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DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid) inhibits an early step of protein translocation across the mammalian ER membrane

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Protein translocation across the endoplasmic reticulum (ER) membrane of yeast can be inhibited by agents believed to specifically affect the transport of ATP through the membrane (Mayinger, P. and Meyer, D I. (1993) EMBO J. 12, 659–666), suggesting the involvement of a translocation component in the lumen of the ER that binds ATP. We demonstrate that one of the inhibitors, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), also affects the translocation of proteins into mammalian microsomes. Translocation is blocked at the point of transfer of the nascent chain from the signal recognition particle (SRP) into the ER-membrane. We also confirm that photoaffinity-labelling of microsomes with 8-azido-ATP inhibits the same early step of protein translocation Since this step is reported to not require ATP, these results raise the possibility that, in both cases, factor(s) other than ATP-binding components of the translocation machinery are perturbed.

Protein translocation; Endoplasmic reticulum; Photoaffinity labelling; Anion transporter

1. INTRODUCTION

The driving force for the transport of proteins across the endoplasmic reticulum (ER) membrane is as yet unknown. One possibility is that an ATP-hydrolyzing component is located in the membrane or in the lumen of the ER membrane in order to push or pull the polypeptide chain across the membrane. Alternatively, ATP-dependent translocation component may not be needed. In the case of cotranslational translocation, the protein-conducting channel in the membrane-bound ribosome may simply be extended by a putative channel formed by proteins in the ER membrane: direction of transport would be determined by the exit from the channel. In the case of posttranslational translocation, the folding of the polypeptide, its modification or its binding to other proteins in the lumen of the ER may bias its diffusion across the membrane [1].

For the mammalian system it has been reported that translocation, per se, does not require ATP [2]. GTP binding, but not hydrolysis, is required for the transfer of the nascent chain from its initial interaction partner in the cytosol, the 54 kDa polypeptide of the SRP (SRP54), into the ER membrane, where it interacts with both the TRAM-protein and Sec61p [3,4]. A short nascent chain can be completely transferred across the membrane after its release from the ribosome by puromycin, without the need for nucleotide triphosphate hydrolysis [2]. GTP-hydrolysis is required for the recycling of SRP so that it is available for subsequent rounds of translocation [5].

On the other hand, evidence for an ATP-binding translocation component has been provided by photoaffinity labelling of mammalian microsomes with 8-azido-ATP [6,7]. The target component is neither cytoplasmic nor identical to the lumenal BiP [8]. Both the posttranslational transport of a small secretory protein and the cotranslational transport of preprolactin were inhibited by 8-azido-ATP. In the latter case, crosslinking of the nascent chain to the TRAM protein [3] was inhibited [8] (then presumed to be the α-subunit of the signal sequence receptor (SSRα), new name: 'translocon-associated protein' (TRAP) [9]). These data indicated that an early step in translocation is inhibited by azido-ATP but, they failed to clarify whether, under these conditions, the nascent chain was indeed targeted to the membrane.

In yeast, the initial membrane insertion of a ribosome-bound nascent chain of prepro-α-factor is also ATP-independent [10]. However, the posttranslational translocation of a larger domain of this protein does not require ATP [11–13]. The ATP does not seem to be needed exclusively for the function of a cytosolic chaperone, since it is required even after denaturation of the polypeptide by urea [14]; a membrane-bound or lumenal ATP-binding component may therefore be involved. This component may be BiP (Kar2), a lumenal ATP-binding chaperone, mutations in which can affect the translocation process [15].

