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A microsomal protein is involved in ATP-dependent transport of presecretory proteins into mammalian microsomes

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Ribonucleoparticle (i.e. ribosome and SRP)-independent transport of proteins into mammalian microsomes is stimulated by a cytosolic ATPase which involves proteins belonging to the hsp70 family. Here we addressed the question of whether there are additional nucleoside triphosphate requirements involved in this transport mechanism. We employed a purified presecretory protein which upon solubilization in dimethyl sulfoxide and subsequent dilution into an aqueous buffer was processed by and transported into mammalian microsomes in the absence of the cytosolic ATPase. Membrane insertion of this precursor protein was found to depend on the hydrolysis of ATP and to involve a microsomal protein which can be photoaffinity inactivated with azido-ATP. Furthermore, a microsomal protein with a similar sensitivity towards photoaffinity modification with azido-ATP was observed to be involved in ribonucleoparticle-dependent transport. We suggest that a novel microsomal protein which depends on ATP hydrolysis is involved in membrane insertion of both ribonucleoparticle-dependent and -independent precursor proteins.

Key words: azido-ATP photoaffinity inactivation/mammalian endoplasmic reticulum/protein transport

Introduction

The decisive initial step in secretion of most eukaryotic proteins is their transport into the lumen of the endoplasmic reticulum (for reviews see Zimmermann and Meyer, 1986; Meyer, 1988; Rapoport, 1990). This event is followed by vesicular transport which eventually leads to the release of the secretory proteins into the extracellular space. A similar mechanism of protein topogenesis operates for resident soluble or membrane proteins of the various organelles which are involved in endo- or exocytosis. A common feature of these proteins is that the microsomal membrane is the only membrane these proteins ever have to insert into or traverse. The membrane insertion or transport of these proteins can be subdivided into the following stages: (i) specific association of the proteins with the membrane; (ii) membrane insertion; and (iii) in the case of soluble proteins, complete transfer across the membrane. The discriminatory mechanism which leads to specificity of membrane insertion or transport of these proteins involves a characteristic amino-terminal signal peptide in the precursor proteins, a signal peptide receptor on the cytosolic phase, and in many cases, signal peptidase on the lumenal phase of the microsomal membrane.

Considerable efforts to elucidate the mechanisms of transport of presecretory proteins into the endoplasmic reticulum have focused on the energy requirements (Zimmermann and Meyer, 1986; Meyer, 1988; Rapoport, 1990). For obvious reasons, this problem could only be addressed independently of translation. Two developments have led to the establishment of transport conditions which are independent of ongoing protein synthesis: (i) the generation of precursor-related peptidyl-tRNAs and their use in the well characterized *in vitro* systems derived from higher eukaryotic organisms (Connolly and Gilmore, 1986; Mueckler and Lodish, 1986a,b; Perara *et al.*, 1986); (ii) the investigation of certain precursor proteins which are able to translocate as completed chains using *in vitro* systems derived from higher (Wiech *et al.*, 1987; Schlenstedt and Zimmermann, 1987; Müller and Zimmermann, 1988; Schlenstedt *et al.*, 1990) or lower eukaryotic organisms (Hansen *et al.*, 1986; Rothblatt and Meyer, 1986; Waters and Blobel, 1986). The common theme emerging from these studies is that nucleoside triphosphates are required for the transport of proteins into microsomes. So far, there are two systems which should be discriminated in this respect: (i) There is a GTP requirement in ribonucleoparticle (i.e. ribosome and SRP)-dependent transport of precursor proteins (Connolly and Gilmore, 1989). This GTP effect is related to the GTP-binding proteins, SRP (54 kDa subunit) and docking protein – SRP receptor (α-subunit) (Connolly and Gilmore, 1989; Römisch *et al.*, 1989; Bernstein *et al.*, 1989); (ii) There is an ATP requirement in ribonucleoparticle-independent transport of precursor proteins (Hansen *et al.*, 1986; Rothblatt and Meyer, 1986; Waters and Blobel, 1986; Waters *et al.*, 1986; Schlenstedt and Zimmermann, 1987; Wiech *et al.*, 1987; Müller and Zimmermann, 1988; Schlenstedt *et al.*, 1990). This ATP effect is related to a cytosolic ATPase which is present in rabbit reticulocyte and yeast lysates (Waters *et al.*, 1986; Rothblatt *et al.*, 1987; Wiech *et al.*, 1987). Heat shock proteins belonging to the hsp70 protein family are part of this ATPase, but at least one additional component is involved (Chirico *et al.*, 1988; Deshaies *et al.*, 1988; Zimmermann *et al.*, 1988).

The present study was begun by asking if there are additional energy requirements in ribonucleoparticle-independent transport of presecretory proteins into mammalian microsomes. We employed a chemically synthesized precursor protein, preprocecropinA, which after solubilization in dimethyl sulfoxide and subsequent dilution into an aqueous buffer was processed by and transported into

dog pancreas microsomes in the absence of cytosolic factors, such as hsp70. We show here that membrane insertion (assayed as removal of the signal peptide by signal peptidase) under these conditions depends on ATP hydrolysis and that ATP is used by a microsomal protein which is sensitive to photoaffinity modification with 2- and 8-azido-ATP. Furthermore, we show that a microsomal protein with the identical azido-ATP sensitivity is involved in ribonucleoparticle-dependent transport of proteins, such as preprolactin, into dog pancreas microsomes. Immunoglobulin heavy chain binding protein (BiP) is disregarded by the present study as being responsible for the observed azido-ATP effects. We therefore suggest that a microsomal protein exists which facilitates membrane insertion of ribonucleoparticle-dependent as well as ribonucleoparticle-independent precursor proteins and which depends on ATP for its action.

Results

Chemically synthesized preprocecropinA can be processed by and transported into mammalian microsomes in the absence of cytosolic factors

We have reported previously that the presecretory protein preprocecropinA (ppcecA, 64 amino acid residues) is efficiently transported into dog pancreas microsomes after synthesis in a rabbit reticulocyte lysate, i.e. under post-translational conditions (Schlenstedt *et al.*, 1990). This transport does not involve the two ribonucleoparticles, ribosome and signal recognition particle (SRP) nor their receptors on the microsomal surface (ribosome receptor and docking protein) but depends on ATP and a microsomal membrane protein which is sensitive to chemical alkylation (Schlenstedt *et al.*, 1990; Zimmermann *et al.*, 1990). In order to characterize the ATP effect(s) further, and as a first step towards a more defined transport reaction, in general, we chemically synthesized preprocecropinA on a large scale and used this synthetic precursor protein, termed ppcecA* solely to indicate its nature, in transport experiments.

