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The membrane proteins TRAMp and sec61αp may be involved in post-translational transport of presecretory proteins into mammalian microsomes

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Abstract

The presecretory protein ppcecDHFR, a hybrid between preprocecropinA and dihydrofolate reductase, is transported into mammalian microsomes post-translationally, i.e. independent of ribosome and signal recognition particle. Here, the involvement of microsomal proteins in ribonucleoparticle-independent transport of ppcecDHFR was analyzed by transport into trypsin-pretreated microsomes and by transport of a truncated version of ppcecDHFR and subsequent chemical cross-linking. We observed that post-translational transport of ppcecDHFR can occur into microsomes which had been pretreated with trypsin (final concentration, 100 μg/ml) and that of the known transport components only TRAMp and sec61αp are still present under these conditions. Furthermore, we found that the truncated ppcecDHFR, ppcecDHFR-98mer', can be cross-linked to 36 kDa microsomal membrane proteins during post-translational transport. Therefore, the two microsomal membrane proteins with molecular masses of about 36 kDa, TRAMp and sec61αp, appear to be involved in the post-translational transport of ppcecDHFR and ppcecDHFR-98mer.

Key words: Mammalian microsome; Post-translational protein transport; Chemical cross-linking

1. Introduction

The initial step in the biogenesis of most eukaryotic secretory proteins is their transport into the lumen of the endoplasmic reticulum [1–3]. Membrane transport of presecretory proteins can be divided into the following stages: (i) specific association of the proteins with the membrane, (ii) membrane insertion, and (iii) complete transfer across the membrane. During the first stage, specificity is facilitated by a characteristic amino-terminal signal peptide in the precursor proteins and by soluble and/or membrane-bound signal peptide binding proteins. The second and third stage are mediated by a translocase in the microsomal membrane which comprises proteins TRAMp (mp39), sec61αp (P37, imp34), sec61βp and sec61γp [4–12].

There are two classes of precursor proteins with respect to their mechanism of transport into mammalian microsomes [2]. Transport of precursor proteins with more than 70 amino acid residues depends on two ribonucleoparticles, the ribosome and the signal recognition particle (SRP), as well as on their receptors on the microsomal surface (ribosome- and SRP receptor, respectively) and involves the hydrolysis of GTP. The other class typically consists of precursor proteins with less than 70 amino acid residues and is transported independently of the ribonucleoparticles and their receptors. Instead, a cytosolic molecular chaperone, i.e. Hsc70, and the hydrolysis of ATP are required [13–15]. Both, ribonucleoparticle-dependent as well as -independent precursor proteins are inserted into the membrane under participation of microsomal proteins which are sensitive to *N*-ethylmaleimide (NEM) treatment [11,16,17] and to photoaffinity labeling with azido-ATP [18–29], respectively. This suggests that the two mechanisms converge at the level of membrane insertion.

We have studied the transport of a synthetic precursor protein (ppcecDHFR, 252 amino acid residues), a hybrid between the presecretory protein preprocecropinA (ppcecA, 64 amino acid residues) and the cytosolic protein dihydrofolate reductase (DHFR), into dog pancreas microsomes [21,22]. Transport of this precursor could proceed under post-translational conditions, i.e. independently of ribosome, SRP, and the α-subunit of SRP receptor (docking protein), and was signal peptide- and ATP-dependent.

Here, the involvement of microsomal proteins in ribonucleoparticle-independent transport of ppcecDHFR was further analyzed by transport into trypsin-pretreated microsomes and by transport of a truncated version of ppcecDHFR (ppcecDHFR-98mer) and subsequent chemical cross-linking.

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2. Materials and methods

2.1. Materials

[^{35}S]Methionine (1,000 Ci/mmol) and ECL Western blotting reagent were obtained from Amersham. *Eco*RI, *Pvu*II, *Sca*I, RNase A, proteinase K, SP6 polymerase and ATP were purchased from Boehringer Mannheim. Cycloheximide, potato apyrase (grade VIII), puromycin and the non-hydrolyzable GTP-analog were obtained from Sigma. X-ray films (X-Omat AR) were from Kodak. The homobifunctional cross-linking reagent, dithiobis(succinimidylpropionate) (DSP), and the heterobifunctional cross-linking reagent, succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), were obtained from Pierce Chemical Co. Phenylmethylsulphonyl fluoride was purchased from Merck.

2.2. In vitro translation and transport

Dog pancreas microsomes were isolated and treated with micrococcal nuclease and EDTA as described [21]. Treatment of microsomes with trypsin, NEM (final concentration 4 mM) and mock-treatment, respectively, were carried out according to published procedures [17,21].