Recently, additional evidence for a lumenal ATP-binding translocation component was provided by experiments in which the transport of prepro-α-factor into

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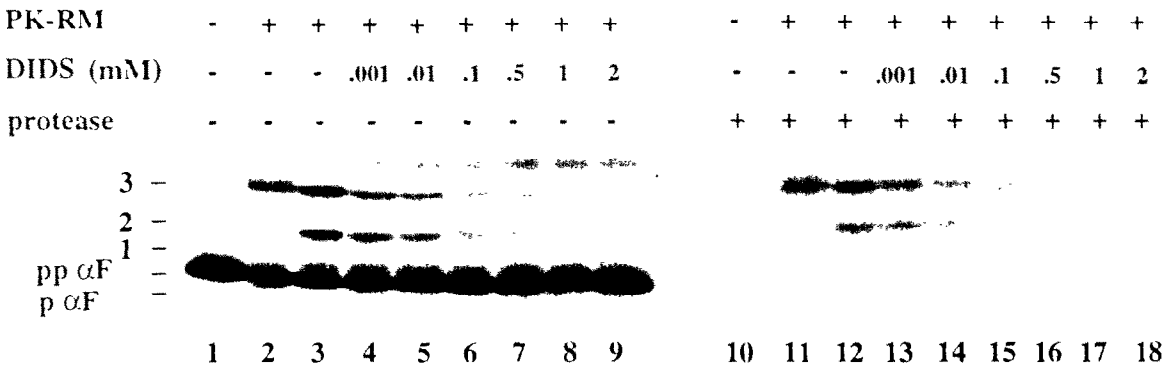

Fig. 1. Inhibition of the translocation of prepro-α-factor by DIDS. The translocation of wild type prepro-α-factor was tested by adding dog pancreatic microsomes, pretreated with different concentrations of DIDS, to an in vitro translation system. Controls were performed with untreated (lanes 2, 11) and mock-treated (lanes 3, 12) microsomes. After translocation, half of the sample was treated with protease (lanes 10–18). The products were analyzed by SDS-gel electrophoresis, followed by fluorography. ppαF, prepro-α-factor; pαF, pro-α-factor respectively; the numbers on the lefthand side of the gel indicate the positions of the glycosylated forms, carrying 1, 2 or 3 carbohydrate chains.

yeast microsomes could be inhibited by reagents that inhibit ATP-translocation through the ER membrane, namely, DIDS and benzoyl-ATP [16,17]. It was therefore surmised that these reagents caused a decrease of the lumenal ATP-concentration which, in turn, inhibited an ATP-binding translocation component. These results raised the possibility that inhibitors of the ATP-translocator may be used to clarify the conflicting situation in the mammalian system.

In this paper, we report that DIDS also inhibits protein translocation into mammalian microsomes. However, it is an early step that is inhibited, i.e. the transfer of the nascent chain from the SRP into the ER membrane, during which lumenal components are unlikely to play a role and which is reported to be ATP-independent [2]. The inhibition by azido-ATP appears to occur at the same step. Our results therefore raise the possibility that both treatments affect factors other than ATP-binding components of the translocation machinery.

2. MATERIALS AND METHODS

2.1. *In vitro transcription*

Transcripts coding for the pPL86mer and for full-length preprolactin were produced with T7 polymerase using the template, plasmid pGEMBP1 [9], linearized with *Pvu*II and *Pst*I, respectively. mRNAs for the wild type prepro-α-factor and the deletion mutant Δ24-89 were synthesized by transcription with SP6 polymerase of the corresponding plasmids (pSP65-αF and pSP65-αF Δ24-89) [18] linearized with *Sal*I. For synthesis of a truncated version of the deletion mutant Δ24-89 that also lacks the C-terminal 5 amino acids, the plasmid was linearized with *Nci*I.

2.2. *In vitro translation and translocation*

Translation in a rabbit reticulocyte lysate system (Promega) was carried out for 20 min at 30°C in a 10 μl volume. Where indicated, dog pancreatic microsomes, stripped of ribosomes by puromycin/high salt treatment (PK-RM) [4], were present during translation at a final concentration of 0.1 eq/μl. After translation, half of the sample was incubated on ice for 30 min with 0.5 mg/ml proteinase K. The reaction was stopped by addition of 5 mM phenylmethylsulfonyl fluoride (PMSF). The proteins were precipitated with ammonium sulfate, washed with trichloroacetic acid and acetone and subjected to SDS-gel electrophoresis (12.5% acrylamide).

