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A stably folded presecretory protein associates with and upon unfolding translocates across the membrane of mammalian microsomes

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Abstract

The presecretory protein ppcecDHFR, a hybrid between preprocecropin A and dihydrofolate reductase, is transported into mammalian microsomes post-translationally, i.e. independently of ribosome and signal recognition particle. Upon staging the transport process, stably folded ppcecDHFR bound to mammalian microsomes and subsequently translocated across the membrane. Membrane association depended on the signal peptide but involved neither ATP nor an *N*-ethylmaleimide-sensitive microsomal protein. Membrane insertion of bound ppcecDHFR did not necessitate unfolding of the DHFR domain but depended on ATP and an *N*-ethylmaleimide-sensitive microsomal protein. Completion of translocation relied on unfolding of the DHFR domain. Thus mammalian microsomes have the capability of transporting a bound and folded precursor protein, i.e. to trigger unfolding of a precursor protein on the membrane surface.

Key words: Protein transport; Mammalian microsome; ATP; Methotrexate

1. Introduction

The decisive initial step in secretion of most eukaryotic proteins is their transport into the lumen of the endoplasmic reticulum [1–3]. Membrane transport of presecretory proteins can be subdivided 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 guaranteed 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 transport machinery, termed translocase, in the microsomal membrane which comprises proteins TRAMp (mp39) and sec61αp (P37, imp34) [4–12].

There are two classes of precursor proteins with respect to their mechanism of transport into mammalian microsomes [2]. One class typically consists of precursor proteins with more than 70 amino acid residues. Transport of these proteins involves two ribonucleoparticles, the ribosome and the signal recognition particle (SRP), and their respective receptors on the microsomal surface. The other class typically consists of precursor proteins with less than 70 amino acid residues and is transported independently of the ribonucleoparticles. Instead, cytosolic molecular chaperones are involved, such as Hsc70 [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 [16,17] and to photoaffinity labeling with azido-ATP [18–20], 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, preprocecropin A (ppcecA), and the cytosolic protein, dihydrofolate reductase (DHFR), into dog pancreas microsomes [21,22]. Transport of this precursor was signal peptide- and ATP-dependent and could proceed under post-translational conditions, i.e. independently of ribosome and SRP. Methotrexate (MTX), a competitive inhibitor of DHFR, bound to ppcecDHFR after it was released from the ribosome, thereby stabilizing the native conformation of the DHFR domain. In this state, membrane insertion of ppcecDHFR was still possible but completion of translocation was blocked.

Here we report that a transport intermediate, ppcecDHFR, which is bound to microsomes and stably folded, can subsequently be chased to inserted pcecDHFR and, eventually, to sequestered pcecDHFR from the bound state. The requirements for the various transport stages have been characterized.

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2. Materials and methods

2.1. Materials

[^{35}S]Methionine (1,000 Ci/mmol) was obtained from Amersham. *Eco*RI, RNase A, proteinase K, SP6 polymerase and ATP were purchased from Boehringer-Mannheim. Cycloheximide, potato apyrase (grade VIII), methotrexate and the non-hydrolyzable ATP analog were obtained from Sigma. X-ray films (X-Omat AR) were from Kodak. Phenylmethylsulphonyl fluoride was from Merck.

