one in the presence of proteinase K (sequestration) for 60 min at 0°C. Following immunoprecipitation the samples were analyzed by gel electrophoresis and fluorography. A control experiment showed the validity of the sequestration assay under these conditions: when Triton X-100 was present during the protease treatment coat protein was degraded (data not shown). CP, creatine phosphate; CK, creatine kinase; RM, dog pancreas microsomes; pc, procoat protein; c, coat protein.

for membrane insertion to an otherwise incompetent form of procoat protein, synthesized in an *E. coli* lysate (Goodman *et al.*, 1981). Procoat protein was synthesized in an *E. coli* lysate for 30 min, then protein synthesis was blocked by incubation in the presence of RNase A for 5 min. Import of procoat protein into microsomes was assayed in the absence and presence of reticulocyte lysate and with respect to ATP dependence. There was a significant stimulation of coat protein formation and sequestration by the reticulocyte lysate (Figure 7, lane 2 versus 5). This stimulation depended on the presence of ATP, or other nucleoside triphosphates (Figure 7, lane 5 versus 3 and 6). Therefore, we conclude that the ATP dependence of the import of procoat protein into microsomes, observed in the reticulocyte lysate, is due to a soluble component of this lysate. Furthermore, this experimental system provides an assay for the isolation of this component.

Discussion

While the endoplasmic reticulum (ER) membrane is the system best understood with respect to specificity of membrane insertion and transport of proteins (SRP/docking protein) (Hortsch and Meyer, 1984; Walter *et al.*, 1984), the general opinion on the mechanisms involved in the actual insertion and transport have recently been revised in the light of new data (Zimmermann and

Meyer, 1986). These processes do not appear to be as different compared to other membrane systems as originally thought. A first hint along these lines came from the observation that M13 procoat protein can be inserted into dog pancreas-ER derived membranes in the absence of protein synthesis (Watts *et al.*, 1983). Similar behaviour occurs with two precursors of eucaryotic secretory proteins having molecular weights similar to that of procoat, namely honeybee prepromelittin (Zimmermann and Mollay, 1986) and frog prepropeptide gla (Schlenstedt and Zimmermann, 1987). These three proteins, however, do not depend on SRP and docking protein either. By employing a yeast cell free translation system and yeast-ER derived membranes it was observed that the precursor of the yeast pheromone α-factor (prepro-α-factor) can be imported into ER-derived vesicles in the absence of protein synthesis (Hansen *et al.*, 1986; Rothblatt and Meyer, 1986a, b; Waters and Blobel, 1986). The most convincing evidence contrary to the cotranslational model for membrane insertion and transport of proteins, however, came from several elegant studies on well established (i.e. SRP and docking protein dependent) secretory and plasma membrane proteins from higher eucaryotic cells and the dog pancreas-ER derived membranes: (i) precursors of the erythrocyte glucose transporter or a carboxy terminally truncated version thereof (peptidyl-tRNA) can be inserted into microsomes post translationally (Mueckler and Lodish, 1986a,b) (ii) precursors of carboxy terminally truncated hybrid proteins consisting of either the pre-β-lactamase signal sequence and the α-globin chain (peptidyl-tRNA) or of an amino terminal domain of bovine rhodopsin and the α-globin chain (peptidyl-tRNA) can be imported into microsomes (Perara *et al.*, 1986); (iii) the precursor of bovine prolactin can be synthesized to completion and still be imported into microsomes, provided the formation of intramolecular disulfide bridges is prevented (Maher and Singer, 1986); (iv) pre-human placental lactogen can be fully synthesized and then imported into microsomes (Caulfield *et al.*, 1986).

The general conclusion from these data is that translation does not provide the driving force for the movement of polar amino acid residues across the apolar core of the phospholipid bilayer in this system. The experimental advantages of being able to study membrane insertion and transport of proteins independently of protein synthesis has already led to the discovery of a couple of interesting details of these events: (i) ATP hydrolysis is required for the insertion of fully synthesized polypeptides (prepro-α-factor, prepropeptide gla, M13 procoat protein) and peptidyl-tRNAs (truncated versions of the glucose transporter, the pre-β-lactamase/α-globin hybrid protein, and the rhodopsin/α-globin hybrid protein) into ER derived membranes, as assayed by removal of the signal peptide or glycosylation or membrane insertion; (ii) this energy requirement does not occur at the level of SRP/docking protein or ribosome/ribosome receptor since prepropeptide gla and M13 procoat protein do not depend on either one of these components, rather it is linked to a previously uncharacterized cytoplasmic component; (iii) the ribosome is required in an unknown fashion to bind to the ER membrane together with the polypeptide chain even in the absence of chain elongation (Caulfield *et al.*, 1986; Mueckler and Lodish, 1986b; Perara *et al.*, 1986). Interestingly, this has only been observed for precursors which also depend on SRP/docking protein.

In summary, there appear to be different requirements for competence for membrane insertion. One requirement is the presence of a signal generally contained in the additional sequences of the precursor molecules. Furthermore, there seem to be requirements for insertion competence which are related to an intrinsic feature of the whole polypeptide chain. The molecules may not be allowed to fold into three-dimensional structures which are thermodynamically more favorable. This may be important so that the signals remain exposed, or so that the polypeptide chain is unfolded to a certain extent during the actual insertion or transport. The findings with mitochondrial (Eilers and Schatz, 1986) and *E. coli* proteins (Randall and Hardy, 1986) can be interpreted in this way; the observation that the formation of disulfide bridges has to be prevented in nascent secretory proteins would also be in agreement (Maher and Singer, 1986). For most of the latter proteins, SRP and docking protein, on the one hand, and the ribosome and a ribosome receptor, on the other, may serve to prevent the proteins from folding. With smaller proteins, such a complicated system may not be required. The ATP requiring step seems to be related to preservation of a competent conformation of precursor proteins.

Materials and methods

Materials

Enzymes used for cloning were purchased from Boehringer Mannheim. 7mGpppG and nucleotriphosphates were obtained from Pharmacia/P-L Biochemicals. SP6 RNA polymerase, RNasin, ribonuclease A, trypsin, soybean trypsin inhibitor and proteinase K were from Boehringer Mannheim. Potato apyrase (grade VIII) and cycloheximide were from Sigma. [^{35}S]methionine (1000 Ci/mmol) was from Amersham Corp. X-ray films (Kodak X-Omat AR) were from Kodak.

Construction of plasmids and in vitro transcription

Construction of the plasmid coding for M13 procoat protein was carried out according to standard procedures (Maniatis *et al.*, 1982). Specifically, a *SalI*/*PstI* fragment, derived from plasmid pQN 805 (Kuhn and Wickner, 1985) was cloned into plasmid pSP 65. *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 use.

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 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 any particular *in vitro* translation mixture was 3.

Analytical procedures

Sequestration assays were carried out as described previously (Zimmermann and Mollay, 1986) except for the proteinase K concentration which was 175 μg/ml. Typically, samples were diluted with an equal volume of double strength sample buffer (Laemmli, 1970) and analyzed on urea-containing SDS−polyacrylamide gels (19%) (Ito *et al.*, 1980). Alternatively, samples were subjected to immunoprecipitation as described (Watts *et al.*, 1983) and analyzed by gel electrophoresis. For fluorography, the gels were treated with sodium salicylate (Chamberlain, 1979), dried, and exposed to X-ray films at −80°C for 1 or 2 days.

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