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Photoaffinity labeling of dog pancreas microsomes with 8-azido-ATP inhibits association of nascent preprolactin with the signal sequence receptor complex

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Transport of bovine preprolactin into dog pancreas microsomes involves a microsomal protein which is sensitive to photoaffinity labeling with azido-ATP and which is distinct from the ATP-binding protein, immunoglobulin heavy chain binding protein. Here we addressed the question of what stage of preprolactin transport is affected. Thus a nascent presecretory protein which is related to preprolactin, termed ppl-86mer, was employed. Here we show that the nascent preprolactin did not become associated with the α-subunit of the signal sequence receptor complex after photoaffinity labeling of microsomes with azido-ATP. Therefore, we conclude that the microsomal protein which is sensitive to photoaffinity labeling with azido-ATP acts prior to the signal sequence receptor complex.

Protein transport; Endoplasmic reticulum

1. INTRODUCTION

The decisive initial step in the biogenesis of most eukaryotic secretory proteins is their transport into the lumen of the endoplasmic reticulum. The discriminatory mechanism which leads to transport specificity typically involves a characteristic amino-terminal signal peptide in the precursor proteins, a putative signal peptide receptor on the cytosolic phase and signal peptidase on the luminal phase of the microsomal membrane [1–4]. There are two classes of signal peptide-containing precursor proteins with respect to their mechanism of transport into mammalian microsomes [4–8]: one class of presecretory proteins typically consists of precursor proteins which contain more than 70 amino acid residues and involve the two ribonucleoparticles, ribosome and signal recognition particle, and their respective receptors on the microsomal surface, ribosome receptor and docking protein (SRP-receptor). The other class typically consists of precursor proteins which comprise less than 70 amino acid residues and do not depend on the ribonucleoparticles and their receptors, but a cytosolic ATPase [9,10]. Ribonucleoparticle-independent precursor proteins are transported as completed polypeptide chains whereas ribonucleoparticle-dependent precursor proteins can only be transported in the absence of ongoing protein synthesis when they are artificially kept on the ribosome [7,8,11,12].

The following order of events has been established for the ribonucleoparticle-dependent transport [3]: SRP binds to signal peptides of nascent chains of large presecretory proteins as soon as they emerge from the ribosome. This interaction leads to a subsequent SRP/ribosome interaction. After interaction of SRP with docking protein the signal peptide is handed over to the so-called signal sequence receptor complex [13,14], and the ribosome binds to the ribosome receptor [15]. We showed previously that transport of ribonucleoparticle-dependent presecretory proteins, such as preprolactin, involves a microsomal protein which is sensitive to photoaffinity labeling with 8-azido-ATP (Klappa, P., Mayinger, P., Pipkorn, R., Zimmermann, M. and Zimmermann, R., submitted for publication). The microsomal ATP-binding protein immunoglobulin heavy chain binding protein (BiP) was disregarded as being responsible for the observed azido-ATP effect. Here we asked at what stage ribonucleoparticle-dependent protein transport is affected.

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2. MATERIALS AND METHODS

2.1. *Materials*

[^{35}S]Methionine was purchased from Amersham. 8-Azido-ATP

and puromycin were obtained from Sigma. The cross-linking reagent *m*-maleimidobenzoyl-*N*-hydroxysulphosuccinimide ester (sulfo-MBS) was from Pierce. Proteinase K, SP6 polymerase, and the restriction enzyme *Pvu*II were purchased from Boehringer Mannheim. X-Ray films (X-Omat AR) were from Kodak.