2.3. *Photocrosslinking*

Photocrosslinking with the pPL86mer and the truncated version of the deletion mutant Δ24-89 of prepro-α-factor was performed essentially as described [19,20]. Briefly, the ribosome/nascent chain/SRP-complexes were formed by translation for 2 min at 26°C in a wheat germ system in the presence of modified lysyl-tRNA, carrying a photoreactive group in the side chain of the amino acid. Further initiation was stopped with 4 μM edeine, and 5 μl translation mixture were then incubated with 1 eq. membranes for 10 min on ice, followed by 4 min at 26°C. The samples were irradiated for 5 min and then submitted to SDS-gel electrophoresis (10–20% acrylamide).

2.4. *Flotation of membranes after crosslinking*

To separate membrane-bound from cytosolic crosslinked products, the samples were adjusted to 2 M sucrose, 5 mM magnesium acetate, 500 mM potassium acetate and layered under a 100 μl cushion containing 1.8 M sucrose, 50 mM HEPES/KOH, pH 7.5, 5 mM magnesium acetate, 500 mM potassium acetate, 1 mM dithiothreitol. 50 μl of 250 mM sucrose in the same buffer was layered on top of the samples in a Beckman polycarbonate tube. The membranes were floated by centrifugation in a Beckman TL100 rotor for 1 h at 100,000 rpm at 4°C.

2.5. *Treatment of PK-RM with azido-ATP and DIDS*

Microsomes were incubated with 8-azido-ATP as described by Klappa et al. [6]. The samples were irradiated for 5 min on ice. Treatment of microsomes with DIDS was performed as described by Mayinger et al. [16].

3. RESULTS AND DISCUSSION

We first tested whether DIDS inhibited translocation of the secretory protein prepro-α-factor into canine pancreatic microsomes in the same manner as it inhibited translocation of the same protein into yeast microsomes [16] (Fig. 1). Prepro-α-factor was synthesized in the reticulocyte lysate system in the presence of dog pancreatic microsomes that had been pretreated with increasing concentrations of DIDS. Translocation, determined by the appearance of *N*-glycosylated polypep-

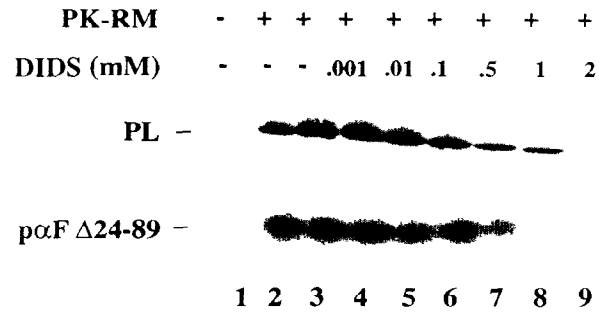

Fig. 2. Inhibition of the translocation of preprolactin and of a deletion mutant of prepro-α-factor Δ24-89 by DIDS. Translocated material was analyzed following proteolysis. The experiments were performed as described in Fig. 1

tides of lower mobility in SDS-gels (Fig. 1, lane 2 vs. 1) and by the occurrence of protease-protected material (lanes 10–18), was clearly inhibited by DIDS. Half-maximum inhibition occurred at about 0.5 mM, in good agreement with the results obtained for yeast microsomes [16]. This suggests that DIDS likely affects a similar step in both systems. In our case, DIDS must have inhibited the cotranslational translocation of prepro-α-factor (translocation of prepro-α-factor in the mammalian system does not occur with nascent chains released from the ribosome [21]), whereas in yeast it might have only influenced the posttranslational mode of translocation, even if the microsomes were added during translation [16].

DIDS also inhibits the translocation into mammalian microsomes of preprolactin and of a short fragment of prepro-α-factor that carries a deletion in its pro-region (Fig. 2) [18]. In both cases, half-maximum inhibition again occurred at about 0.5 mM.