In our first set of experiments we addressed the question of whether ppcecA* behaves like the *in vitro* synthesized ppcecA with respect to its requirements for transport into dog pancreas microsomes. For radiolabeling the chemically synthesized and HPLC-purified precursor was derivatized on lysine residues with either [^{14}C]methyl groups or ^{35}S-labeling reagent as described in Materials and methods. After solubilization in aqueous buffers the radiolabeled ppcecA* was neither processed by nor transported into microsomes under the conditions which were previously established for post-translational transport, i.e. in the presence of reticulocyte lysate (Schlenstedt *et al.*, 1990) (data not shown). However, when the radiolabeled ppcecA* was first solubilized in dimethyl sulfoxide and subsequently incubated with microsomes under similar conditions, membrane insertion (i.e. processing by signal peptidase) and transport (i.e. sequestration) of ppcecA* occurred (Figure 1 A, lanes 1 through 4). In other transport systems solubilization of the precursor proteins in denaturants, such as urea, and subsequent dilution into an aqueous solution were found to circumvent the need for certain soluble factors (reviewed by Wiech *et al.*, 1990). Therefore, we asked whether in our system solublization of ppcecA* in dimethyl sulfoxide had a similar effect. Membrane insertion and transport of ppcecA* also occurred in the absence of reticulocyte lysate (Figure 2 A, lanes 1–3 versus 7–9). The presence of lysate, however, was observed to have a stimulatory effect (Figure 1B). Up to 40% of ppcecA* were transported under

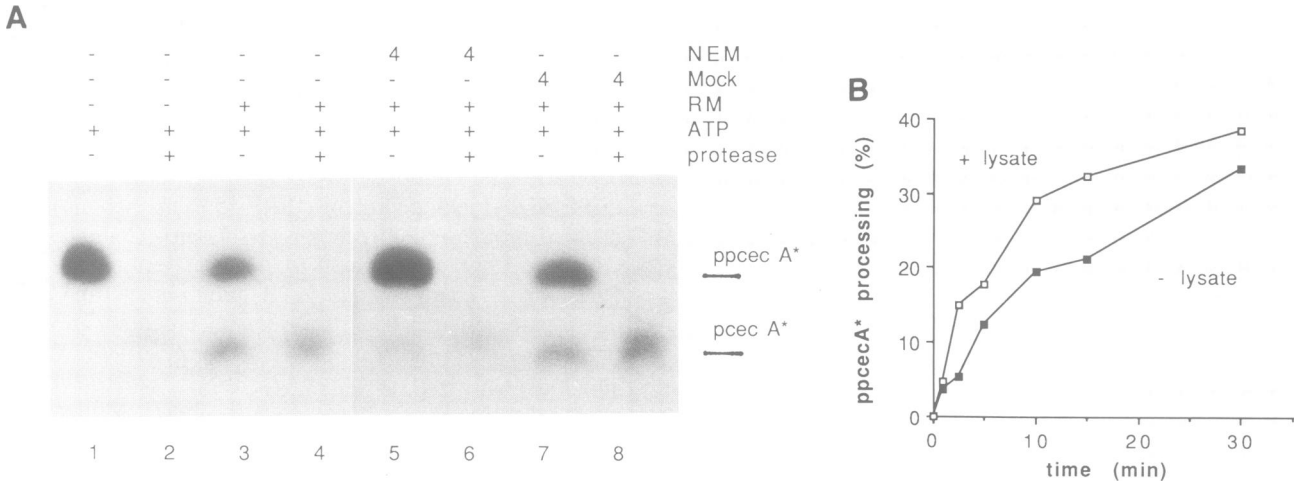

Fig. 1. Transport of chemically synthesized preprocecropinA (ppcecA*) into dog pancreas microsomes in the presence of rabbit reticulocyte lysate. **(A)** ^{35}SLR-labeled ppcecA* (50 ng solubilized in 0.44 μl dimethyl sulfoxide) was added to 40 μl of rabbit reticulocyte lysate translation mix without exogenously added mRNA and [^{35}S]methionine. The mixture was divided into four aliquots which were supplemented with RM-buffer, microsomes or microsomes which were NEM- or mock-treated as described in Materials and methods (final concentration of microsomal protein: 1 mg/ml, final volume: 11 μl). After an incubation for 30 min at 37°C each transport reaction was divided into two halves; one half was incubated further in the absence of protease, the other one in the presence of protease as described in Materials and methods. The samples were analyzed by gel electrophoresis and fluorography. The concentration of *N*-ethylmaleimide which was used for the treatment of microsomes is given in mmol/l. **(B)** ^{35}SLR-labeled ppcecA* (80 ng solubilized in 0.88 μl dimethyl sulfoxide) was added to 80 μl of rabbit reticulocyte lysate translation mix or 80 μl of transport buffer which contained ATP. The two mixtures were supplemented with microsomes (final concentration of microsomal protein: 1 fmg/ml) and each one was divided into eight aliquots (final volume: 11 μl). Incubation of the various aliquots at 37°C was carried out for the times indicated. The samples were analyzed by gel electrophoresis and fluorography. The amounts of ppcecA* as well as of pcecA* were quantified by laser densitometry of the fluorograph, and the efficiencies of processing of ppcecA* (given as pcecA* in percentage of ppcecA* plus pcecA*) were plotted against the incubation time.

both conditions and the transport efficiency showed a dependency on the concentration of microsomal protein (up to 1.5 mg/ml, data not shown) and on the incubation time (Figure 1B).

The pcecA*, produced under both conditions, was identified as the expected mature form by comigration with the product after processing by signal peptidase and with the processing product of the *in vitro* synthesized precursor (data not shown). Two lines of evidence suggest that pcecA* was transported into the lumen of the microsomes: (i) in contrast to the precursor, the mature form was protected against externally added protease in the absence of detergent but degraded in the presence of detergent (Figure 2A, lanes 8 versus 9); (ii) the mature form pelleted with the microsomes during centrifugation at neutral pH, but was recovered in the supernatant after alkaline extraction and subsequent centrifugation (Figure 2B).

We conclude from these data that after solubilization in dimethyl sulfoxide the chemically synthesized and radiolabeled preprocecropinA is a substrate for transport into dog pancreas microsomes and that soluble factors are not essential for but can stimulate transport.

Based on these observations we asked next whether ppcecA* transport requires the microsomal protein which is sensitve to *N*-ethylmaleimide treatment (Zimmermann *et al.*, 1990). Microsomes pretreated with *N*-ethylmaleimide were found to be inactive (Figure 1A, lanes 5–8). Furthermore, we asked whether ppcecA* transport depends on the signal peptide. Chemically synthesized procecropinA, pcecA* (Boman *et al.*, 1989), was radiolabeled by reductive methylation, solubilized in dimethyl sulfoxide, and incubated with microsomes. The signal peptide deficient protein was neither bound to nor transported into microsomes (data not shown). Therefore, the possibility that solubilization of ppcecA* in dimethyl sulfoxide facilitated spontaneous membrane insertion of the precursor protein can be excluded. Similar to biosynthetic ppcecA (Schlenstedt *et al.*, 1990), the transport of ppcecA* into trypsin pretreated microsomes (i.e. in the absence of the α-subunit of docking protein) occurred with the same efficiency as into untreated microsomes (i.e. in the presence of the α-subunit of docking protein) (Figure 2A, lanes 7–12).