2.2. *Preparation of microsomes and photoaffinity labeling with azido-ATP*

Rough microsomes were prepared from dog pancreas and treated with micrococcal nuclease and EDTA as described [16]; 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). Treatment of microsomes with trypsin (final concentration: 3 μg/ml) or elastase (final concentration: 0.75 μg/ml) was carried out according to published procedures [17]. Photoaffinity labeling of microsomes with 8-azido-ATP was carried out as described previously (Klappa, P., Mayinger, P., Pipkorn, R., Zimmermann, M. and Zimmermann, R., submitted for publication); microsomes were transferred into the wells of a microtiter plate which was placed on ice and were supplemented with ATP or 8-azido-ATP which had been dissolved in a HEPES-KOH buffer. After preincubation for 5 min at 0°C the aliquots were irradiated individually with a 90 W Hg-lamp at 0°C. 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 [18]. We showed previously that the photoaffinity inactivation of microsomes by azido-ATP was not due to any secondary effects of products of the photoactivation of azido-ATP and that ATP but not adenine was able to prevent the photoaffinity inactivation by azido-ATP (Klappa, P., Mayinger, P., Pipkorn, R., Zimmermann, M. and Zimmermann, R., submitted for publication).

2.3. *Other methods*

Plasmid pSPB4 [19] was linearized with *Pvu*II and transcribed with SP6 polymerase [20]. Translation in nuclease-treated rabbit reticulocyte lysates in the presence of [^{35}S]methionine was performed at 30°C as described [8,15]. Sequestration assays were performed for 60 min at 0°C in 80 mM sucrose and proteinase K (final concentrations: 175 μg/ml); where indicated Triton X-100 was added at a final concentration of 0.5%. Proteolysis was stopped by addition of phenylmethylsulphonyl fluoride (final concentration: 10 mM) and further incubation for 5 min at 0°C. Chemical cross-linking was carried out according to published procedures [21]: prior to cross-linking microsomes were reisolated under high salt conditions. The cross-linking products were subjected to carbonate extraction. Samples were boiled in sample buffer [22] and subjected to electrophoresis in high Tris/urea/SDS-polyacrylamide gels [8]. For fluorography, the gels were treated with 1 M sodium salicylate [23], dried and exposed to X-ray films.

3. RESULTS

3.1. *Signal sequence receptor complex-associated ppl-86mer can be translocated into mammalian microsomes after termination of protein synthesis with puromycin*

It was shown previously that translation of a truncated mRNA, related to bovine preprolactin mRNA, in the presence of dog pancreas microsomes leads to the SRP and docking protein-dependent binding of ribosomes which contain the nascent preprolactin chain, termed ppl-86mer [12]. Furthermore, it was shown that the nascent chain becomes associated with the signal sequence receptor complex under these conditions [13] and subsequently can be translocated across the microsomal membrane, i.e. can be converted to sequestered pl-56mer, by release of the nascent chain from the ribosome with puromycin [12].

In order to set the stage for the subsequent work, experiments were carried out to reproduce these findings. The results demonstrate that the ppl-86mer bound to microsomes in a fashion where the bound precursor was resistant to protease in the absence as well as in the presence of detergent and where the bound precursor could be chemically cross-linked to the α-subunit (34 kDa-subunit) of the signal sequence receptor complex (Fig. 1A and B). Furthermore, the results show that the ppl-86mer was chased to pl-56mer upon addition of puromycin in a fashion where the mature form was resistant to protease in the absence of detergent but sensitive to protease in the presence of detergent (i.e. where the pl-56mer was sequestered) and where the mature form could not be cross-linked to the signal sequence receptor complex (Fig. 1A and B). Control experiments established that the observed cross-link was correct [13,24–26]: the cross-linking product was of the right size as well as resistant to carbonate extraction (Fig. 1B) and was trypsin resistant as well as binding to concanavalin A (data not shown).

In order to demonstrate the SRP and docking protein-dependence, pretrypsinized microsomes, which were depleted of a functional docking protein, were analyzed. The results demonstrate that productive binding of the ppl-86mer was prevented by pretrypsinization of microsomes. The trypsin pretreatment prevented protease resistant binding (Fig. 2A), cross-linking to the signal sequence receptor complex (Fig. 1B), and chase to sequestered pl-56mer (Fig. 2A).