We then determined at which step translocation is inhibited by DIDS. To this end, a fragment of the deletion mutant of prepro-α-factor was synthesized, containing photoreactive lysine derivatives instead of unmodified lysines in the polypeptide chain. This was then photocrosslinked in the absence or presence of microsomes (Fig. 3A) [22,23]. In the absence of membranes, crosslinks to SRP54 were observed (lane 1), whereas in the presence of untreated microsomes, most crosslinks were to the membrane protein Sec61p (lane 2), as identified by immunoprecipitation with specific antibodies (data not shown). Pretreatment of the microsomes with increasing concentrations of DIDS caused the gradual disappearance of the Sec61p-crosslinks and a concomitant increase of SRP54-crosslinks (lanes 3–8). These changes occurred within the range of DIDS-concentrations that caused inhibition of translocation (Figs. 1 and 2B). Our data indicate that DIDS affects translocation by inhibiting the transfer of nascent chains from the SRP into the ER membrane.

These results were confirmed by the use of a protease-protection assay for the membrane insertion of nascent chains (Fig. 3B) [2]. In the absence of membranes (lane 1) or in the presence of membranes pretreated with high concentrations of DIDS (lanes 6–8), only a low molecular weight peptide was protected from the protease attack. The fragment is believed to be the nascent chain within the ribosome [2]. In the presence of active microsomes, the complete fragment was protected (lanes 2–5), indicating that in this case, membrane proteins shield the nascent chain as well. Again, inhibition of membrane insertion of the nascent chain occurred at approximately the same concentration of DIDS at which inhibition of translocation had been observed.

To confirm these results, a second protein was investigated. An 86 amino acid fragment of preprolactin (pPL86mer), containing the photoreactive probes exclusively in its signal sequence, was employed in the crosslinking assay (Fig. 4). In the absence of microsomes, the signal sequence is crosslinked to SRP54 (lane

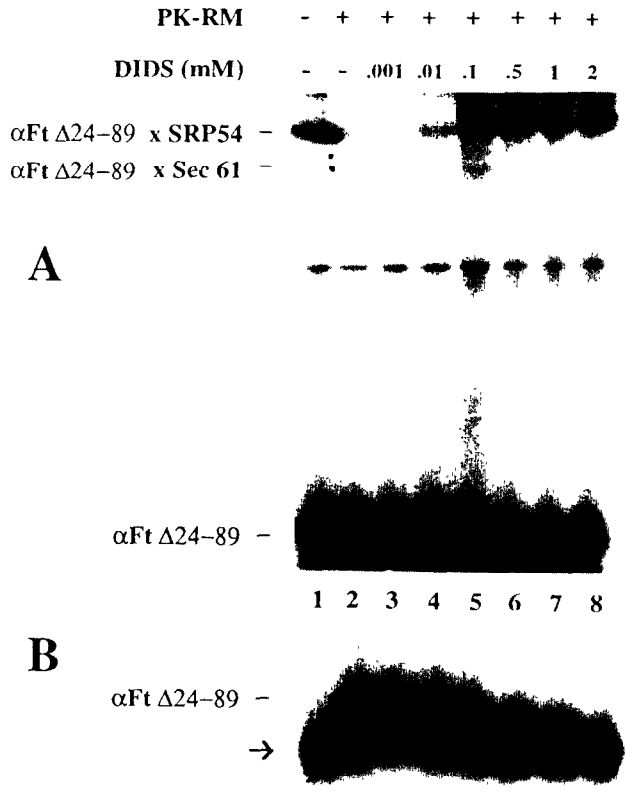

Fig. 3. Inhibition by DIDS of the membrane insertion of a short prepro-α-factor chain. (A) A truncated version of the deletion mutant of prepro-α-factor Δ24-89 (αF Δ24-89) was synthesized in vitro in the presence of incorporated photoreactive lysine derivatives. Crosslinking was analysed with both microsomes pretreated with different concentrations of DIDS (lanes 3–8) and with untreated microsomes (lane 2). A second control was carried out in the absence of microsomes (lane 1). Crosslinks to the 54 kDa polypeptide of SRP (SRP54) and to Sec61p (dots) are as indicated. (B) The insertion of the truncated nascent chain αF Δ24-89 into microsomes was confirmed by treatment with protease. The arrow indicates the position of the fragment of the nascent chain that is presumably exclusively protected by the ribosome.