In summary we conclude that ppcecA* is transported into dog pancreas microsomes following the same path as the *in vitro* synthesized ppcecA.

Membrane insertion of ppcecA depends on the hydrolysis of ATP*

Experiments were then performed to determine if ppcecA* transport requires ATP. Strikingly, membrane insertion of ppcecA* was observed to be stimulated by the addition of ATP even in the absence of cytosolic factors (Figure 2A, lane 4 versus 7). The low rate of transport in the absence of externally added ATP must be due to endogenous ATP since addition of an energy regenerating system had a similar effect to the addition of ATP (Table I). This basal transport could not be reduced by the addition of apyrase (data not shown). This suggests that the endogenous ATP was bound to proteins with high affinity. Since the transport of biosynthetic ppcecA was sensitive to apyrase (Schlenstedt *et al.*, 1990), we assume that hsp70 was involved in the transport of biosynthetic ppcecA and of ppcecA* in the presence of reticulocyte lysate.

The concentration of ATP which was necessary for half-maximal transport stimulation was in the order of 10 μM (Figure 3). At this concentration other nucleotides, such as GTP, were unable to substitute for ATP (Table I). Furthermore, the non-hydrolyzable ATP analogs AMP-PCP and AMP-PNP could not substitute for ATP (Table I). Since these analogs competed with ATP when added together with ATP one can conclude that the hydrolysis of ATP is required. At higher concentrations other nucleotides, such as GTP, were able to substitute for ATP. Strikingly, the 8-azido derivative of ATP was able to substitute for ATP.

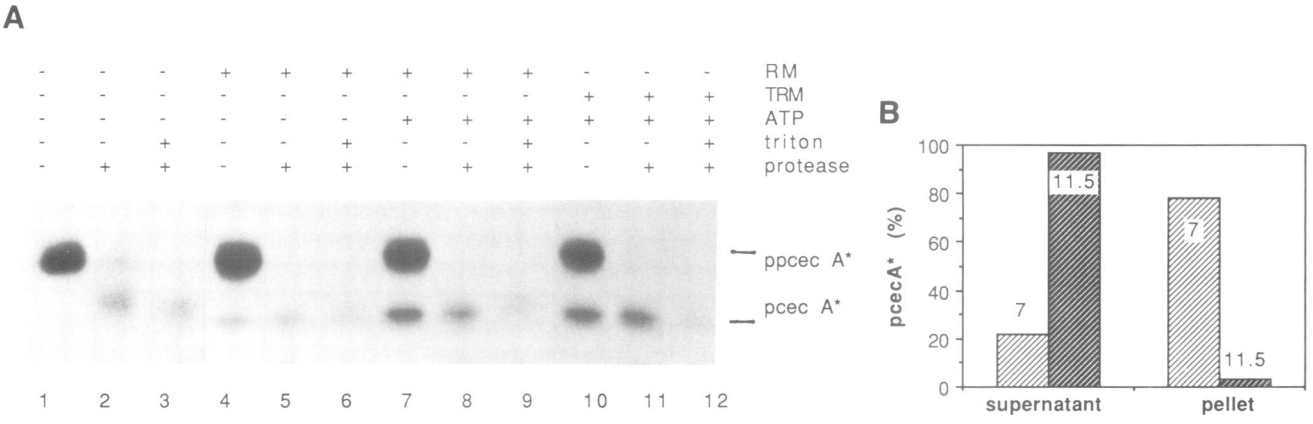

Fig. 2. Transport of ppcecA* into microsomes in the absence of rabbit reticulocyte lysate. (**A**) ^{14}C-labeled ppcecA* (700 ng solubilized in 0.67 μl dimethyl sulfoxide) was added to 80 μl of transport buffer which did not contain ATP. The mixture was divided into four aliquots and the aliquots were supplemented with RM-buffer, microsomes or microsomes which were pretreated with trypsin as described in Materials and methods (final concentration of microsomal protein: 1 mg/ml) plus water or ATP (final volume: 22 μl). After an incubation for 30 min at 37°C each transport reaction was divided into three aliquots; one aliquot was incubated further in the absence of protease, one in the presence of protease and one in the presence of protease plus detergent as described in Materials and methods. The samples were analyzed by gel electrophoresis and fluorography. TRM, trypsin-pretreated microsomes. (**B**) ^{35}SLR-labeled ppcecA* (20 ng solubilized in 0.1 μl dimethyl sulfoxide) was added to 40 μl of transport buffer which contained ATP. The mixture was supplemented with microsomes (final concentration of microsomal protein: 1 mg/ml, final volume: 44 μl). After an incubation for 30 min at 37°C the transport reaction was divided into three aliquots; one aliquot was kept on ice; the other two aliquots were subjected to subfractionation at pH values of 7 and 11.5, respectively, as described previously (Schlenstedt *et al.*, 1990). The samples were analyzed by gel electrophoresis and fluorography. The amounts of pcecA* in the various fractions were quantified by laser densitometry of the fluorograph.

Membrane insertion of ppcecA depends on a microsomal protein which is sensitive to photoaffinity modification with azido-ATP*

In order to determine independently whether the microsomes contain an ATP-binding site which is involved in ppcecA* transport we asked whether irreversible binding of ATP would inactivate microsomes. Therefore, microsomes were photoaffinity modified with 8-azido-ATP at different concentrations of the ATP analog as described in Materials and methods. These microsomes were then analyzed with respect to their ability to transport ppcecA* in the presence of ATP. Photoaffinity modification of microsomes with 8-azido-ATP for 1 min at 0°C at concentrations > 100 μM led to inactivation of the microsomes (Figure 4A and B); the effect was on membrane insertion. The concentration which was necessary to result in half-maximal inhibition was determined to be in the order of 500 μM (Figure 4B). Photoaffinity modification with 2-azido-ATP yielded a similar result (data not shown). The modification with azido-ATP must have been a specific one since UV irradiation of the microsomes in the absence of the ATP analog and incubation with the analog in the absence of irradiation had little effect and, furthermore, since the effect of irradiation in the presence of azido-ATP was prevented by the presence of a 5-fold molar excess of ATP but not of adenine (data not shown, see below).