Plasmid pCA2 containing the ppcecDHFR coding region downstream of the SP6 promoter was linearized with *Eco*RI and transcribed with SP6 polymerase [21]. Translation in rabbit reticulocyte lysate in the presence of in vitro transcript and [^{35}S]methionine (final concentration 1.4 mCi/ml) and post-translational transport were carried out as described previously [21]. For post-translational transport of truncated ppcecDHFR, plasmid pCA2 was linearized with *Sca*I and transcribed with SP6 polymerase. The transcript which coded for the 98 amino-terminal amino acid residues of ppcecDHFR and lacked a termination codon was used to program a reticulocyte lysate. After translation for 20 min at 30°C, puromycin (final concentration 1.25 mM) was added and the incubation was continued for 5 min. Then, dog pancreas microsomes were added and a subsequent incubation was carried out for 10 min at 30°C.

Plasmid pB4 which contains the preprolactin (ppl) coding for the region downstream of the SP6 promoter was linearized with *Pvu*II and transcribed with SP6 polymerase as described [17]. The transcript which coded for the 86 amino-terminal amino acid residues of ppl and lacked a termination codon was used to program a reticulocyte lysate.

2.3. Chemical cross-linking

Cross-linking was carried out after re-isolation of microsomes with either dithiobis DSP or SMCC as cross-linking reagents. Typically, transport reactions were diluted with an equal volume of XL-buffer (50 mM HEPES-KOH, pH 7.5, 50 mM K-acetate, 2 mM Mg-acetate, 200 mM sucrose) and microsomes were subsequently re-isolated by centrifugation (125.000 × *g* for 10 min at 2°C). The microsomal pellet was resuspended in 25 μl of XL-buffer and supplemented with 0.25 μl of the cross-linking stock solution (5 mg/ml DMSO). Cross-linking was carried out for 20 min at 0°C and terminated by the addition of SDS-sample buffer.

2.4. Analytical procedures

Sequestration assays were performed for 60 min at 0°C in 80 mM sucrose and proteinase K (50 μg/ml). The controls received water instead of protease. Proteolysis was stopped by addition of phenylmethylsulphonyl fluoride (10 mM) and further incubation for 5 min at 0°C. Samples were subjected to electrophoresis in high Tris/urea/SDS-polyacrylamide gels (SDS-PAGE) [21]. In the case of DSP-cross-linked samples, β-mercaptoethanol was omitted from the SDS-sample buffer. The gels were treated with 1 M sodium salicylate, dried and exposed to X-ray films. Densitometric analysis was performed with a LKB Ultrascan XL laser densitometer.

3. Results

3.1. Transport of ppcecDHFR into trypsin-pretreated microsomes

It has previously been shown that ribosome, SRP, and the α-subunit of SRP receptor are not involved in post-translational transport of ppcecDHFR into dog pancreas microsomes [21]. In order to gain further insight into which microsomal components are involved in the transport of ppcecDHFR under these conditions, microsomes were pretreated with increasing concentrations of trypsin and analyzed with respect to their content of certain microsomal membrane proteins as well as with respect to their transport activity (Table 1).

After synthesis in reticulocyte lysate, transport of ppcecDHFR occurred with a higher than 50% efficiency (i.e. as compared to untreated microsomes) even after pretreatment of microsomes with trypsin at a final concentration of 100 μg/ml and subsequent inhibition of trypsin. The 23 kDa-subunit of signal peptidase as well as the signal peptidase activity were more or less unaffected by this treatment, indicating that the microsomes were almost intact. It can be concluded that, under the experimental conditions used, the α- and β-subunit of the SRP receptor [23] and the putative ribosome receptor ERp180 [24] are not necessary for transport of ppcecDHFR. These observations are of particular relevance since these three proteins were described to have an affinity for nucleoside triphosphates [19,25]. Further-

Table 1
The role of microsomal proteins in the transport of ppcecDHFR under post-translational conditions

	Residual amount of intact protein and of activity, respectively (% of control)					
Trypsin (μg/ml)	0.3	1.0	3.0	10	30	100
SRP receptor α-subunit	80	5	0	0	0	0
SRP receptor β-subunit	89	80	47	29	0	0
Ribosome receptor (ERp180)	n.d.	1	3	0	0	0
TRAMp (mp39)	50	0	0	0	0	0
sec61p (P37)	98	116	100	81	64	24
SPase 23 kDa-subunit	86	103	108	93	114	111
SPase activity	90	85	81	65	n.d.	63
Transport activity	92	118	130	94	89	58