3.2. *An azido-ATP sensitive protein of the microsomes is involved in productive binding of the ppl-86mer*

The observed inhibition of preprolactin-transport by photoaffinity labeling of microsomes with azido-ATP (Klappa, P., Mayinger, P., Pipkorn, R., Zimmermann, M. and Zimmermann, R., submitted for publication) allowed us to ask at what stage ribonucleoparticle-dependent transport of presecretory proteins is affected. The same photoaffinity labeled microsomes which were used previously for preprolactin transport were analyzed with respect to their ability to transport the ppl-86mer. The results demonstrate that productive binding was prevented by photoaffinity labeling of microsomes with 8-azido-ATP. Photoaffinity labeling with 8-azido-ATP, but not UV-irradiation or incubation with azido-ATP, prevented protease resistant binding (Fig. 2B), cross-linking to the signal sequence receptor complex (data not shown), and chase to sequestered pl-56mer (Fig. 2B).

If one takes into account that the α-subunit of docking protein has an affinity for ATP [27], the effect of photoaffinity labeling of microsomes with azido-ATP could be interpreted by concluding that the α-subunit of docking protein was affected under these conditions

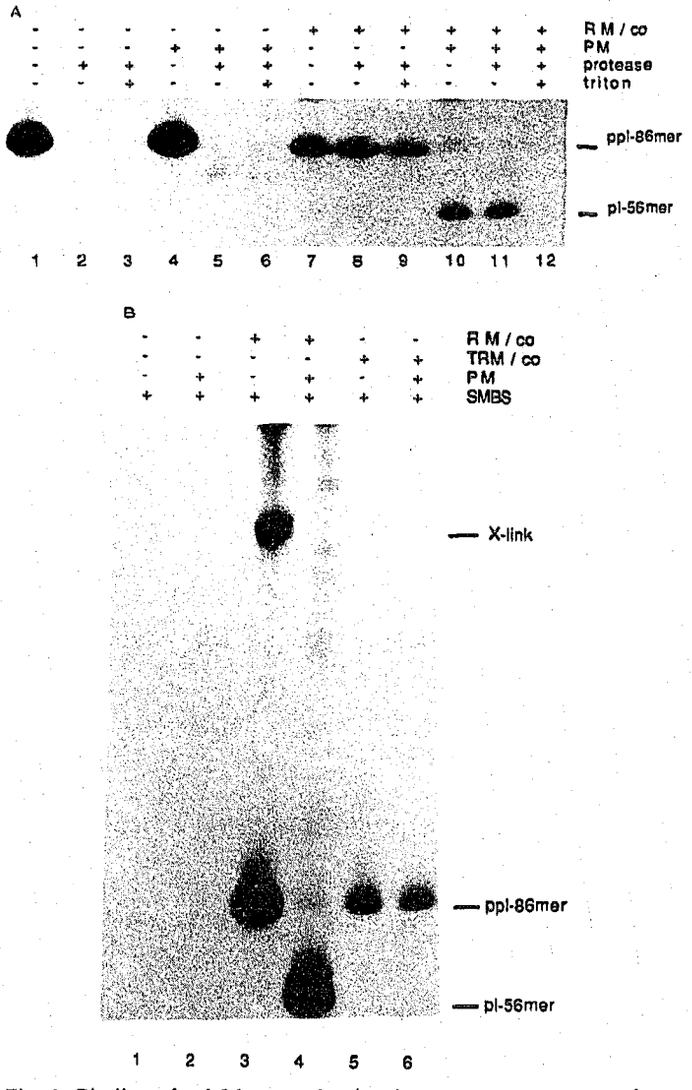

Fig. 1. Binding of ppl-86mer to the signal sequence receptor complex and subsequent transport into mammalian microsomes. The ppl-86mer was synthesized for 20 min in rabbit reticulocyte lysates in the absence of microsomes or in the presence of dog pancreas microsomes (RM) or in the presence of microsomes which had been pretreated with trypsin (TRM). After supplementation of part of the samples with puromycin (PM) and further incubation for 10 min each reaction was divided into four aliquots; one aliquot was incubated further in the absence of protease, the second one in the presence of protease, and the third one in the presence of protease plus Triton X-100 (A). The fourth aliquot was subjected to centrifugation and subsequent cross-linking with sulfo-MBS (SMBS) which was followed by carbonate extraction (B). All samples were analyzed by gel electrophoresis and fluorography.

and that, therefore, ppl-86mer did not reach the signal sequence receptor complex.