Table I. The hydrolysis of ATP is involved in the transport of ppcecA* into microsomes

Addition	ppcecA* processing (%)			
	10 μM	25 μM	100 μM	100 μM + 10 μM ATP
ATP	24	46	40	–
8-azido-ATP	8	34	40	–
AMP-PCP	5	10	11	17
AMP-PNP	4	11	11	12
GTP	6	17	28	–
GMP-PCP	–	11	14	33
GMP-PNP	–	11	12	30
CP	7	–	–	–
CP+CK	41	–	–	–
No addition	6	–	–	–

^{14}C-labeled ppcecA* was added to transport buffer as described in the legend to Figure 3. The mixture was divided into aliquots and the aliquots were supplemented with microsomes (final concentration of microsomal protein: 0.6 mg/ml) plus various amounts of different nucleotides (the final concentrations of nucleotides are given in μmol/l). After an incubation for 30 min at 37°C the samples were analyzed by gel electrophoresis and fluorography. The amounts of ppcecA* as well as of pcecA* were quantified by laser densitometry of the fluorograph, and the efficiencies of processing of ppcecA* were determined (given as pcecA* in percent of ppcecA* plus pcecA*). CP, creatine phosphate; CK, creatine kinase (final concentration: 50 μg/ml).

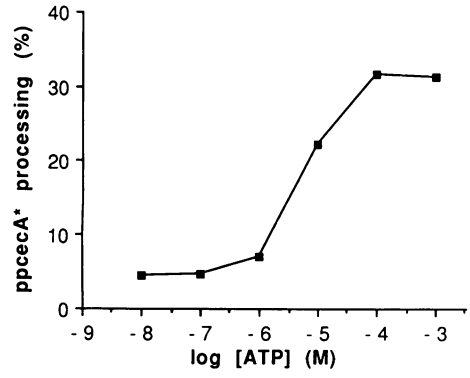

Fig. 3. ATP stimulates the transport of ppcecA* into microsomes. ^{14}C-labeled ppcecA* (780 ng solubilized in 0.93 μl dimethyl sulfoxide) was added to 150 μl of transport buffer which did not contain ATP. The mixture was divided into seven aliquots and the aliquots were supplemented with microsomes in RM-buffer (final concentration of microsomal protein: 0.6 mg/ml) plus various amounts of ATP (final volume: 22 μl). After an incubation for 30 min at 37°C each transport reaction was divided into two halves; one half was incubated further in the absence of protease and one in the presence of protease. The samples were analyzed by gel electrophoresis and fluorography. The amounts of ppcecA* as well as of pcecA* were quantified by laser densitometry of the fluorograph, and the efficiencies of processing of ppcecA* (given as pcecA* in percent of ppcecA* plus pcecA*) were plotted against the ATP concentration.

M13 procoat protein is known to become inserted into *N*-ethylmaleimide pretreated microsomes (Zimmermann *et al.*, 1990) as well as into protein-free liposomes (Geller and Wickner, 1985) and to be protected against externally added protease. Therefore, this protein was used as a control for the integrity of the microsomes after photoaffinity labeling with 8-azido-ATP. Since in this respect there was no effect detectable for the various treatments (Figure 4B) we conclude that the photoaffinity labeling with azido-ATP did not damage the phospholipid bilayer to any significant extent.

In summary, we conclude that there is a microsomal component which is required for membrane insertion of ppcecA* and that this component is sensitive to modification with 8-azido-ATP. The requirement for relatively high concentrations of azido-ATP may be due to endogenous ATP which was bound to microsomal proteins and which had to be displaced by azido-ATP. For comparison, in the analogous experiments in the bacterial system 1–15 mM azido-ATP was applied for 10–15 min (Chen and Tai, 1986 and 1987; Lill *et al.*, 1989).

The azido-ATP sensitive protein is distinct from BiP

Recent results on protein transport into yeast microsomes have suggested a role for immunoglobulin heavy chain binding protein (BiP) in protein transport across the yeast microsomal membrane (Vogel *et al.*, 1990). Since BiP belongs to the hsp70 protein family and has a high-affinity binding site for ATP an ATP requirement of protein transport at the level of BiP is conceivable (Munro and Pelham, 1986; Kassenbrock and Kelly, 1989). However, BiP was found not to be limiting for transport of ribonucleoparticle-dependent precursor proteins into mammalian microsomes (Bulleid and Freedman, 1988; Yu *et al.*, 1990; Zimmerman and Walter, 1990; Nicchitta and Blobel, 1990). Roughly 100- to 1000-fold higher amounts of ppcecA* are transported compared with *in vitro* synthesized precursors. Therefore, we asked whether with ppcecA* BiP might become limiting. Microsomes were depleted of their lumenal proteins by treatment with increasing concentrations of octyl glucoside as described by Zimmerman and Walter (1990). After removal of the detergent the reconstituted microsomes were analyzed for their BiP-content and for their ability to process and sequester three different precursor proteins. Again M13 procoat protein processing and coat protein sequestration

served as a control for the integrity of the microsomes (see above).

When a concentration of octyl glucoside of 22.5 mM was used during the pretreatment of microsomes, >90% of BiP was removed (Figure 5B). However, the activity of the microsomes with respect to processing of any of the three precursor proteins was unaffected (Figure 5A and B). The concentration of ppcecA* was kept constant at about 1 μM while the concentration of BiP was varied between ~200 nM and 20 nM. Although this result does not exclude the possibility that BiP was involved in the transport of ppcecA* one can conclude that BiP was not limiting even under conditions where the BiP content was reduced 10-fold. Since photoaffinity modification of microsomes with azido-ATP is not likely to lead to more than 90% derivatization of BiP (Potter and Haley, 1982) modification of BiP is probably