Dog pancreas microsomes were subjected to trypsin treatment at the indicated final concentrations of TPCK-trypsin as described previously [21]. The trypsin-pretreated microsomes were analyzed with respect to their content of SRP receptor α- and β-subunit, ERp180, TRAMp, sec61αp, and signal peptidase (SPase) 23 kDa subunit by Western blotting and decoration with specific antibodies and an appropriate second antibody–peroxidase conjugate. Detection of antibody was performed by coupled chemiluminescence (ECL) and quantified by laser densitometry of the X-ray films. Furthermore, these microsomes were characterized with respect to their abilities to process ppcecDHFR under different conditions: (i) signal peptidase assays were carried out in 0.25% Triton X-100 and quantified (SPase activity) as described [21]; (ii) translation of ppcecDHFR in the presence of [^{35}S]methionine was carried out in the rabbit reticulocyte lysate for 15 min at 37°C. Translation was terminated by addition of cycloheximide and RNase A. Aliquots were further incubated for 30 min at 37°C in the presence of various microsomes. Each reaction was divided into two halves and incubated further in the presence or absence of protease. The samples were analyzed by SDS-PAGE and fluorography. The efficiencies of processing and sequestration, respectively, were quantified by laser densitometry of the autoradiographs. The sequestration efficiencies are shown (transport activity).

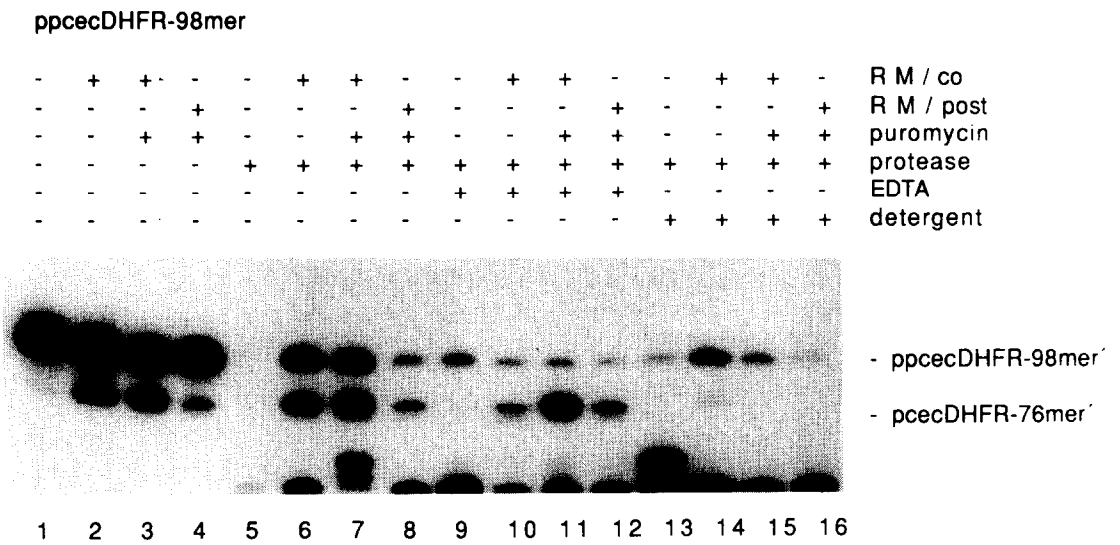


Fig. 1. Transport of truncated ppcecDHFR, ppcecDHFR-98mer, into microsomes. Plasmid pCA2 was linearized within the coding region and transcribed with SP6 polymerase. After translation in rabbit reticulocyte lysate for 20 min at 30°C, the translation mixture was divided into four aliquots. These aliquots were supplemented with water (lane 1), microsomes (RM/co) (lanes 2 and 3) or puromycin (final concentration 1.25 mM) (lane 4). Following a subsequent incubation of samples 2–4 for 5 min, puromycin or microsomes (RM/post) were added to samples 3 and 4, respectively, and the transport reaction was carried out for 5 (lane 3) or 10 min (lane 4) at 30°C. Then, all samples were divided into four aliqouts. One aliquot was incubated in the absence of protease (lanes 1–4), a second aliquot in the presence of protease (lanes 5–8), a third one in the presence of protease plus EDTA (final concentration 5 mM) to release the ribosomes from the membrane (lanes 9–12), and a fourth aliquot in the presence of protease plus Triton X-100 (final concentration 2.5%) (lanes 13–16). All samples were analyzed by SDS-PAGE [21] and fluorography.

