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WALTER NEUPERT and ROLAND LILL

Institut für Physiologische Chemie, Physikalische Biochemie und Zellbiologie, Ludwig-Maximilians-Universität München, Goethestraße 33, 8000 München 2, Germany

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CHAPTER 12

Consecutive steps of nucleoside triphosphate hydrolysis are driving transport of precursor proteins into the endoplasmic reticulum

PETER KLAPPA, GÜNTER MÜLLER[#], GABRIEL SCHLENSTEDT*, HANS WIECH and RICHARD ZIMMERMANN

Zentrum Biochemie/Abteilung Biochemie II der Universität, Gosslerstraße 12d, W-3400 Göttingen, Germany

Abstract

Transport of secretory proteins into the mammalian endoplasmic reticulum can be visualized as a sequence of various steps which include membrane association, membrane insertion and completion of translocation. It turns out that this transport depends on the hydrolysis of nucleoside triphosphates at various stages: (i) There is a GTP requirement in ribonucleoparticle-dependent transport. This GTP effect is related to the GTP binding proteins signal recognition particle (SRP) and docking protein. (ii) There is an ATP requirement in ribonucleoparticle-independent transport. This ATP effect is related to the cytosolic (termed cis-acting) molecular chaperone hsp70. (iii) Recently we addressed the question of whether there are additional nucleoside triphosphate requirements in protein transport into mammalian microsomes. We observed that a microsomal protein which depends on ATP hydrolysis is involved in membrane insertion of both, ribonucleoparticle-dependent and -independent precursor proteins. The azido-ATP sensitive protein was shown to be distinct from the lumenal (termed trans-acting) molecular chaperone BiP.

1. Introduction

Every polypeptide has a unique intra- or extracellular location where it fulfills its function. The following facts complicate our attempts to understand this situation:

[#]*Present address:* Hoechst AG, W-6230 Frankfurt am Main 80, Germany.
**Present address:* Department of Molecular Biology, Princeton University, Princeton, New Jersey, USA.

(i) most proteins are synthesized in the cytosol, however, non-cytosolic proteins must subsequently be directed to a variety of different subcellular locations, and (ii) in the case of non-cytosolic proteins the sites of synthesis and of functional location are separated by at least one biological membrane. Consequently, mechanisms exist which ensure the specific transport of proteins across membranes. Here we discuss the mechanisms involved in export of newly synthesized secretory proteins.

There appear to be different ATP-dependent transport mechanisms for protein export [1]. One can distinguish between transport mechanisms involving signal peptides and those that do not. The signal peptide-independent mechanism takes place at the plasma membrane. It involves transport components which are related to the multiple drug resistance proteins, i.e. a family of ATP-dependent membrane proteins. The signal peptide-dependent mechanism, however, operates at the level of the membrane of the endoplasmic reticulum. From there, secretory proteins reach the extracellular space by vesicular transport. There are at least two different mechanisms for the transport of secretory proteins into the mammalian endoplasmic reticulum. Both mechanisms depend on the presence of a signal peptide on the respective precursor protein and involve a signal peptide receptor on the cytosolic surface of the membrane and a membrane component that is sensitive towards photoaffinity modification by azido-ATP. The decisive feature of the precursor protein with respect to which of the two mechanisms is used is the chain length of the polypeptide. The critical size seems to be around 70 amino acid residues (including the signal peptide). One mechanism is used by precursor proteins larger than about 70 amino acid residues and relies on the hydrolysis of GTP and two cytosolic ribonucleoparticles (ribosome and signal recognition particle) and their receptors on the microsomal surface (ribosome receptor and docking protein). The other mechanism is used by small precursor proteins and involves the hydrolysis of ATP and cytosolic molecular chaperones such as hsp70.

2. Results

We focus on the following presecretory proteins as tools for gaining insight into the molecular details of how proteins are transported into the mammalian endoplasmic reticulum: preprocecropin A [2–4], prepromelittin [5–7], and prepropeptide GLa [8]. All three precursor proteins contain a cleavable signal peptide and about 70 amino acid residues (including the signal peptide). We employ in vitro systems which are derived from mammalian organisms such as rabbit reticulocyte lysates and dog pancreas microsomes.