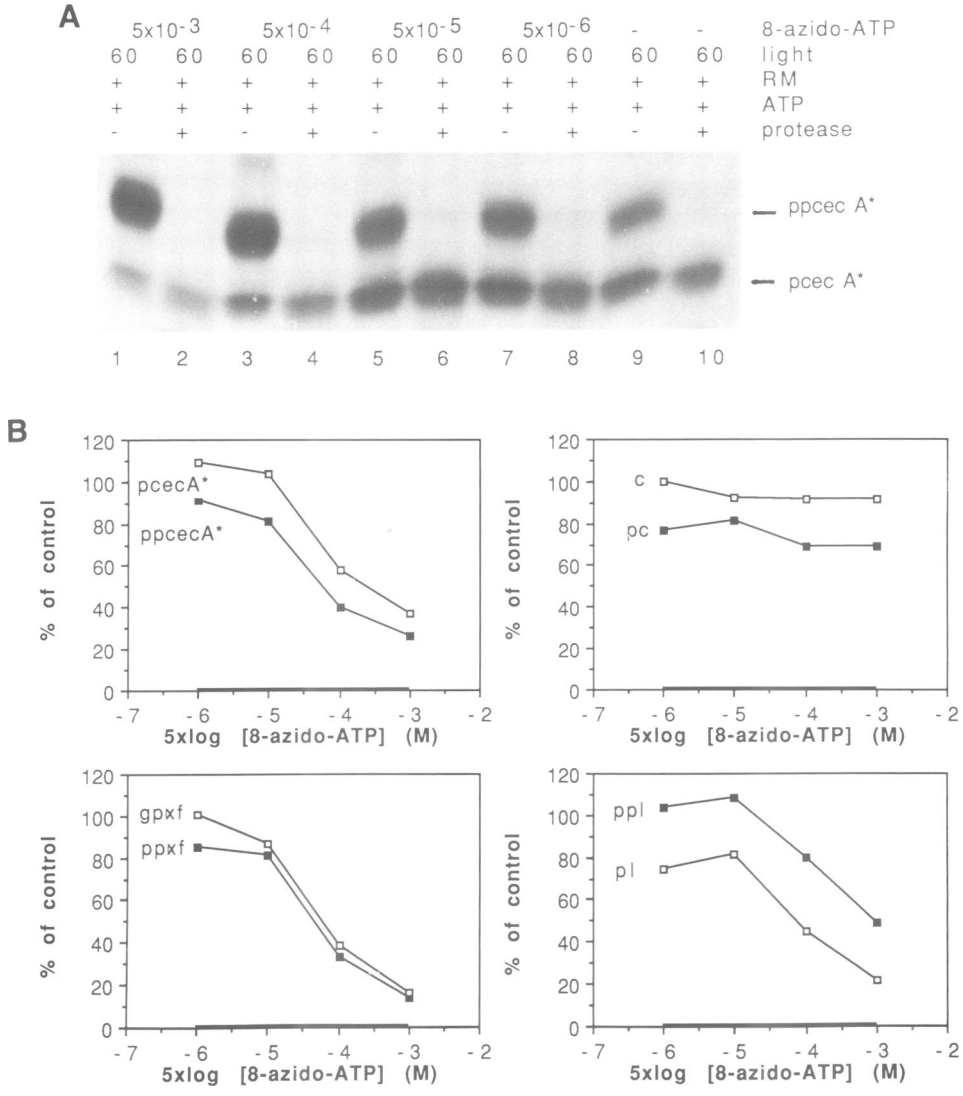

Fig. 4. Photoaffinity modification of microsomes with 8-azido-ATP leads to inactivation of the microsomes with respect to membrane insertion of ppcecA* (**A**) ^{14}C-labeled ppcecA* (500 ng solubilized in 1.2 μl dimethyl sulfoxide) was added to 120 μl of transport buffer which contained ATP. The mixture was divided into five aliquots and the aliquots were supplemented with microsomes which were pretreated as described in Materials and methods (final concentration of microsomal protein: 1 mg/ml, final volume: 24 μl). After an incubation for 15 min at 37°C the microsomes were reisolated by centrifugation (5 min, 4°C, Eppendorf Microfuge). The microsomes from each transport reaction were resuspended in RM-buffer and divided into two halves; one half was incubated further in the absence of protease and one in the presence of protease. The samples were analyzed by gel electrophoresis and fluorography. The amounts of ppcecA* as well as of pcecA* and sequestered pcecA* were quantified by laser densitometry of the fluorograph, and the efficiencies of processing of ppcecA* (ppcecA*) and sequestration of pcecA* (pcecA*) were plotted against the concentration of 8-azido-ATP which was used for the photoaffinity modification of the microsomes (both given as a percentage of the values which were obtained for untreated microsomes) (shown in B). Note that shortly exposed fluorographs which did not show any pcecA*-like material in the absence of microsomes were used for the quantification of sequestered pcecA*. The concentrations of azido-ATP are given in mol/l; the times of UV irradiation (light) are given in seconds. (**B**) M13 procoat protein, bovine preprolactin and yeast prepro-α-factor, respectively were synthesized in rabbit reticulocyte lysates in the presence of [^{35}S]methionine and of different microsomes which were present at concentrations within the linear range (Schlenstedt *et al.*, 1990). Each translation reaction was divided into two halves; one half was incubated further in the absence of protease, the other one in the presence of protease. The samples were analyzed by gel electrophoresis and fluorography. The amounts of precursor as well as of mature and sequestered mature protein were quantified by laser densitometry of the fluorograph, and the efficiencies of processing (pc, ppl and ppαf, respectively) and sequestration (c, pl and gpαf, respectively) were plotted against the concentration of 8-azido-ATP which was used for the photoaffinity modification of the microsomes.