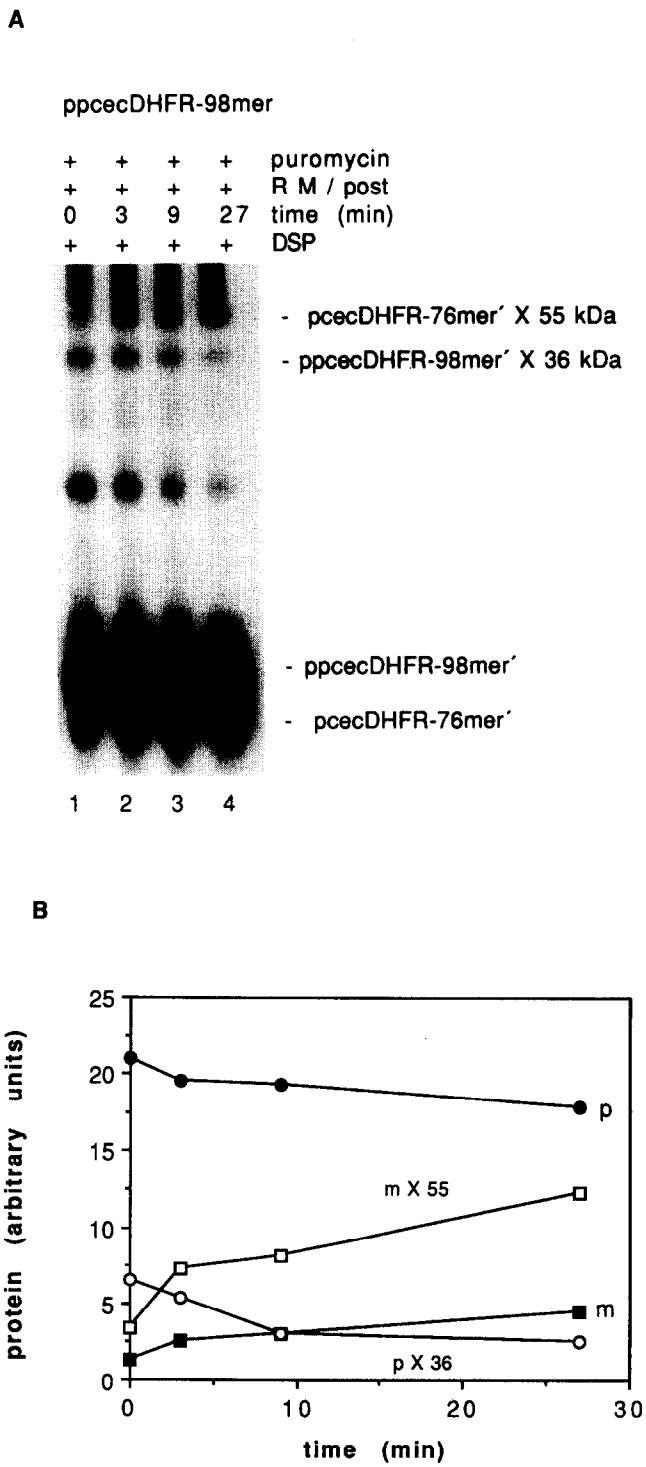
more, one could conclude from these data that intact TRAMp [4,6,12] is not essential for transport of ppcecDHFR. However, in this particular case only a small C-terminal cytosolic domain (recognized by the antibody, raised against a C-terminal peptide) may have been destroyed by trypsin and this domain is not required for TRAMp function [4]. On the basis of its relative protease-resistance, sec61αp [5,7–9,11] may be involved in transport of ppcecDHFR.

3.2. *ATP-depletion or NEM pretreatment of microsomes inhibit association of ppcecDHFR-98mer with the microsomal membrane proteins, TRAMp and sec61αp*

It has previously been shown that nascent chains (i.e. peptidyl-tRNAs) of ribonucleoparticle-dependent precursor proteins which are in transit into mammalian microsomes (such as a nascent preprolactin chain, termed ppl-86mer) are in close contact with the microsomal membrane proteins TRAMp (which is glycosylated) and sec61αp (which is not glycosylated) [5–12]. Therefore, we asked here whether this is true for ppcecDHFR in transit into the microsomal lumen under post-translational conditions and at what stage ATP-depletion or NEM-pretreatment of microsomes inhibit transport.