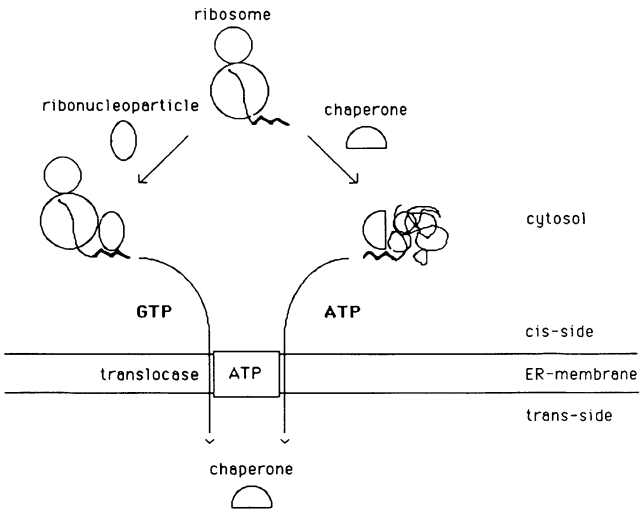

Fig. 1. Signal peptide-dependent transport of secretory proteins into the mammalian endoplasmic reticulum involves nucleoside triphosphate hydrolysis. Refer to Results for details.

2.1. Ribonucleoparticles versus molecular chaperones

It is clear that precursor proteins are not transported in their native (i.e. folded) state and that signal peptides are involved in preserving the unfolded state as well as in facilitating membrane recognition. Furthermore, it appears that there are two mechanisms preserving transport competence in the cytosol (Fig. 1, Table I). The mechanisms differ in how transport competence is preserved. In the first case protein synthesis is slowed down, in the second case protein folding and/or aggregation is slowed down. The first mechanism involves the hydrolysis of GTP and ribonucleoparticles and their receptors on the microsomal surface, the second mechanism does not involve ribonucleoparticles and their receptors but depends on the hydrolysis of ATP and on molecular chaperones. Small presecretory proteins (i.e. precursor proteins which contain less than 75 amino acid residues) such as preprocecropin A are the best substrates for the latter mechanism.

The ribonucleoparticle-dependent pathway seems to be used by the majority of presecretory proteins and has been analyzed in great detail (refer to Chapters 9 and 10 for references). It involves SRP and its receptor in the microsomal membrane, docking protein (SRP receptor) and the ribosome and its receptor. In addition, ribophorins I and II seem to be involved in this mechanism [9]. There is a GTP requirement in the transport of ribonucleoparticle-dependent precursor proteins [10–12]. This GTP effect is related to the GTP binding proteins, SRP and docking protein [11,13,14].

The first observations with respect to ribonucleoparticle-independent transport

TABLE I
Components involved in protein transport into the mammalian endoplasmic reticulum

Signal recognition particle	7S RNA
	SRP 72 kDa subunit
	SRP 68 kDa subunit
	SRP 54 kDa subunit
	SRP 19 kDa subunit
	SRP 14 kDa subunit
	SRP 9 kDa subunit
SRP receptor	DPα subunit
	DPβ subunit
Ribosome	
Ribosome receptor	
cis-Acting chaperone	hsp70
Translocase	Signal peptide receptor
	NEM-sensitive component
	Azido-ATP-sensitive component
	SSRα subunit
	SSRβ subunit
trans-Acting chaperone	BiP

were that the loosely folded (unfolded, denatured) precursor is the best substrate for transport and that the hydrolysis of ATP by cytosolic factors is involved in preserving this state [2,7,8,15]. In collaboration with M. Lewis and H. Pelham, we were able to demonstrate that hsp70 is part of what we had termed a cytosolic ATPase and that a second cytosolic protein (which in contrast to hsp70 is NEM-sensitive) is involved [16]. Our current working model proposes that hsp90 may be the protein of interest, the main reason being that it is enriched in a fraction that contains the desired activity (Fig. 2). We find this to be an attractive hypothesis for two reasons: (i) hsp70 and hsp90 were shown to cooperate with respect to hormone receptors and (ii) BiP (grp78, a member of the hsp70 family) and grp94 (a member of the hsp90 family) are present in the microsomal lumen.