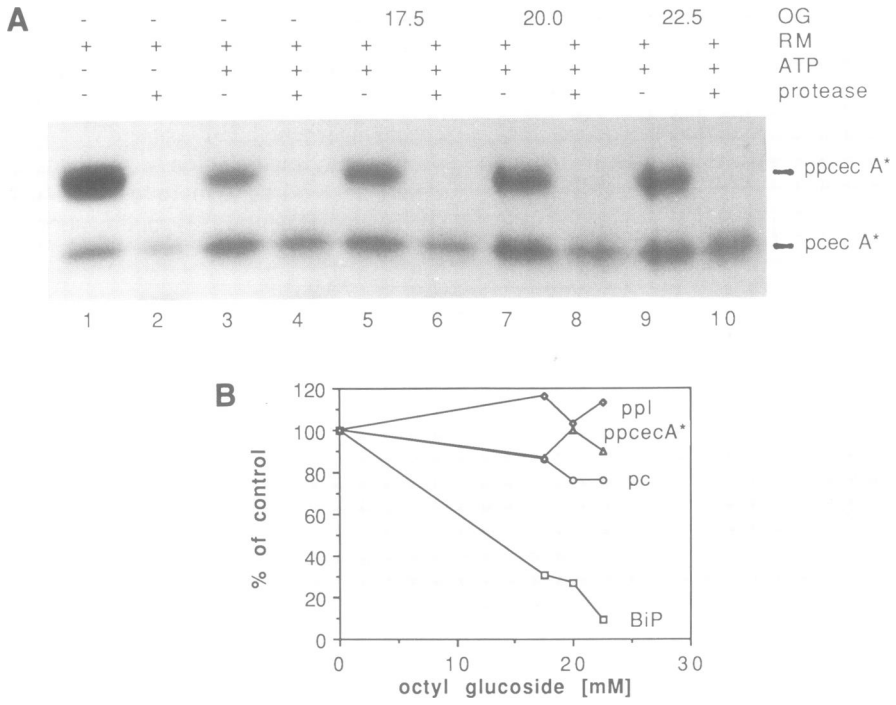

Fig. 5. Depletion of microsomes with respect to luminal proteins does not lead to inactivation of the microsomes with respect to membrane insertion of ppcecA*. (**A**) ^{14}C-labeled ppcecA* (970 ng solubilized in 0.90 μl dimethyl sulfoxide) was added to 125 μl of transport buffer which did not contain ATP. The mixture was divided into five aliquots and the aliquots were supplemented with microsomes which were pretreated as described in Materials and methods (final concentration of microsomal protein: 0.5 mg/ml) plus water and ATP (final volume: 25 μl). After an incubation for 15 min at 37°C the microsomes were reisolated by centrifugation (5 min, 4°C, Eppendorf Microfuge). The microsomes from each transport reaction were resuspended in RM-buffer and divided into two halves; one half was incubated further in the absence of protease and one in the presence of protease. The samples were analyzed by gel electrophoresis and fluorography. The amounts of ppcecA* as well as of pcecA* were quantified by laser densitometry of the fluorograph, and the efficiencies of processing of ppcecA* were plotted against the concentration of octyl glucoside which was used for the pretreatment of the microsomes (given as a percentage of the values which were obtained for untreated microsomes) (shown in B). The concentrations of octyl glucoside are given in mmol/l. (**B**) M13 procoat protein and bovine preprolactin, respectively were synthesized in rabbit reticulocyte lysates in the presence of [^{35}S]methionine and of different microsomes which were present at concentrations within the linear range (Schlenstedt *et al.*, 1990). Each translation reaction was divided into two halves; one half was incubated further in the absence of protease, the other one in the presence of protease. The samples were analyzed by gel electrophoresis and fluorography. The amounts of precursor as well as of mature protein were quantified by laser densitometry of the fluorograph, and the efficiencies of processing were plotted against the concentration of octyl glucoside which was used for the pretreatment of the microsomes. Furthermore, aliquots of the different microsomes were analyzed with respect to their BiP content by Western blotting and decoration with rat monoclonal antibody 7.10 (which recognizes proteins belonging to the hsp70 family) and anti-rat IgG peroxidase conjugate. The quantitation was based on ECL Western blotting detection system (Amersham) and laser densitometry of the X-ray films.

not responsible for the observed inactivation of microsomes. However, alternative interpretations have to be considered (see Discussion).

The azido-ATP sensitive protein is involved in membrane insertion of ribonucleoparticle-dependent presecretory proteins

Is an azido-ATP sensitive site involved in the ribonucleoparticle-dependent transport of presecretory proteins? Azido-ATP modified microsomes were analyzed with respect to their ability to transport bovine preprolactin or yeast prepro-α-factor under cotranslational conditions. Membrane insertion of both precursor proteins was affected to a similar extent (Figure 4B). Furthermore, the dose–response curves for these two precursor proteins paralleled the curve which was obtained for ppcecA* (Figure 4B). Neither UV irradiation nor addition of azido-ATP alone had any significant effect on the transport activity of the microsomes (Figure 6A). The photoaffinity inactivation of microsomes by azido-ATP was not due to secondary effects of products of the photoactivation of azido-ATP, i.e. mixing of untreated microsomes with microsomes which had been photoaffinity labeled with 2- or 8-azido-ATP did not affect the transport acivity of the untreated microsomes (Figure 6B). Furthermore, ATP but not adenine was able to prevent the photoaffinity inactivation by azido-ATP (Figure 6C). Therefore, we conclude that transport of preprolactin and prepro-α-factor depend on a microsomal protein which is sensitive to photoaffinity modification with ATP analogs. As in the case of ppcecA*-transport, membrane insertion was affected. Because of the similarities between the dose–response curves (Figure 4B) we suggest that the same microsomal protein component is involved in both pathways.

Discussion

The purified precursor protein ppcecA is a substrate for transport into mammalian microsomes*

Chemically synthesized preprocecropinA is a substrate for signal peptidase and dipeptidyl aminopeptidase (Boman *et al.*, 1989). The processing product (in contrast to the precursor protein) has the antibacterial activity which has been ascribed to cecropinA (Boman and Hultmark, 1981,

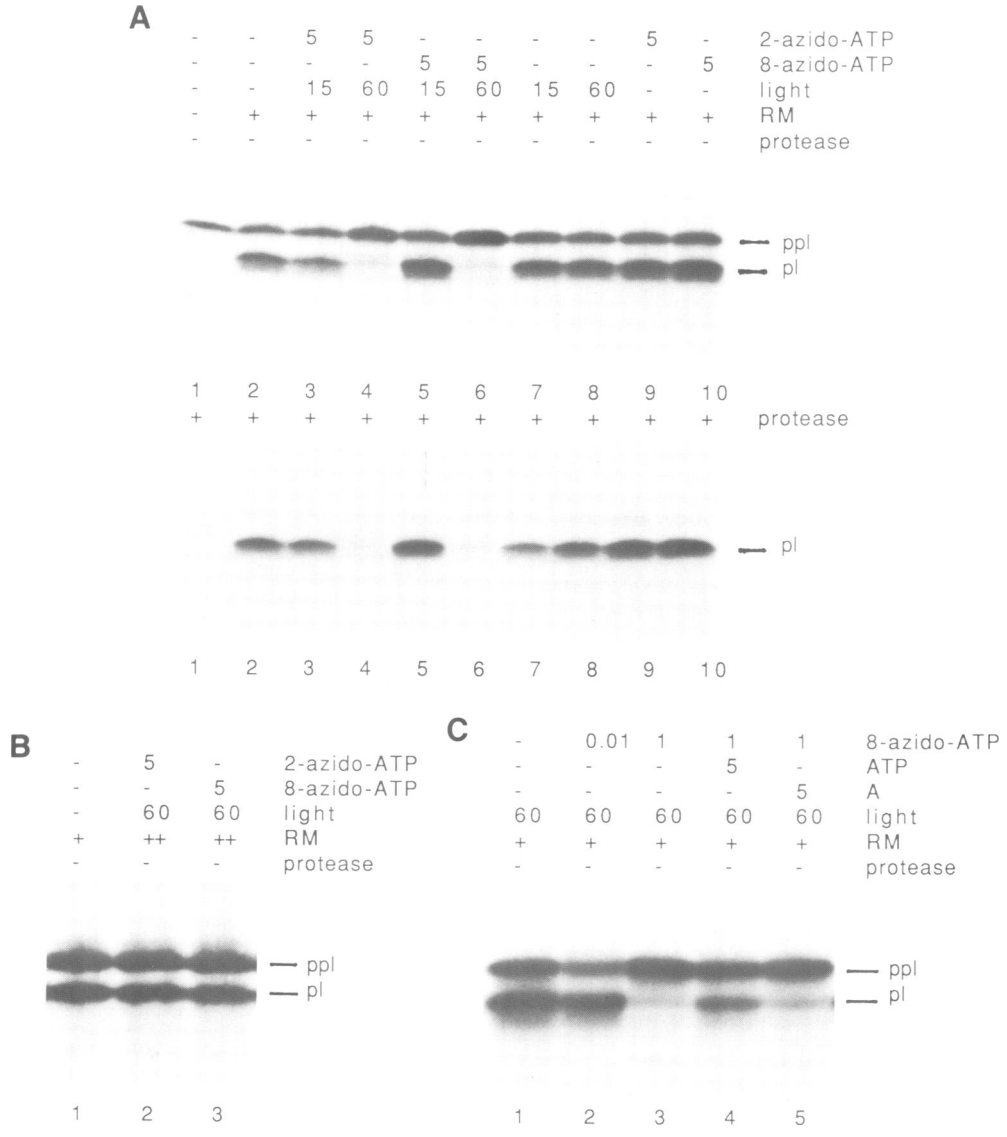

Fig. 6. Photoaffinity modification of microsomes with 8-azido-ATP leads to inactivation of the microsomes with respect to membrane insertion of preprolactin. Bovine preprolactin was synthesized in rabbit reticulocyte lysates in the presence of [^{35}S]methionine and different microsomes which were present at concentrations within the linear range (Schlenstedt *et al.*, 1990). In the experiment which is depicted in lanes 2 and 3 of B, untreated microsomes (as shown in A, lane 2) and microsomes which had been photoaffinity modified with either 2- or 8-azido-ATP (as shown in A, lanes 4 and 6, respectively) were present simultaneously (++). Each translation reaction was divided into two halves; one half was incubated further in the absence of protease, the other one in the presence of protease (data not shown in B and C). The samples were analyzed by gel electrophoresis and fluorography. The concentrations of azido-ATP, ATP and adenine (A) are given in mmol/l; the times of UV irradiation (light) are given in seconds.