For this purpose a nascent chain which is related to ppcecDHFR (ppcecDHFR-98mer) was generated in the reticulocyte lysate and subsequently released from ribosomes, i.e. converted to the puromycin-containing derivative (ppcecDHFR-98mer') either prior to or after addition of microsomes (Fig. 1). When ppcecDHFR-98mer was incubated with microsomes in the absence of puromycin, a fraction of the ribosome-bound precursors was protected against protease by the ribosome, i.e. was protease sensitive in the presence of EDTA (ppcecDHFR-98mer' in lanes 6 and 10). It was chased to the processed and sequestered form by release from the ribosome (pcecDHFR-76mer' in lanes 7 and 11). Another fraction of the ribosome-bound precursors was processed to pcecDHFR-76mer' which was protease-sensitive in the presence of EDTA (lane 6 vs. 10). It was chased to the sequestered form by release from the ribosome (lanes 7 vs. 11). Furthermore, there was processing and sequestration when microsomes were added after release of the precursor form from the ribosome (Fig. 1, lanes 4, 8, 12, 16). Transport under these ribosome-independent conditions (post-translational conditions) occurred even after pre-trypsinization of microsomes (data not shown), i.e. in the absence of docking protein (Table 1). When ppl-86mer was incubated with microsomes in the absence of puromycin, ribosome-bound precursor was protected against protease by the ribosome, i.e. protease-sensitive in the presence of EDTA, and was chased to the processed and sequestered mature form by release from the ribosome (data not shown). However, there was no processing and sequestration when microsomes were added after release of the precursor form from the ribosome, which demonstrated the quantitative effect of puromycin (data not shown). Thus, in contrast to ppl-86mer and full-length ppl, ppcecDHFR-98mer can be transported

Fig. 2. Chemical cross-linking of ppcecDHFR-98mer to 36 kDa microsomal membrane proteins. The truncated presecretory protein ppcecDHFR-98mer was synthesized in vitro as described in the legend to Fig. 1. After puromycin-treatment, microsomes were added and the mixture was divided into four aliquots on ice. The transport reaction was carried out for 0, 3, 9, or 27 min at 30°C. Afterwards the samples were kept on ice. After re-isolation, the microsomes were incubated with the cleavable cross-linker DSP and subsequently subjected to SDS-PAGE and fluorography (A). The precursor and mature forms, cross-linked and non-cross-linked, were quantified by laser densitometry of the autoradiographs (the arbitrary units for the cross-linked forms have to be divided by ten in order to be compared directly with the non-cross-linked forms) (B). We note that, simultaneously with processing of ppcecDHFR-98mer to pcecDHFR-76mer, a cross-linking product between pcecDHFR-76mer′ and two soluble proteins in the microsomal lumen appeared (i.e. after re-isolation of the cross-linking product (which was termed pcecDHFR-76mer′ X 55 kDa (m X 55 in B)) from the gel and subsequent SDS-PAGE in the presence of β-mercaptoethanol, pcecDHFR-76mer′ was detected).



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into mammalian microsomes post-translationally, i.e. it behaves like full-length ppcecDHFR [21].

Under these post-translational transport conditions ppcecDHFR-98mer′ could be cross-linked to 36 kDa microsomal protein(s) (Fig. 2A). We note that (i) the appearance of the cross-linking product, termed ppcecDHFR-98mer′ X 36 kDa (p X 36 in Fig. 2B) on the basis of its co-migration with marker proteins, depended on the presence of transport-competent microsomes (Fig. 5) as well as on cross-linker (Figs. 3 and 5), and that (ii) ppcecDHFR-98mer′ was detected after re-isolation of the cross-linking product from the gel and subsequent SDS-PAGE in the presence of β-mercaptoethanol. This cross-linking product was also observed under co-translational transport conditions, such as described in Fig. 1, lanes 2, 6, 10, 14 (data not shown). Under post-translational conditions it was observed only transiently, i.e. it was detected shortly after addition of microsomes and disappeared with increasing incubation time, concomitant with the appearance of sequestered pcecDHFR-76mer′ (Fig. 2A and B). Thus, ppcecDHFR-98mer, bound to the microsomal 36 kDa proteins, represents a transport intermediate. The cross-linking product resembled that of ppl-86mer (i.e. in the absence of puromycin, data not shown) in several respects: (i) it behaved as a membrane protein, i.e. it could not be extracted by alkaline treatment of the membrane (data not shown); (ii) it was partially retained on concanavalin A–agarose (data not shown), suggesting a glycoprotein; and (iii) it could be partially immunoprecipitated by antibodies which were directed against TRAMp and sec61αp, respectively (data not shown). The cross-linking product of ppcecDHFR-98mer′ was visible both at 0°C (where transport did not occur) and at 30°C (where transport occurred) (Fig. 3, left panel). On the basis of these characteristics, the microsomal cross-linking partners of ppcecDHFR-98mer′ appear to be identical to the constituents of the translocase, TRAMp (glycoprotein) and sec61αp (non-glycoprotein).