3.3. *The azido-ATP sensitive protein of the microsomes is distinct from the α-subunit of docking protein and acts prior to the signal sequence receptor complex*

Therefore, we addressed the question whether there is a target of photoaffinity labeling of microsomes with

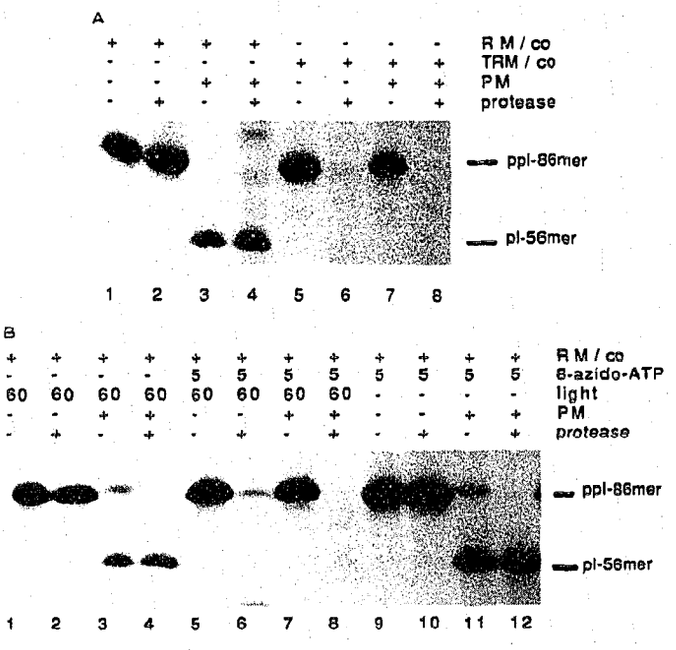

Fig. 2. Photoaffinity labeling of microsomes with 8-azido-ATP leads to inactivation of the microsomes with respect to productive binding of ppl-86mer. The ppl-86mer was synthesized for 20 min in rabbit reticulocyte lysates in the presence of microsomes which had been pretreated under various conditions. After supplementation of part of the samples with puromycin (PM) and further incubation for 10 min each 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 concentration of 8-azido-ATP is given in mmol/l; the time of UV-irradiation (light) is given in seconds (B).

azido-ATP which is distinct from the α-subunit of docking protein. In this approach trypsinized microsomes which are unable to facilitate transport of ribonucleoparticle-dependent precursor proteins, such as preprolactin, are functionally reconstituted with the cytosolic domain of the docking protein α-subunit. Specifically we asked whether the pretrypsinized microsomes are sensitive to photoaffinity labeling with azido-ATP. Furthermore, we asked at what stage preprolactin transport is affected after photoaffinity labeling of pretrypsinized microsomes with azido-ATP and subsequent reconstitution with the cytosolic domain of the α-subunit of docking protein. The results demonstrate that productive binding of the ppl-86mer was prevented by azido-ATP photoaffinity labeling of pretrypsinized microsomes when assayed in the presence of an elastase extract of microsomes. 8-Azido-ATP prevented protease resistant binding (Fig. 3A), cross-linking to the α-subunit of the signal sequence receptor complex (Fig. 3B), and chase to sequestered pl-56mer (Fig. 3B) to a comparable extent. According to quantification by laser densitometry of the fluorographs, shown in Fig. 3A and B, there was a 60% inhibition for all three events caused by the photoaffini-