2.2. In vitro assay

Dog pancreas microsomes were isolated and treated with micrococcal nuclease and EDTA as described [21]. Treatment of microsomes with NEM (final concentration: 4 mM) and mock-treatment, respectively, were carried out according to published procedures [17]. Plasmid pCA2, which contains the ppcecDHFR coding region behind the SP6 promoter [21], and pCA25, which codes for the signal peptide deletion mutant pcecDHFR [22], respectively, were linearized with *Eco*RI and transcribed with SP6 polymerase as described [21]. Translation in rabbit reticulocyte lysate was performed at 37°C in the presence of in vitro transcript and [^{35}S]methionine (final concentration: 1.4 mCi/ml) as described [21]. For staging of post-translational transport the translation was terminated after 15 min at 37°C by the addition of cycloheximide (final concentration: 100 μg/ml) plus RNaseA (80 μg/ml) and a further incubation for 5 min at 37°C; then binding of ppcecDHFR to microsomes (A_{280}: 4) was carried out for 10 min at 37°C in the presence of MTX (50 nM). Then the microsomes were re-isolated by centrifugation for 5 min at 4°C in an Eppendorf Microfuge. For the determination of microsome-bound and soluble ppcecDHFR, supernatant and pellet were boiled in sample buffer and subjected to electrophoresis. For chase of the bound ppcecDHFR, the pellet was resuspended in the original volume with T-buffer (50 mM TEA-HCl, pH 7.5, 50 mM K-acetate, 2 mM Mg-acetate, 1 mM DTT, 200 mM sucrose). After a second centrifugation the microsomes were resuspended in the original volume with T-buffer and subsequently incubated for 30 min at 0 or 37°C in the absence or presence of ATP (5 mM). Where indicated MTX (50 nM) was present during the chase.

2.3. 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. The samples were subjected to electrophoresis in high Tris/urea/SDS-polyacrylamide gels [21]. The gels were treated with 1 M sodium salicylate, dried and exposed to X-ray films. Densitometric analysis was performed with an LKB Ultrascan XL laser densitometer.

3. Results and discussion

3.1. Specific binding of ppcecDHFR to microsomes

The hybrid presecretory protein, ppcecDHFR, was synthesized in reticulocyte lysate. For subsequent membrane association, ppcecDHFR was incubated with dog pancreas microsomes for 10 min at 37°C in the presence of MTX under the conditions which we have established for post-translational transport [21]. Then the microsomes were re-isolated by centrifugation and their ppcecDHFR content was determined. During the binding reaction about 50% of ppcecDHFR became insoluble, i.e. were recovered with the microsomal pellet upon centrifugation (typical result shown in Fig. 1A, ppcecDHFR). Of the ppcecDHFR in the pellet about 60% was associated with microsomes, whereas 40% aggregated or bound to tube walls (i.e. was recovered in the pellet also after incubation in the absence of microsomes). When the microsomes after re-isolation were