Again, as in the case of cross-linking of ppl-86mer (Fig. 4), the cross-linking product of ppcecDHFR-98mer′ was not detected after apyrase treatment (Fig. 3, left panel) or NEM-pretreatment of microsomes (Fig. 5), i.e. under non-transport conditions. However, the targets of nucleoside triphosphate depletion have to be dif-

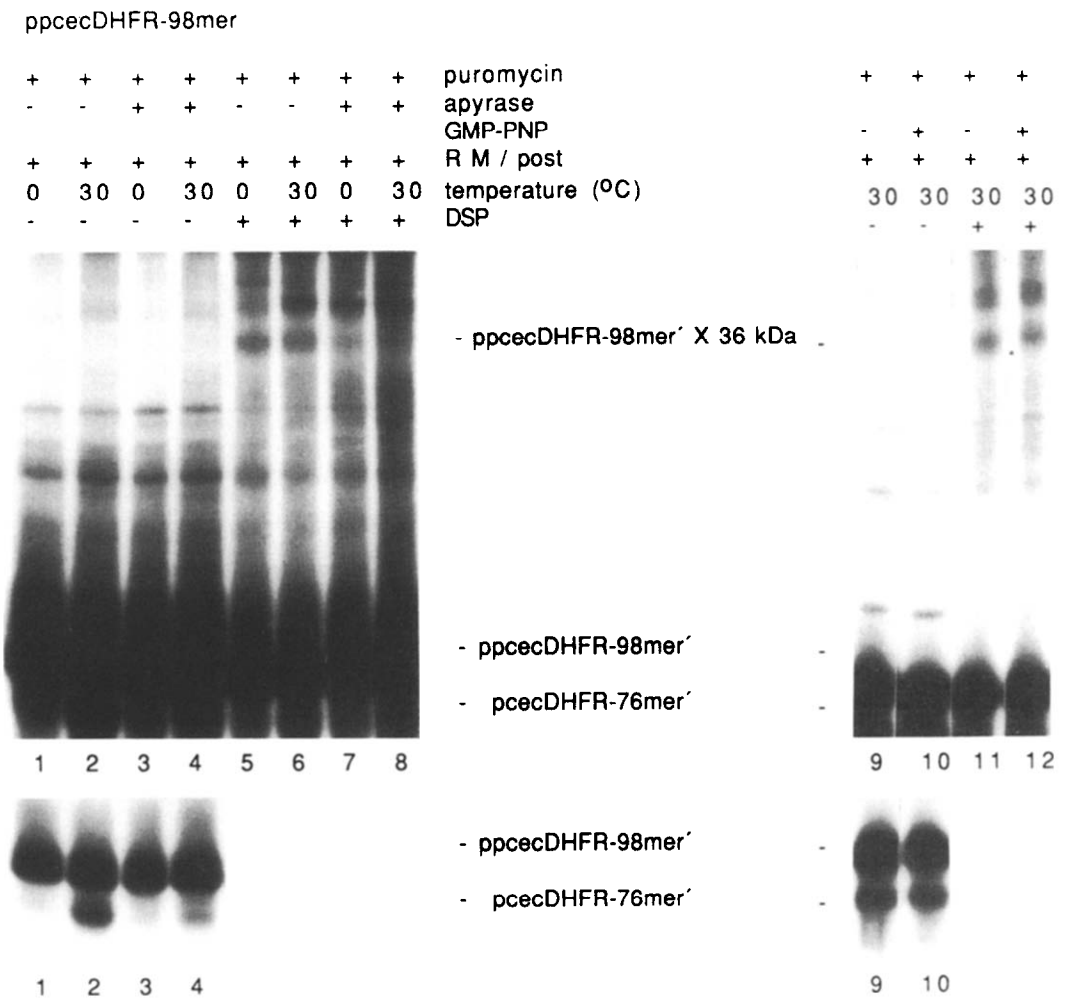

Fig. 3. Chemical cross-linking of ppcecDHFR-98mer to 36 kDa microsomal membrane proteins is prevented by apyrase pretreatment but not by addition of GMP-PNP. The truncated presecretory protein ppcecDHFR-98mer was synthesized in vitro and the translation mixture was divided into six aliqouts and supplemented with puromycin plus either water, or apyrase (final concentration 100 U/ml), or GMP-PNP (final concentration 1.25 mM). Following incubation for 5 min, microsomes were added and the transport reaction was carried out for 10 min at 0 or 30°C as indicated. After re-isolation, the microsomes were incubated in the absence or presence of DSP and subsequently subjected to SDS-PAGE and fluorography. The lower part of the figure represents a short exposure (15 h) of the upper parts area of interest (150 h, left panel; 75 hrs, right panel).