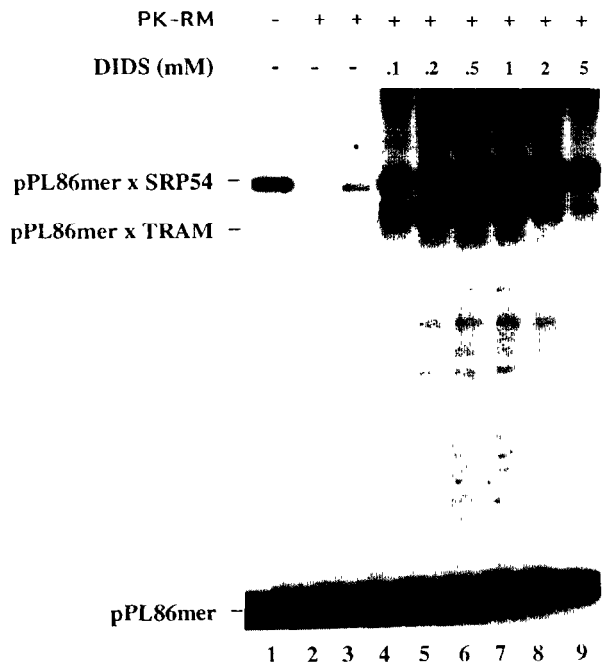

Fig. 4. Inhibition by DIDS of the membrane insertion of a short fragment of preprolactin. A fragment of preprolactin containing 86 amino acids (pPL86mer) was synthesized in vitro in the presence of photoreactive lysine derivatives. The experiment was performed as in Fig. 3A. The crosslinks to the TRAM protein are as indicated

1) [24,25], whereas in the presence of untreated microsomes crosslinking occurs predominantly to the TRAM-protein (86mer × TRAM; lane 2) [3]. Pretreatment of microsomes with increasing concentrations of DIDS resulted in a parallel increase in the intensity of the SRP54-crosslinks (lanes 4–9). Crosslinking to the membrane proteins also changed dramatically; those to the TRAM protein disappeared at low DIDS concentrations (verified by binding of the products to concanavalin A; data not shown) and other (non-glycosylated) crosslinks were seen in an intermediate range of DIDS concentrations. Half-maximum effects of DIDS on the insertion of the nascent chain were seen at about the same concentration needed for half-maximum inhibition of translocation of full-length preprolactin (cf. Fig. 2A). The inhibition of membrane insertion of the 86mer of preprolactin could also be confirmed by the protease-protection assay (data not shown).

Similar observations were made with microsomes pretreated by irradiation in the presence of 8-azido-ATP. These membranes were not competent for protein translocation (half-maximum inhibition at about 2–4 mM for preprolactin and for prepro-α-factor; data not shown), as reported by others [7,8]. It should be noted, however, that in the case of the prepro-α-factor deletion mutant, the inhibition was not complete (data not shown). Crosslinking of the 86mer of preprolactin was again used to monitor the membrane insertion of the nascent chain (Fig. 5C). TRAM-crosslinks disappeared with microsomes photolyzed in the presence of 8-azido-ATP (lanes 3–6), whereas the SRP54 crosslinks reap-