1987; Boman *et al.*, 1989). Here we showed that the precursor form (in contrast to procecropinA) also has biological activity in a different respect, i.e. it can be processed by, and transported into, vesicles derived from mammalian rough endoplasmic reticulum. Solubilization in dimethyl sulfoxide had to be employed to make the synthetic ppcecA* a substrate for transport. This behavior was not unexpected since a similar requirement has been observed with other purified precursor proteins in other systems (Wiech *et al.*, 1990). Apparently, precursor proteins have to be taken through an unfolding step prior to transport in order to mimic the conditions of classical post-translational transport reactions. Furthermore, soluble cytosolic factors are not essential for transport when the denatured precursor is employed (Wiech *et al.*, 1990). Thus purified precursor proteins can be used for protein transport into mammalian microsomes.

A microsomal protein with a high affinity for ATP and a sensitivity towards photoaffinity modification with azido-ATP is involved in ribonucleoparticle-independent protein transport

Ribonucleoparticle (i.e. ribosome and SRP)-independent transport of proteins into mammalian microsomes is stimulated by cytosolic factors which include proteins belonging to the hsp70 family and which require the hydrolysis of ATP (Zimmermann *et al.*, 1988; Schlenstedt *et al.*, 1990). We have now shown that there is an additional ATP requirement involved in this transport mechanism. This ATP requirement occurs at the microsomal level as was demonstrated by two independent lines of experimentation: (i) in the absence of cytosolic factors the chemically synthesized precursor protein ppcecA* was processed by and transported into mammalian microsomes in an ATP dependent manner; (ii) photoaffinity modification of

microsomes with azido-ATP led to inactivation of the microsomes with respect to their ability to facilitate membrane insertion of this precursor protein. SRP and docking protein can be disregarded as targets of the azido-ATP inactivation with respect to ppcecA* transport since ppcecA transport does not involve SRP and docking protein under post-translational transport conditions (Schlenstedt *et al.*, 1990). Furthermore, BiP which has a high affinity for ATP (Kassenbrock and Kelly, 1988) and was shown to play a role in protein transport across the yeast microsomal membrane (Vogel *et al.*, 1990) was shown here to be an unlikely candidate for the observed azido-ATP inactivation. However, BiP cannot unequivocally be disregarded when all available data on the yeast system are taken into account. In yeast microsomes BiP seems to interact with a membrane protein, the sec63 gene product (also termed ptl1 and npl1 gene product), and defects in both BiP and sec63 protein result in a translocation defect (Toyn *et al.*, 1988; Rothblatt *et al.*, 1989; Sadler *et al.*, 1989; Vogel *et al.*, 1990). Thus, the lack of ATP or the azido-ATP photoaffinity modification of BiP (although it is not quantitative) could result in a trapping of BiP molecules at a putative mammalian equivalent of the sec63 protein and thereby lead to a translocation defect. This point will have to be resolved in further work employing a reconstituted system such as the one described by Nicchitta and Blobel (1990).

A microsomal protein with a sensitivity towards photoaffinity modification with azido-ATP is involved in ribonucleoparticle-dependent protein transport

The ribonucleoparticle-independent and the dependent pathway were found to involve a microsomal protein with the identical sensitivity towards photoaffinity modification with azido-ATP. In ribonucleoparticle-dependent protein transport BiP was observed not to be limiting under the *in vitro* transport conditions (Bulleid and Freedman, 1988; Nicchitta and Blobel, 1990; Yu *et al.*, 1990; Zimmerman and Walter, 1990; and shown here) and, therefore, appears as an unlikely candidate for the observed azido-ATP inactivation. Thus a so far unidentified nucleoside triphosphate-dependent component of the mammalian microsomes appears to be the target of the observed azido-ATP inactivation.

Membrane insertion of ribonucleoparticle-dependent precursor proteins, such as preprolactin, required only the addition of non-hydrolyzable GTP analogs (Connolly and Gilmore, 1986). However, this was under conditions where only one translocation cycle took place. In the case of ppcecA* there was a basal level of transport in the absence of exogenously added ATP (which was due to endogenous ATP, bound to protein) and multiple transport cycles occurred in the presence of exogenous ATP. Apparently, a requirement for ATP hydrolysis is observed only under conditions where multiple transport cycles occur.

We speculate that there is a microsomal protein involved in facilitating membrane insertion of both ribonucleoparticle-dependent and -independent precursor proteins and that this protein depends on ATP for its function. What could be the role of this protein? The azido-ATP sensitive protein could be directly involved in facilitating membrane insertion in a fashion similar to secA protein of the bacterial protein export apparatus. Protein secA hydrolyzes ATP in facilitating membrane insertion of precursor proteins, it is sensitive to photoaffinity modification with azido-ATP (Chen and Tai, 1986, 1987; Lill *et al.*, 1989). Alternatively, the ATP could be used by a protein kinase which may regulate the action of transport components such as the signal sequence–receptor complex (Hartmann *et al.*, 1989). The α-subunit of this complex was shown to be partially phosphorylated (Prehn *et al.*, 1990). Current experiments are directed towards the question of whether the signal sequence–receptor complex is involved in the transport of ribonucleoparticle-independent presecretory proteins.

Materials and methods

Materials

The ^{35}S-labeling reagent ^{35}SLR (*t*-butoxycarbonyl-L-[^{35}S]methionine-*N*-hydroxy succinimidyl ester) (specific radioactivity: 800 Ci/mmol), [^{35}S]methionine (specific radioactivity: 1000 Ci/mmol) and ECL Western blotting detection system were purchased from Amersham Corp., [^{14}C]formaldehyde (specific radioactivity: 50 mCi/mmol) was from New England Nuclear. 8-azido-ATP and the non-hydrolyzable ATP and GTP analogs were obtained from Sigma; dimethyl sulfoxide was from Merck. ATP and GTP were purchased from Boehringer-Mannheim; X-ray films (X-Omat AR) were from Kodak. 2-azido-ATP was synthesized as described previously (Mayinger *et al.*, 1989).