ferent for both presecretory proteins. In the case of ppl-86mer, the effects are obviously due to the inactivation of the GTP-dependent SRP receptor α-subunit. However, as shown in Table 1, SRP receptor α-subunit is not involved in the transport of ppcecDHFR-98mer'. This view is further substantiated by the experiments in which the non-hydrolyzable GTP-analog guanosine 5'-(β,γ-imido)-triphosphate (GMP-PNP) was employed. In the case of ppl-86mer, as expected [26], (i) a reduction of transport, (ii) a decrease in the extent of cross-linking to the 36 kDa proteins, and (iii) a concomitant appearance of a cross-linking product between ppl-86mer and the 54 kDa subunit of SRP were observed (Fig. 4). However, in the case of ppcecDHFR-98mer' none of these effects were observed (Fig. 3, right panel). On the other hand, the targets of NEM treatment of microsomes may be identical for ppl-86mer and ppcecDHFR-98mer' [11]. With respect to the ATP requirement, we cannot distinguish between two possibilities: either ATP is used by the microsomal protein which is sensitive towards azido-ATP photoaffinity modification, or it is used by this microsomal protein and Hsc70 which may be associated with ppcecDHFR-98mer'.

4. Discussion

The results obtained can be interpreted as follows. Since post-translational transport of ppcecDHFR can occur into trypsin-pretreated microsomes (final concentration 100 μg/ml) we conclude that of the known transport components only TRAMp and sec61αp appear to be candidates which could be involved in transport of ppcecDHFR. From the fact that truncated ppcecDHFR, ppcecDHFR-98mer', can be cross-linked to 36 kDa microsomal membrane proteins during post-translational

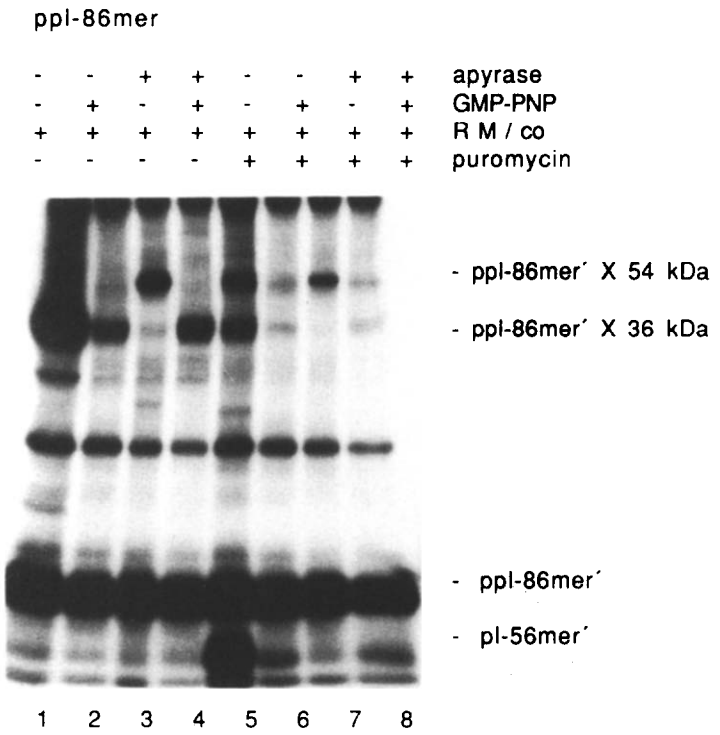

Fig. 4. Chemical cross-linking of ppl-86mer to 36 kDa microsomal membrane proteins is prevented by apyrase pretreatment and by addition of GMP-PNP. The truncated presecretory protein ppl-86mer was synthesized in vitro (see section 2) and the translation mixture was divided into four aliqouts and supplemented with water, apyrase (final concentration 100 U/ml) and GMP-PNP (final concentration 1.25 mM), respectively, as indicated. Following incubation for 5 min, microsomes were added and the targeting reaction was carried out for 5 min at 30°C. Each targeting reaction was divided into two aliqouts, supplemented with water or puromycin as indicated, and the transport reaction was carried out for 5 min at 30°C. After re-isolation, the microsomes were incubated in the presence of SMCC and subsequently subjected to SDS-PAGE and fluorography. We note that simultaneously with processing of ppl-86mer to pl-56mer, a cross-linking product between pl-56mer and two soluble proteins in the microsomal lumen appeared (lane 5; Klappa and Zimmermann, manuscript in preparation).

transport, one can conclude that transport of ppcecDHFR-98mer may actually involve both microsomal membrane proteins with molecular masses of about 36 kDa, i.e. TRAMp and sec61αp. Since the post-translational transport of both, ppcecDHFR and ppcecDHFR-98mer' (i) occurs after release from the ribosome, (ii) does not involve the GTP-dependent SRP/SRP receptor system, (iii) is inhibited by ATP-depletion, and (iv) is inhibited by NEM-pretreatment of microsomes, it seems clear that the full-length precursor and its truncated derivative are transported in a similar or even identical fashion.