The decisive feature of the precursor protein with respect to which of the two mechanisms is used is the chain length of the polypeptide. This conclusion was based on the observation that carboxy-terminal extension of a small precursor protein in size, typically leads to the phenotype of a large precursor protein [6,8]. If one takes into account that approximately 40 amino acid residues of a nascent polypeptide chain are buried in the ribosome [17–19] and that a signal peptide contains 20–30 amino acid residues [20–22] and, furthermore, that SRP can bind to signal peptides

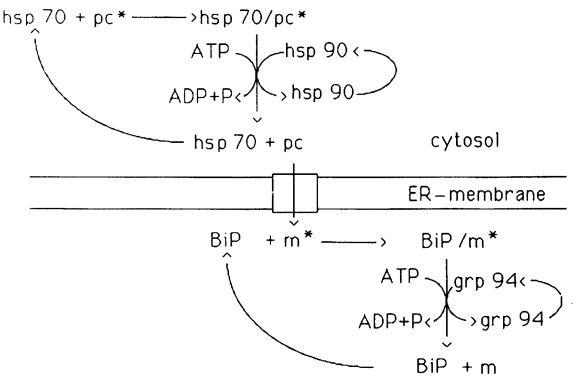

Fig. 2. Molecular chaperones are involved in ribonucleoparticle-independent transport. pc, precursor after release from cis-acting molecular chaperone (molten globule state); pc*, precursor during or after release from ribosome; m, mature protein after release from trans-acting molecular chaperone (native state); m*, mature protein during or after release from translocase. Refer to Results for details.

only as long as they are presented by a ribosome [23,24], one can imagine that precursor proteins with less than 60–70 amino acids cannot make use of the two ribonucleoparticles; they are released before SRP can bind to the signal peptide. However, the ribonucleoparticle-independent mechanism can also be used by a large precursor protein [2]. A synthetic hybrid between preprocecropin A and dihydrofolate reductase, translocates post-translationally (without the involvement of signal recognition particle and ribosome). This was directly demonstrated by adding methotrexate to the translocation reaction. Methotrexate and related drugs bind to ppcecDHFR after it is completed and released from the ribosome, stabilize the native conformation of the DHFR domain and allow membrane insertion but block completion of translocation.

2.2. Translocase

We assume that the two pathways converge at the level of a putative signal peptide receptor which may be identical to the 45 kDa protein that was characterized as a signal sequence binding protein in microsomal membranes [25]. Besides this protein, biochemical evidence points to additional membrane proteins as parts of a general translocase (Table I).

There is an ATP-requiring step at the microsomal level which is involved in both mechanisms and which is not related to the lumenal molecular chaperone BiP [4].

After solubilization in DMSO and subsequent dilution into an aqueous buffer, the transport of the chemically synthesized and purified precursor protein preprocecropin A* occurs in the absence of molecular chaperones but depends on the hydrolysis of ATP. The concentration of ATP that leads to half-maximal stimulation is in the order of 10 μM. At this concentration other nucleotides cannot substitute for ATP. In

other words, the effect appears to be specific for ATP. Furthermore, non-hydrolyzable ATP analogs, such as AMP-PCP or AMP-PNP, cannot substitute for ATP. Since these analogs compete with ATP, one can conclude that the hydrolysis of ATP is required. Photoaffinity modification of dog pancreas microsomes with 8-azido-ATP leads to inactivation of the microsomes with respect to membrane insertion of preprocecropin A* as well as of prepro-α-factor and preprolactin. Therefore, we concluded that a hitherto unknown microsomal protein that depends on ATP hydrolysis is involved in membrane insertion of both ribonucleoparticle-dependent and -independent precursor proteins (Fig. 1, Table I). We are currently employing a combination of two approaches in order to identify the ATP-dependent component of interest: photoaffinity modification of microsomal proteins with ^{32}P-8-azido-ATP and affinity purification of ATP-binding proteins from microsomal extracts.