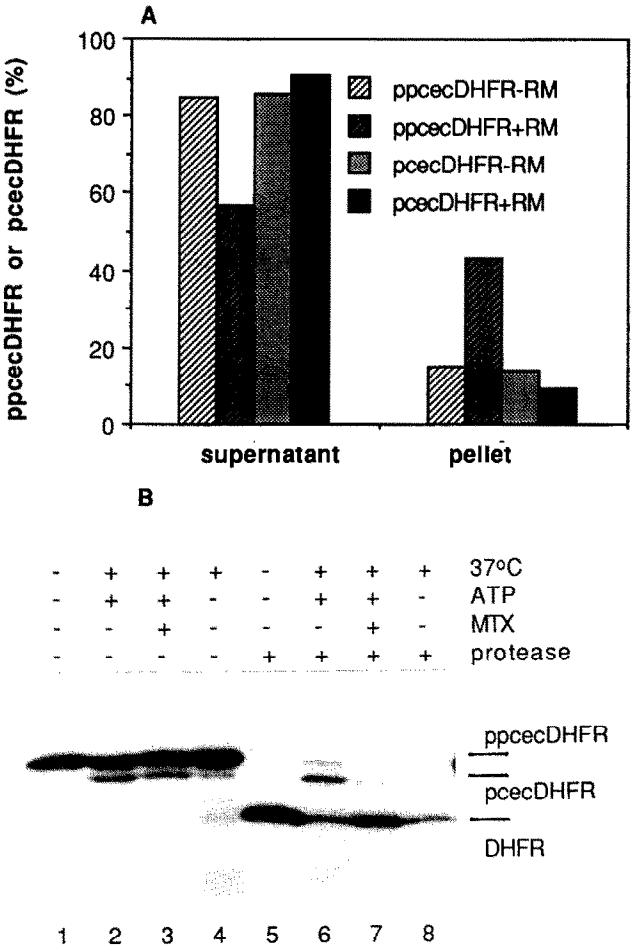

Fig. 1. Signal peptide-dependent binding of ppcecDHFR to dog pancreas microsomes and chase to sequestered pcecDHFR. Plasmid pCA2 (A,B), which contains the ppcecDHFR coding region behind the SP6 promoter, and pCA25 (A), which codes for the signal peptide deletion mutant pcecDHFR, respectively, were linearized and transcribed. Translation in rabbit reticulocyte lysate was performed for 15 min at 37°C. For staging of post-translational transport, translation was terminated by the addition of cycloheximide plus RNaseA and incubation for 5 min at 37°C; then binding of ppcecDHFR to microsomes was carried out for 10 min at 37°C in the presence of MTX. Then the microsomes were re-isolated by centrifugation. (A) For the determination of microsome-bound and soluble ppcecDHFR, supernatant and pellet were subjected to electrophoresis. (B) For chase of the bound ppcecDHFR the pellet was resuspended in the original volume with T-buffer. After a second centrifugation the microsomes were resuspended in the original volume with T-buffer and subsequently were incubated for 30 min at 0 or 37°C in the absence or presence of ATP. Where indicated MTX was present during the chase. Sequestration assays were performed and the samples were subjected to electrophoresis.

incubated for 30 min at 37°C in buffer which contained ATP, about 20% of ppcecDHFR in the pellet (i.e. approximately 30% of ppcecDHFR associated with microsomes) was processed by and transported into the microsomes (typical result shown in Fig. 1B, lanes 2 and 6).

Binding of ppcecDHFR to dog pancreas microsomes was specific since (i) it involved the signal peptide and (ii) ppcecDHFR, which was bound to microsomes, was

processed to pcecDHFR and transported into the microsomal lumen from the bound state.

When the signal peptide-deficient mutant of the hybrid, pcecDHFR [22], was synthesized in reticulocyte lysate and was incubated with microsomes, as described for ppcecDHFR, it did not bind to microsomes to a significant extent (Fig. 1A, pcecDHFR). This protein lacks the entire signal peptide and was previously shown to be incapable of ribonucleoparticle-dependent or independent protein transport [22].

In order to demonstrate that transport occurred from the bound state we studied the sensitivity of this reaction to dilution (data not shown). Diluting a complete post-translational transport reaction, i.e. prior to membrane association of ppcecDHFR, impaired the processing to pcecDHFR. The chase reaction, however, was much less sensitive to dilution (after membrane association). A 7-fold dilution of the complete transport reaction led to 50% inhibition, while a 90-fold dilution was required to reduce the efficiency of the chase by 50%.

3.2. *Binding is ATP-independent and NEM-resistant*

Association of ppcecDHFR with microsomes was studied after depletion of translation reactions of ATP as well as under conditions where the microsomes had been NEM-pretreated.

Apyrase, an enzyme which hydrolyzes ATP and ADP, was used at increasing concentrations to deplete, after translation, the reticulocyte lysate of ATP and, due to the action of nucleoside diphosphate kinases, other nucleotides [21]. Then the efficiencies of transport, membrane association and chase of bound ppcecDHFR, respectively, were evaluated in the presence or absence of exogenously added ATP (Fig. 2A). While the complete transport reaction was very sensitive to apyrase treatment (transport) and was partially restored by the addition of ATP (transport + ATP), there was hardly any effect of ATP depletion on membrane association of ppcecDHFR (binding). A significant fraction of the ppcecDHFR which associated with microsomes in the absence of ATP was found to become chased to sequestered pcecDHFR in the presence of ATP (chase + ATP). Thus, ATP is not required for membrane association but for a subsequent step of transport.