In summary, we conclude that post-translational transport of ppcecDHFR occurs via the translocase which is involved in ribonucleoparticle-dependent transport, and that a microsomal protein which is sensitive towards treatment with *N*-ethylmaleimide is involved in mediating association of the presecretory protein in transit with the components of translocase, i.e. TRAMp and sec61αp. A similar NEM sensitivity had been reported for preprolactin [11]. Furthermore, one can conclude that a cytosolic and/or microsomal protein which depend(s) on ATP is involved in mediating association of ppcecDHFR in transit with components of the translocase. Again, a similar conclusion was reached previously for preprolactin after photoaffinity modification of microsomes with azido-ATP [20]. Furthermore, this situation is very similar to what has been observed for post-translational transport of proteins into yeast microsomes [27,28].

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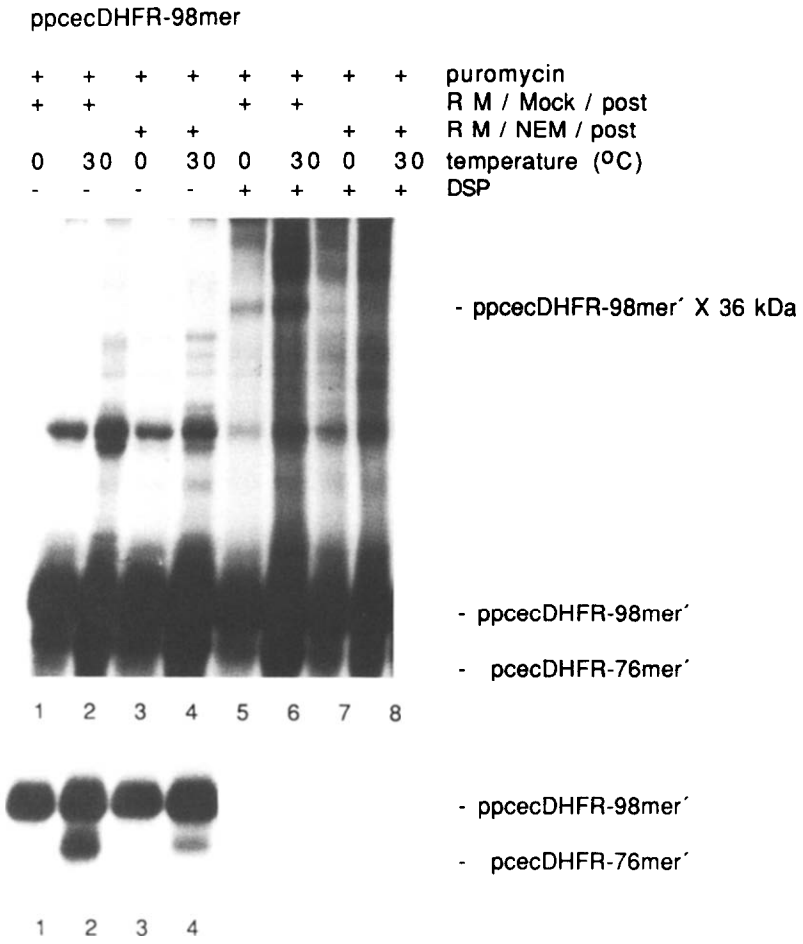

Fig. 5. Chemical cross-linking of ppcecDHFR-98mer to 36 kDa microsomal membrane proteins is prevented by pretreatment of microsomes with NEM. The truncated presecretory protein ppcecDHFR-98mer was synthesized in vitro as described in the legend to Fig. 1 and the translation mixture was divided into four aliqouts. Following incubation with puromycin for 5 min, microsomes which had been mock-treated or pretreated with *N*-ethylmaleimide (final concentration 4 mM) were added and the transport reaction was carried out for 10 min at 0 or 30°C as indicated. After re-isolation, the microsomes were incubated in the absence or presence of DSP and subsequently subjected to SDS-PAGE and fluorography. The lower part of the figure represents a short exposure (15 h) of the upper parts area of interest (75 h).

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