Although BiP is an ATP-binding protein and is modified by azido-ATP, it appears to be distinct from the azido-ATP sensitive component that is involved in protein transport. Treatment of dog pancreas microsomes with octyl glucoside and subsequent removal of the detergent leads to depletion of the lumenal content. Under these conditions more than 90% of BiP is removed. Protein transport, however, is unaffected. Since it is very unlikely that photoaffinity modification leads to more than 90% derivatization of its targets, BiP cannot be the target of the observed inhibition of protein transport after photoaffinity modification of microsomes. However, this result does not rule out the possibility that BiP is involved in protein transport under these conditions.

In addition, ribonucleoparticle-independent transport of presecretory proteins involves a membrane component which is sensitive to chemical alkylation with *N*-ethylmaleimide, i.e. which has an essential sulfhydryl [3]. The sulfhydryl is cytoplasmically exposed and is involved in membrane insertion but not in membrane binding of the precursor proteins (M. Zimmermann, unpublished observation). This component may be identical to an *N*-ethylmaleimide-sensitive component which acts past docking protein and ribosome receptor in ribonucleoparticle-dependent transport [26,27].

The so-called SSR subunits appear to be part of the translocase and can be expected to be generally involved [28–32]. We addressed the question of what stage of ribonucleoparticle-dependent transport is affected after photoinactivation of microsomes by azido-ATP [33]. Thus, a nascent presecretory protein was employed. We observed that the nascent precursor protein does not become associated with the SSR complex after photoaffinity labeling of microsomes with azido-ATP. We concluded that the microsomal protein, which is sensitive to photoaffinity labeling with azido-ATP, acts prior to the SSR complex.

3. Discussion

3.1. Components involved in protein transport into yeast endoplasmic reticulum

With respect to yeast microsomes, genetic and biochemical evidence demonstrate a role for the cis-acting chaperone hsp70 and a second, NEM-sensitive, protein [34,35]. However, there also is ribonucleoparticle-dependent protein transport in yeast [36–38]. We assume that the two pathways converge at the level of a putative signal peptide receptor [39]. Genetic evidence suggests that the membrane proteins sec61, sec62 and sec63 (also termed ptl1 or npl1) are generally involved in protein transport [40–43]. Biochemical evidence suggests that the sec61, sec62 and sec63 proteins transiently form complexes with a 31.5 kDa glycoprotein and a 23 kDa protein, i.e. two proteins that are reminiscent of two mammalian ER proteins which have been termed SSR α- and β-subunit [44]. Furthermore, the trans-acting chaperone BiP (KAR2 gene product) has been shown to have a role in transport [45].

3.2. Model for ribonucleoparticle-independent transport

It is clear that precursor proteins have to be unfolded to be translocated and that unfolding has to occur on the cis-side of the respective membrane (Fig. 3). It ap-

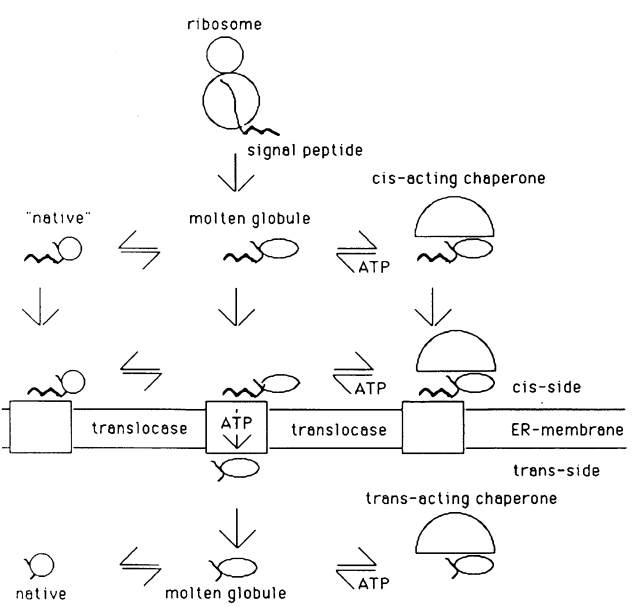

Fig. 3. Model for ribonucleoparticle-independent transport of presecretory proteins into the endoplasmic reticulum. Refer to Discussion for details.