It has previously been shown that a microsomal protein which is NEM-sensitive is involved in protein transport into mammalian microsomes [16,17]. To address the question of which stage of ppcecDHFR transport is af-

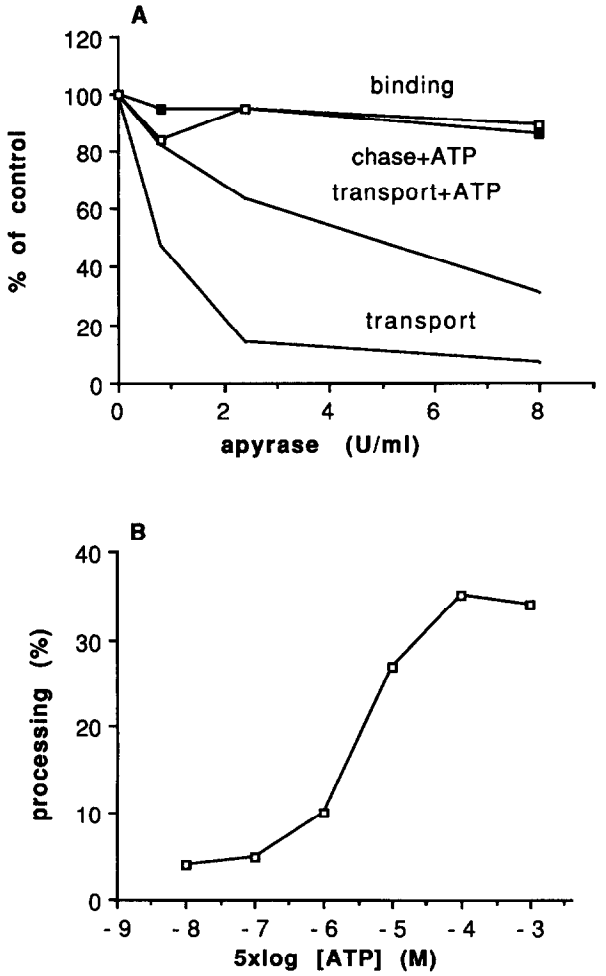

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Fig. 2. The role of ATP in binding of ppcecDHFR to microsomes and subsequent chase to sequestered pcecDHFR. Translation in rabbit reticulocyte lysate was performed for 15 min at 37°C. (A) For ATP depletion and subsequent post-translational transport, translation was terminated by the addition of cycloheximide plus RNaseA and apyrase (final concentrations as indicated) and incubation for 5 min at 37°C; then microsomes were added and the samples were incubated for 30 min at 37°C (A, transport). Where indicated, ATP was present during the chase (A, transport + ATP) together with creatine phosphate (10 mM) plus creatine kinase (50 μg/ml). The samples were subjected to electrophoresis. For staging of post-translational transport the translation was terminated and the ATP depleted as described above; then binding of ppcecDHFR to microsomes was carried out for 10 min at 37°C in the presence of MTX. Then the microsomes were re-isolated by centrifugation. For the determination of bound ppcecDHFR the pellets were subjected to electrophoresis (A, binding). For chase of the bound ppcecDHFR the pellets were resuspended in the original volume with T-buffer. After a second centrifugation the microsomes were resuspended in the original volume with T-buffer and subsequently were incubated for 30 min at 37°C in the presence of ATP (A, chase + ATP) together with creatine phosphate plus creatine kinase. The samples were subjected to electrophoresis. Note that apyrase was still present in 'transport + ATP' but was absent from 'chase + ATP', due to the re-isolation of microsomes. (B) For the characterization of the ATP requirement the translation was terminated as described above; then binding of ppcecDHFR to microsomes was carried out for 10 min at 37°C in the presence of MTX. Then the microsomes were re-isolated by centrifugation and resuspended in the original volume with T-buffer. After a second centrifugation the microsomes were resuspended in the original volume with T-buffer and subsequently incubated for 30 min at 37°C in the presence of ATP (at final concentrations as indicated). The samples were subjected to electrophoresis.

fected, microsomes were NEM- or mock-treated and analyzed with respect to membrane association of ppcecDHFR after various incubation times and at different concentrations of microsomes (Fig. 3, A and B). Both, efficiency and rate, of membrane association were not affected by NEM-treatment of microsomes. Thus, a NEM-sensitive component of microsomes is not involved in membrane association of ppcecDHFR but in a subsequent step. This is consistent with the results which were obtained for SRP-dependent transport [16].

3.3. *Membrane insertion is ATP-dependent and NEM-sensitive*

In contrast to membrane association, membrane insertion of ppcecDHFR was inhibited by NEM-pretreatment of the microsomes and required the presence, and most likely hydrolysis, of ATP.