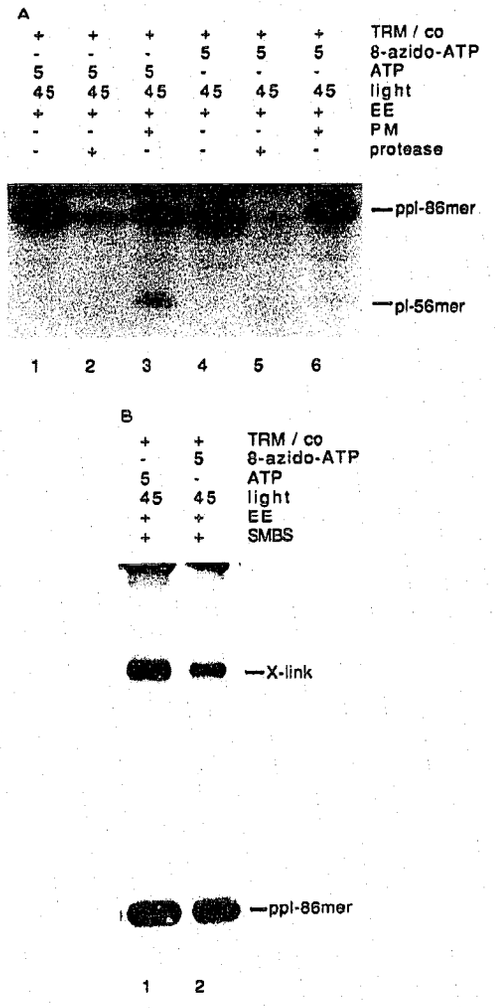

Fig. 3. Photoaffinity labeling of microsomes with 8-azido-ATP leads to inactivation of the microsomes with respect to a microsomal protein which is distinct from docking protein. The ppl-86mer was synthesized for 20 min in rabbit reticulocyte lysates in the presence of elastase extracts of microsomes (EE) and pretrypsinized microsomes (TRM) which had been irradiated in the presence of either ATP or 8-azido-ATP. After supplementation of part of the samples with puromycin (PM) and further incubation for 10 min each reaction was divided into three aliquots; one aliquot was incubated further in the absence of protease and the second one in the presence of protease (A). The third aliquot was subjected to centrifugation and subsequent cross-linking with sulfo-MBS (SMBS) which was followed by carbonate extraction (B). All samples were analyzed by gel electrophoresis and fluorography. The concentration of 8-azido-ATP is given in mmol/l; the time of UV-irradiation (light) is given in seconds. Note that for pretrypsinized microsomes the time of UV-irradiation had to be reduced as compared to untreated microsomes. This led to incomplete photoinactivation by 8-azido-ATP.

ty labeling of pretrypsinized microsomes with 8-azido-ATP.

Therefore, we conclude that the nucleotide-requirement in ribonucleoparticle-dependent protein transport into mammalian microsomes is distinct from α-subunit of docking protein and acts prior to the signal sequence receptor complex. This conclusion is supported by our observations that photoaffinity labeling with azido-ATP, carried out after binding of the ppl-86mer, did not give rise to protease sensitivity of the ppl-86mer and did not inhibit subsequent chase to sequestered pl-56mer (data not shown).

4. DISCUSSION

We showed previously that a microsomal protein with a sensitivity towards photoaffinity labeling with azido-ATP is involved in ribonucleoparticle-dependent transport of presecretory proteins into mammalian microsomes. The α-subunit of docking protein has been shown to have an affinity for GTP as well as ATP [27]. Furthermore, the 54 kDa subunit of SRP has been suggested to have a GTP-binding site [28,29]. SRP can be ruled out as a potential candidate for the observed photoaffinity inactivation with respect to productive membrane association of the ppl-86mer because the binding experiments were carried out in the reticulocyte lysate (i.e. in the presence of an excess of SRP). The α-subunit of docking protein was disregarded as the target of the observed photoaffinity inactivation by azido-ATP by the experiments which involved pretrypsinized microsomes plus the cytosolic domain of the α-subunit of docking protein. Furthermore, we showed here that the nascent preprolactin did not become associated with the signal sequence receptor complex after photoaffinity labeling of pretrypsinized microsomes with azido-ATP and subsequent reconstitution with the soluble elastase fragment of the α-subunit of docking protein. Thus we conclude that the azido-ATP sensitive component of mammalian microsomes is distinct from the docking protein α-subunit and is acting at a stage prior to the signal sequence receptor complex. This is consistent with the recent proposal that the signal sequence receptor complex is involved in precursor translocation rather than precursor recognition [13,14]. Thus 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 [30–32]. Alternatively, the ATP could be used by a protein kinase which may regulate the action of the so-called signal sequence receptor complex [21]. The α-subunit of this complex was shown to be partially phosphorylated [26].

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