pears that the signal peptide interferes with folding of the precursor to the native conformation of the mature part to a certain degree. Therefore, precursor proteins interact with molecular chaperones at some stage of their synthesis. This interaction has to be reversible, however, in order to eventually allow translocation. This may represent the point where ATP hydrolysis and the additional component come into action. Membrane association of the precursor proteins occurs via a putative signal peptide receptor. At this stage the precursor may be in a native-like folding state or in the molten globule state; it may be free or bound to a molecular chaperone.

With the help of the translocase, the signal peptides are then inserted into the membrane, most likely in the form of a loop structure which is made up by the signal peptide plus the amino terminus of the mature part. The ATP hydrolysis at the microsomal level seems to be directly providing the energy for membrane insertion. In order to become inserted, the precursor has to unfold at least partially, starting at its amino terminus. The question is where does the energy for unfolding come from. Practically all precursor proteins carry signal peptides that are cleaved off during or after translocation by signal peptidase. Thus, in principle, the differences between the free energies of precursor versus mature forms of a protein could be sufficient to drive unfolding at the surface. Furthermore, the energy for complete unfolding of a precursor protein may be as low as 10 kcal/mol, i.e. the initial hydrolysis of one ATP could be sufficient to drive such an unfolding reaction.

In order for translocation to progress, the protein on the cis-side has to unfold further. Again, the question is where does the energy for unfolding come from. A possible answer to this question may reside in the recent observation that protein transport into yeast microsomes involves the trans-acting molecular chaperone BiP. However, a similar requirement for BiP in mammalian microsomes has not yet been observed. It is tempting to speculate that binding of the precursor protein in transit to the trans-acting molecular chaperone provides the energy. Alternatively, completion of translocation may be driven by spontaneous refolding on the trans-side of the target membrane.

3.3. Open questions

Even 20 years after the signal hypothesis was first put forward, one of the major open questions is whether the components of the translocase form a pore, i.e. an aqueous channel that the precursor protein in transit passes through or whether the translocase is a set of enzymes that facilitates translocation at a lipid/protein interface.