After binding of ppcecDHFR to microsomes and subsequent re-isolation of the microsomes the chase incubation was carried out in the absence of ATP or in the presence of increasing concentrations of ATP (Fig. 2B, see also Fig. 1B, lanes 2,6 vs. 4,8). ATP stimulated the chase of bound ppcecDHFR to sequestered pcecDHFR, half-maximal stimulation being observed at about 10 μM ATP. The non-hydrolyzable ATP analog, adenosine 5'-(β,γ-methylene)-triphosphate (AMP-PCP), could not substitute for ATP in this respect. Both results are consistent with our observations on transport of a purified and denatured presecretory protein (ppcecA) in the absence of molecular chaperones [18]. This consistency also refers to the observed NEM-sensitivity of the chase (Fig. 3C).

We conclude, therefore, that ATP and a microsomal protein which is NEM-sensitive are involved in membrane insertion of ppcecDHFR. A similar conclusion was reached previously for post-translational transport of precursor proteins into yeast microsomes [23]. With respect to the ATP requirement, however, we cannot distinguish between two possibilities. Either the ATP at this stage is used by the microsomal protein which is sensitive to photoaffinity modification with azido-ATP [18,19] or it is used by this microsomal protein and Hsc70 [13,14] which may have been associated with bound ppcecDHFR.

3.4. *Unfolding of the DHFR domain is not required for membrane insertion but for completion of translocation*

Concerning the complete post-translational transport reaction, we observed previously that at least partial unfolding of the DHFR domain is essential [21]. Thus we addressed the question of at what stage unfolding has to occur, i.e. whether folded ppcecDHFR, stabilized by the presence of MTX, not only associates with microsomes but can also be inserted into the membrane, as would be indicated by processing to pcecDHFR. The data demon-