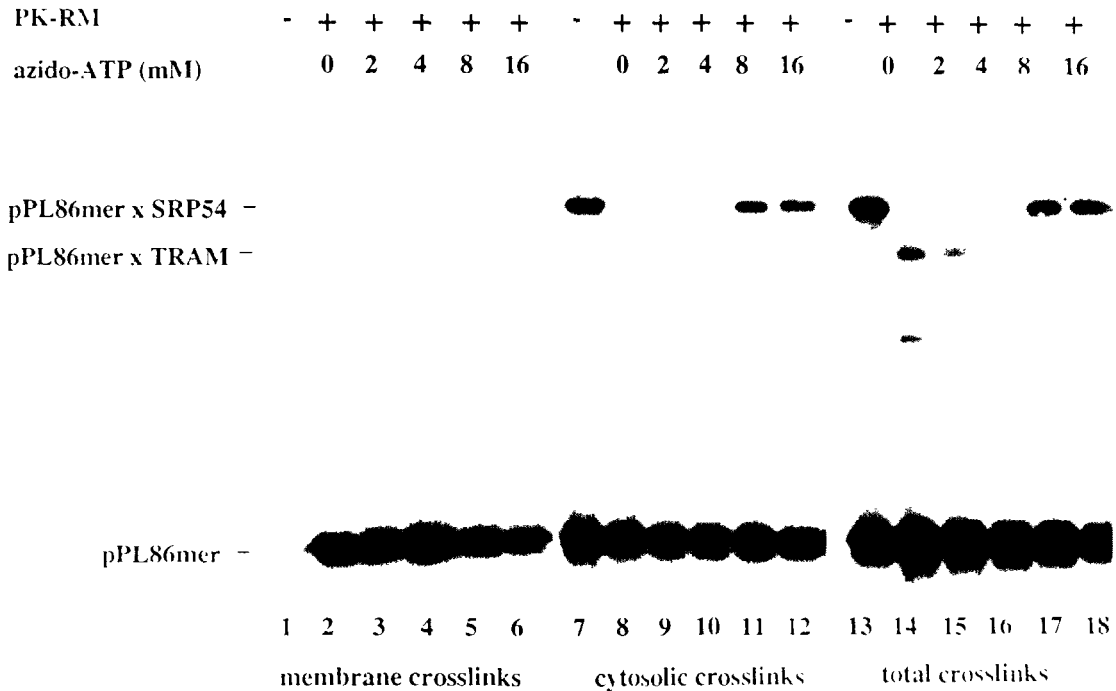

Fig. 5. Inhibition of the membrane insertion of a short fragment of preprolactin by azido-ATP. A fragment of 86 amino acids of preprolactin (pPL86mer) was synthesized in the presence of photoreactive lysine derivatives. Microsomes were pretreated by irradiation in the presence of different concentrations of 8-azido-ATP. Photocrosslinked products of the nascent chain were either analyzed directly by SDS-gel electrophoresis and fluorography (total crosslinks) or first separated by flotation into membrane-bound (membrane crosslinks) and cytosolic (cytosolic crosslinks) fractions. The crosslinks to SRP54 and to the TRAM-protein are as indicated.

peared. Most of the SRP54-crosslinks were found in the cytosolic compartment after flotation of the membranes (Fig. 5B), whereas the TRAM-crosslinks were found associated with the membranes (Fig. 5A). These data show that inactivation of microsomes by photolabelling with 8-azido-ATP results in inhibition of the transfer of the nascent chain from the cytosolic SRP into the ER membrane.

In conclusion, both treatment with DIDS and photolabelling with 8-azido-ATP inhibit a translocation step that has been reported to not require ATP [2]. Also, components of the lumen of the ER are not likely to play a role during this early translocation phase when only a few, if any, amino acids of the translocated polypeptide have reached the lumenal space.

The simplest interpretation of our results is that DIDS and azido-ATP affect in addition to anion transporters and ATP-binding proteins, respectively, the SRP-receptor, the TRAM protein or Sec61p. Both treatments result in covalent modifications of proteins and, in the case of photolabelling with azido-ATP, the reactive nitrene labels many proteins, including the α-subunit of TRAP [7], which does not contain an ATP-binding domain. On the other hand, the nucleotide binding site of the α-subunit of the SRP-receptor does have a low affinity for ATP [2]. However, the inhibition of targeting has been observed even in the presence of a large excess of GTP [7], suggesting that modification of this site is not responsible.

An alternative interpretation is that an ATP-requiring component functioning at a later stage of the process is indeed inhibited exclusively, but that the translocation intermediates accumulate. Although we cannot rule out this possibility, we consider it unlikely since one would have expected to detect membrane-inserted intermediates and thus crosslinks to Sec61p and TRAM-protein (in addition to SRP54-crosslinks), which are the first membrane proteins encountered by the translocating nascent chains. Contrary to this expectation, there was always a reciprocal relationship between the crosslinks to SRP54 and the membrane proteins.

In conclusion, our results indicate that, contrary to previous interpretations, the effects of DIDS and azido-ATP cannot be considered as firm evidence for the existence of ATP-binding translocation components.

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