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References

1. Wiech, H., Klappa, P. and Zimmermann, R. (1991) FEBS Lett. 285, 182–188.
2. Schlenstedt, G., Gudmundsson, G.H., Boman, H.G. and Zimmermann, R. (1990) J. Biol. Chem. 265, 13960–13968.
3. Zimmermann, R., Sagstetter, M. and Schlenstedt, G. (1990) Biochimie 72, 95–101.
4. Klappa, P., Mayinger, P., Pipkorn, R., Zimmermann, M. and Zimmermann, R. (1991) EMBO J. 10, 2795–2803.
5. Zimmermann, R. and Mollay, C. (1986) J. Biol. Chem. 261, 12889–12895.
6. Müller, G. and Zimmermann, R. (1987) EMBO J. 6, 2099–2107.
7. Müller, G. and Zimmermann, R. (1988) EMBO J. 7, 639–648.
8. Schlenstedt, G. and Zimmermann, R. (1987) EMBO J. 6, 699–703.
9. Yu, Y., Sabatini, D. and Kreibich, G. (1990) J. Cell Biol. 111, 1335–1342.
10. Connolly, T. and Gilmore, R. (1986) J. Cell Biol. 103, 2253–2261.
11. Connolly, T. and Gilmore, R. (1989) Cell 57, 599–610.
12. Connolly, T., Rapiejko, P.J. and Gilmore, R. (1991) Science 252, 1171–1173.
13. Römisch, K., Webb, J., Herz, J., Prehn, S., Frank, R., Vingron, M. and Dobberstein, B. (1989) Nature 340, 478–482.
14. Bernstein, H.D., Poritz, M.A., Strub, K., Hoben, P.J., Brenner, S. and Walter, P. (1989) Nature 340, 482–486.
15. Wiech, H., Sagstetter, M., Müller, G. and Zimmermann, R. (1987) EMBO J. 6, 1011–1016.
16. Zimmermann, R., Sagstetter, M., Lewis, J.L. and Pelham H.R.B. (1988) EMBO J. 7, 2875–2880.
17. Malkin, L.I. and Rich, A. (1967) J. Mol. Biol. 26, 329–346.
18. Blobel, G. and Sabatini, D.D. (1970) J. Cell Biol. 45, 130–145.
19. Bernabeu, C. and Lake, J.A. (1982) Proc. Natl. Acad. Sci. USA 79, 3111–3115.
20. von Heijne, G. (1981) Eur. J. Biochem. 116, 419–422.
21. Perlman, D. and Halvorson, H.O. (1983) J. Mol. Biol. 167, 391–409.
22. von Heijne, G. (1984) EMBO J. 3, 2315–2318.
23. Ainger, K.J. and Meyer, D.I. (1986) EMBO J. 5, 951–955.
24. Wiedmann, M., Kurzchalia, T.V., Bielka, H. and Rapoport, T.A. (1987) J. Cell Biol. 104, 201–208.
25. Robinson, A., Kaderbhai, M.A. and Austen, B.M. (1987) Biochem. J. 242, 767–777.
26. Hortsch, M., Avossa, D. and Meyer, D.I. (1986) J. Cell Biol. 103, 241–253.
27. Nicchitta, C.V. and Blobel, G. (1989) J. Cell. Biol. 108, 789–795.
28. Wiedmann, M., Kurzchalia, T.V., Hartmann, E. and Rapoport, T.A.(1987) Nature 328, 830–833.
29. Krieg, U.C., Johnson, A.E. and Walter, P. (1989) J. Cell Biol. 109, 2033–2043.
30. Hartmann, E., Wiedmann, M. and Rapoport, T.A. (1989) EMBO J. 8, 2225–2229.
31. Görlich, D., Prehn, S., Hartmann, E., Herz, J., Otto, A., Kraft, R., Wiedmann, M., Knespel, S., Dobberstein, B. and Rapoport, T. (1990) J. Cell Biol. 111, 2283–2294.

32. Prehn, S., Herz, J., Hartmann, E., Kurzchalia, T.V., Frank, R., Roemisch, K., Dobberstein, B. and Rapoport, T.A. (1990) Eur. J. Biochem. 188, 439–445.
33. Zimmermann, R., Zimmermann, M., Mayinger, P. and Klappa, P.(1991) FEBS Lett. 286, 95–99.
34. Chirico, W.J., Waters, G.M. and Blobel, G. (1988) Nature 332, 805–810.
35. Deshaies, R.J., Koch, B.D., Werner-Washburne, M., Craig, E.A. and Schekman, R. (1988) Nature 332, 800–805.
36. Ribes, V., Dehaux, P. and Tollervey, D. (1988) EMBO J. 7, 231–237.
37. Poritz, M.A., Siegel, V., Hansen, W. and Walter, P. (1988) Proc. Natl. Acad. Sci. USA 85, 4315–4319.
38. Hann, B.C., Poritz, M.A. and Walter, P. (1989) J. Cell Biol. 109, 3223–3230.
39. Sanz, P. and Meyer, D.I. (1989) J. Cell Biol. 108, 2101–2106.
40. Deshaies, R.J. and Schekman, R. (1987) J. Cell Biol. 105, 633–645.
41. Deshaies, R.J. and Schekman, R. (1989) J. Cell Biol. 109, 2653–2664.
42. Sadler, I., Chiang, A., Kurihara, T., Rothblatt, J., Way, J. and Silver, P. (1989) J. Cell Biol. 109, 2665–2675.
43. Toyn, J., Hibbs, A.R., Sanz, P., Crowe, J. and Meyer, D.I. (1988) EMBO J. 7, 4347–4353.
44. Deshaies, R.J., Sanders, S.L., Feldheim, D.A. and Schekman, R. (1991) Nature 349, 806–808.
45. Vogel, J.P., Misra, L.M. and Rose, M.D. (1990) J. Cell Biol. 110, 1885–1895.