*Chemical synthesis and purification of ppcecA**

For solid phase synthesis of preprocecropinA we employed the Fmoc-strategy in a continuous flow automated synthesizer (Milligen 9050). The synthesis was carried out on 0.1 mmol Fmoc-Tyr(Bu^t)-polyamide resin using an 8-fold excess of the Fmoc amino acids. As coupling agent benzotriazolyl-oxy-tris-(dimethylamino)-phosphonium-hexafluorophosphate (BOP) was used. The following side chain protecting groups were employed: Lys(Boc), Thr(Bu^t), Glu(OtBu), Ser(Bu^t), Asp(OtBu), Arg(Pmc), and Cys(Trt). Asn and Gln were coupled as Pfp-esters. The protected peptidyl resin was treated with 20% piperidin in dimethylformamide for 12 min and then washed thoroughly with dimethylformamide. Cleavage and deprotection of the peptide resin were effected by treatment with 90% trifluoroacetic acid, 5% ethanedithiol, 2.5% thioanisol, 2.5% phenol (v/v/v/v) for 2.5 h at room temperature. The product was precipitated in ether. A portion (100 mg) of the crude material was purified by preparative HPLC on an 120-5 Nucleosil C_4 reverse phase column (20×250 mm) using an eluant of 0.1% trifluoroacetic acid in water (A) and 60% acetonitrile in water (B). The ppcecA* was eluted with a successive linear gradient of 50% B to 90% B in 40 min at a flow rate of 20 ml/min. The fractions corresponding to the purified protein were lyophilized (overall yield: 12.8 mg, i.e. 9.5%). The purified material was characterized with analytical HPLC and amino acid analysis. The purified ppcecA* was indistinguishable with respect to purity as well as electrophoretic mobility from the material which had been synthesized in Dr Bruce Merrifield's laboratory and was described previously (Boman *et al.*, 1989).

Preparation of microsomes and photoaffinity modification with azido-ATP

Rough microsomes were prepared from dog pancreas and treated with micrococcal nuclease and EDTA as described (Watts *et al.*, 1983); they were stored in RM-buffer (20 mM HEPES–KOH pH 7.5, 50 mM KCl, 2 mM Mg-acetate, 1 mM DTT, 200 mM sucrose) at an absorbance at 280 nm of 40, as measured in 2% SDS. Treatment of microsomes with *N*-ethylmaleimide (final concentration: 4 mM), trypsin (final concentration 3 μg/ml) and octyl glucoside (final concentration: 17.5–22.5 mM), respectively, was carried out according to published procedures (Zimmermann *et al.*, 1990; Schlenstedt *et al.*, 1990; Zimmerman and Walter, 1990).

Prior to photoaffinity modification the microsomes were reisolated by centrifugation for 5 min at 4°C in an Eppendorf Microfuge and resuspended in the original volume of RM-buffer minus DTT. After further centrifugation the microsomes were resuspended in the original volume of RM-buffer minus DTT. Aliquots were transferred into the wells of a microtiter plate which was placed on ice and were supplemented with different nucleotides which had been dissolved in a HEPES–KOH buffer (0.1 M, pH 7.5). After preincubation for 5 min at 0°C the aliquots were irradiated individually with a 90 W Hg lamp at 0°C following the procedure which was described by E.Winkler and M.Klingenberg (manuscript in preparation). To protect the microsomes against photooxidation and UV damage the different wells were

flushed with nitrogen prior to irradiation and a chloroform filter (1 cm) was placed between light source and sample (Mayinger *et al.*, 1989).

Radioactive labeling of ppcecA* and transport into microsomes

In order to be used in transport experiments ppcecA* was radiolabeled by reductive methylation and ^{35}SLR-labeling, respectively. For reductive methylation ppcecA* (16 μg) was solubilized in 200 μl of sodium phosphate buffer (0.1 M, pH 7) and incubated in the presence of [^{14}C]formaldhyde (10 μCi) and sodium cyanoborohydride (100 μg) for 2 h at 25°C. The specific radioactivity of the product was determined to be in the order of 1.7 Ci/mmol. For ^{35}SLR-labeling ppcecA* (16 μg) was solubilized in 3 μl of sodium borate buffer (0.1 M, pH 8.5) and incubated in the vessel which contained the dried ^{35}SLR (500 μCi) for 30 min at 0°C. The incubation was terminated by the addition of 100 μl of a glycine solution (0.2 M in sodium borate buffer). The specific radioactivity of the product was determined to be in the order of 6 Ci/mmol. ^{14}C- and ^{35}S-labeled ppcecA* were reisolated by precipitation with trichloroacetic acid (which led to the removal of the *t*-butoxycarbonyl group in the case of ^{35}S-labeled ppcecA*) in the presence of bovine serum albumin and subsequently solubilized in dimethyl sulfoxide. Note that in ^{14}C-labeled ppcecA* each lysine residue was methylated 3-fold which resulted in a positive charge in the modified amino acid side chain irrespective of the pH and that in ^{35}S-labeled ppcecA* only one lysine residue was modified per labeled precursor molecule. Since the precursors produced by both procedures had the same behaviour it is very unlikely that the modifications influenced the transport properties of the respective precursor molecules.

For transport of ppcecA* into mammalian microsomes the ppcecA* solution was diluted into transport-buffer [50 mM TEA–HCl pH 7.5, 50 mM K-acetate, 2 mM Mg-acetate, 2 mM DTT, 200 mM sucrose, 0.02% bovine serum albumin and 2 mM Mg-ATP (where indicated)]. Subsequently, microsomes were added resulting in a final concentration of dimethyl sulfoxide of 1%, and the samples were incubated for 15 or 30 min at 37°C. Control experiments showed that the microsomes can tolerate dimethyl sulfoxide to a final concentration of 2% without any loss of transport activity. The concentration of ppcecA* in transport reactions was between 0.1 μM ([^{35}SLR]ppcecA*) and 1 μM ([^{14}C]ppcecA*).

Analytical procedures

Sequestration assays were performed for 60 min at 0°C in 80 mM sucrose and proteinase K plus trypsin (final concentrations: 50 μg/ml); where indicated Triton X-100 was added at a final concentration of 0.5%. The minus-protease controls received water instead of protease. Proteolysis was stopped by addition of phenylmethylsulfonyl fluoride (final concentration: 10 mM) and further incubation for 5 min at 0°C. Finally, samples were boiled in sample buffer (Laemmli, 1970) and subjected to electrophoresis in high Tris–urea SDS–polyacrylamide gels (Schlenstedt *et al.*, 1990). For fluorography, the gels were treated with 1 M sodium salicylate (Chamberlain, 1979), dried and exposed to X-ray films at −80°C. Densitometric analysis of X-ray films was performed with an LKB Ultrascan XL laser densitometer.

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