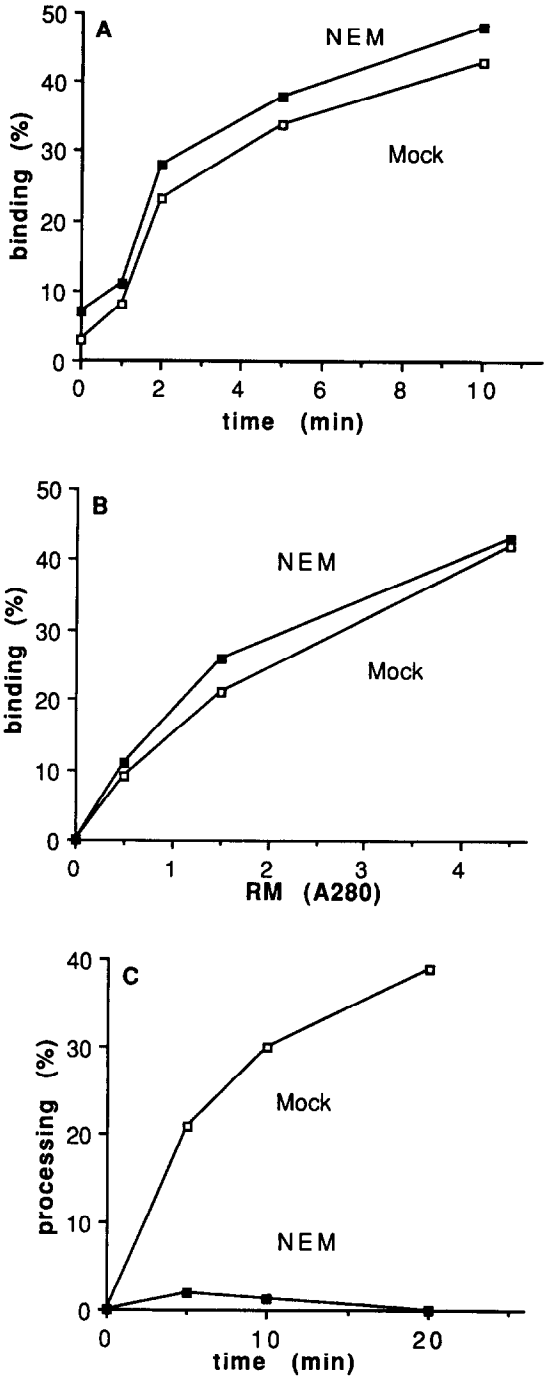

Fig. 3. The effect of NEM treatment of microsomes on binding of ppcecDHFR to microsomes and subsequent chase to sequestered pcecDHFR. Binding of ppcecDHFR to mock-treated microsomes or NEM-pretreated microsomes (at final concentrations as indicated) was carried out for the times indicated at 37°C in the presence of MTX. Then the microsomes were re-isolated by centrifugation. (A and B) For the determination of bound ppcecDHFR the pellets were subjected to electrophoresis. Note that the binding data were corrected for ppcecDHFR which aggregated or bound to tube walls. (C) For chase of the bound ppcecDHFR the pellet was resuspended in the original volume of T-buffer. After a second centrifugation the microsomes were resuspended in the original volume of T-buffer and subsequently incubated for 30 min at 37°C in the presence of ATP. Sequestration assays were performed and the samples were subjected to electrophoresis.

strate that ppcecDHFR was chased to protease-sensitive pcecDHFR when binding and subsequent chase were carried out in the presence of MTX (Fig. 1B, lanes 3 and 7). Thus, the unfolding of the DHFR domain is not essential for membrane insertion of ppcecDHFR but for completion of translocation.

It was shown above that ppcecDHFR, which associated with microsomes in the presence of MTX, was chased to sequestered pcecDHFR when the chase incubation was carried out in the absence of MTX (Fig. 1B, lanes 2 and 6). The effect of MTX on the protease resistance of the DHFR domain served as a monitor for the action of the drug and the folding state of the DHFR domain [21]. When ppcecDHFR after binding to microsomes was kept at low temperature and subjected to protease treatment, ppcecDHFR was quantitatively converted into the protease-resistant DHFR domain (lane 5). At higher temperatures, protease resistance of the DHFR domain was partially maintained only in the presence of MTX (lane 7). In the absence of MTX the DHFR domain was protease-sensitive irrespective of the presence of ATP (lanes 6 and 8).

These results can be explained as follows. The microsome-associated ppcecDHFR molecules had a folded DHFR domain with bound MTX. This domain was protease protected when, after membrane association, the microsomes were kept at low temperature throughout re-isolation, chase incubation and protease treatment, or when fresh MTX was added back to the microsomes for the chase incubation at elevated temperature. However, when the microsomes were re-isolated and the chase incubation was carried out at 37°C in the absence of fresh MTX, apparently most ppcecDHFR molecules released MTX and the DHFR domain became protease-sensitive, i.e. started to unfold. This was temperature-dependent but independent of ATP. On the other hand, membrane insertion occurred only when ATP was present and irrespective of whether MTX was present or not. Sequestration of pcecDHFR occurred only when MTX was absent. In conclusion, bound ppcecDHFR with a folded DHFR domain was inserted into the membrane in an ATP-dependent manner and subsequently was chased into the microsomal lumen under conditions where the DHFR domain was at least partially unfolded. Since we did not detect a stable transmembrane form of pcecDHFR [21] we were unable to address the question as to whether completion of unfolding requires ATP.

In summary it seems reasonable to assume that membrane association of ppcecDHFR occurs via a putative signal peptide binding protein. Upon contact with the translocase the signal peptide is then inserted into the membrane, most likely in the form of a loop structure which is made up of the signal peptide plus the amino-terminus of the pcecA part. We assume that at this point the ATP-hydrolyzing and NEM-sensitive subunit(s) of the translocase come(s) into action. Furthermore, if Hsc70 is still associated with the pcecA part at this stage it has to be released. In order for translocation to progress the DHFR domain on the *cis*-side of the membrane has to unfold at least partially. Since the energy for complete unfolding of a precursor protein may be as low as 10 kcal/mol, the initial hydrolysis of one ATP, necessary for membrane insertion, could, in principle, be sufficient to trigger such an unfolding reaction. Completion of translocation, possibly tightly coupled to unfolding, may be driven by binding to molecular chaperones on the *trans*-side of the membrane, such as BiP, or by spontaneous refolding on the *trans*-side of the membrane. Thus, the protein transport apparatus in the microsomal membrane has a similar capability of translocating a formerly folded substrate protein as the machineries in the mitochondrial membranes and the bacterial plasma membrane [24–26].

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References

[1] Zimmermann, R. and Meyer, D.I. (1986) Trends Biochem. Sci. 11, 512–515.
[2] Wiech, H., Klappa, P. and Zimmermann, R. (1991) FEBS Lett. 285, 182–188.
[3] Klappa, P., Zimmermann, M., Dierks, T. and Zimmermann, R. (1993) Subcellular Biochemistry 21, 17–40.
[4] Wiedmann, M., Kurzchalia, T.V., Hartmann, E. and Rapoport, T.A. (1987) Nature 328, 830–833.
[5] Görlich, D., Harmann, E., Prehn, S. and Rapoport, T.A. (1992) Nature 357, 47–52.
[6] Görlich, D., Prehn, S., Hartmann, E., Kalies, K.-U. and Rapoport, T.A. (1992) Cell 71, 489–503.
[7] Görlich, D. and Rapoport, T.A. (1993) Cell 75, 615–630.
[8] High, S., Görlich, D., Wiedmann, M., Rapoport, T.A. and Dobberstein, B. (1991) J. Cell Biol. 113, 35–44.
[9] High, S., Andersen, S.S.L., Görlich, D., Hartmann, E., Prehn, S., Rapoport, T.A. and Dobberstein, B. (1993) J. Cell Biol. 121, 743–750.
[10] Thrift, R.N., Andrews, D.W., Walter, P. and Johnson, A.E. (1991) J. Cell Biol. 112, 809–821.
[11] Kellaris, K.V., Bowen, S. and Gilmore, R. (1991) J. Cell Biol. 114, 21–33.
[12] Krieg, U.C., Johnson, A.E. and Walter, P. (1989) J. Cell Biol. 109, 2033–2043.
[13] Wiech, H., Sagstetter, M., Müller, G. and Zimmermann, R. (1987) EMBO J. 6, 1011–1016.
[14] Zimmermann, R., Sagstetter, M., Lewis, J.L. and Pelham, H.R.B. (1988) EMBO J., 7, 2875–2880.
[15] Wiech, H., Buchner, J., Zimmermann, M., Zimmermann, R. and Jakob, U. (1993) J. Biol. Chem. 268, 7414–7421.
[16] Nicchitta, C.V. and Blobel, G. (1989) J. Cell Biol. 108, 789–795.
[17] Zimmermann, R., Sagstetter, M. and Schlenstedt, G. (1990) Biochimie 72, 95–101.
[18] Klappa, P., Mayinger, P., Pipkorn, R., Zimmermann, M. and Zimmermann, R. (1991) EMBO J. 10, 2795–2803.
[19] Zimmermann, D.L. and Walter, P. (1991) Cell Reg. 2, 851–859.
[20] Zimmermann, R., Zimmermann, M., Mayinger, P. and Klappa, P. (1991) FEBS Lett. 286, 95–99.

[21] Schlenstedt, G., Gudmundsson, G.H., Boman, H.G. and Zimmermann, R. (1990) J. Biol. Chem. 265, 13960–13968.
[22] Schlenstedt, G., Gudmundsson, G.H., Boman, H.G. and Zimmermann, R. (1992) J. Biol. Chem. 267, 24328–24332.
[23] Sanz, P. and Meyer, D.I. (1989) J. Cell Biol. 108, 2101–2106.
[24] Eilers, M., Hwang, S. and Schatz, G. (1988) EMBO J. 7, 1139–1145.
[25] Rassow, J., Guiard, B., Wienhues, U., Herzog, V., Hartl, F.-U. and Neupert, W. (1989) J. Cell Biol. 109, 1421–1428.
[26] Arkowitz, R.A., Joly, J.C. and Wickner, W. (1993) EMBO J. 12, 243–253.