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Transport of prepro-albumin into inverted vesicles prepared from the inner membrane of rat liver mitochondria

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When inverted vesicles prepared from the inner membrane of rat liver mitochondria were incubated with prepro-rat serum albumin, considerable amounts of prepro-albumin and pro-albumin were recovered with the inverted vesicles re-isolated by centrifugation. Pro-albumin was resistant to trypsin, but prepro-albumin was completely digested by trypsin, indicating that prepro-albumin was transported into the vesicles and concomitantly converted to pro-albumin. This transport process required ATP, but not a membrane potential. These results suggest that some export machinery for a protein having an amino acid sequence in its N-terminal portion similar to the signal sequence of secretory protein exists in the inner mitochondrial membrane.

Mitochondria; Protein transport; Transport machinery; Re-translocation

1. INTRODUCTION

Precursor proteins destined for the intermembrane space of fungus mitochondria, such as cytochrome b_2 and c_1, are synthesized on cytoplasmic ribosomes with a large presequence, which has two amino-terminal signals arranged in tandem. The first signal has the features of a typical matrix targeting signal, whereas the second signal contains a hydrophobic stretch with a similar feature to those of bacterial export signals and eukaryotic secretion signals [1–4]. The precursors are first translocated to translocation contact sites by the first signal, then they are transported into the intermembrane space. For transport into the intermembrane-space from the contact sites, two contrasting models, a conservative or re-export model [3–7] and a stop-transfer model [8–10] have been proposed.

According to the former case, the precursor is translocated into matrix, where its first signal is cleaved by a processing protease to be an intermediate-sized form, then transported into the intermembrane space followed by further processing to mature protein. In the latter one, the transfer of the precursors into the matrix stops when the second signal enters the inner membrane. After the first signal was cleaved off, an intermediate-sized protein attached across the inner membrane moves to the proper site of the inner membrane, resulting in transport of protein into the intermembrane space. Although there have been many reports to support these two models, it remains uncertain which model is more likely.

As mitochondria are considered to be organelles of endosymbiotic origin, it is expected that the inner mitochondrial membrane has some export machinery similar to the bacterial export machinery, which has been preserved during evolution. If a conservative model is the case, secretory proteins that have a similar presequence to the second presequence of precursors to the intermembrane space may be transported from the matrix into the intermembrane space. In this communication, we report that prepro-rat serum albumin (preproRSA) is post-translationally transported into inverted vesicles prepared from inner membranes of rat liver mitochondria, and discuss the possibility that the inner membrane is equipped with export machinery functioning for transport of the intermembrane space proteins.

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Abbreviations: preproRSA, prepro-rat serum alubumin; proRSA, pro-rat serum albumin.

2. MATERIALS AND METHODS

2.1. *Preparation of mitochondria and mitoplasts*

Mitochondrial and microsomal fractions were prepared from rat liver [11] and each fraction was suspended in Buffer A (220 mM mannitol, 70 mM sucrose, 10 mM HEPES-KOH (pH 7.6)). To prepare mitoplasts, mitochondria were suspended in a hypotonic solution of 10 mM potassium phosphate buffer (pH 7.5), kept for 30 min on ice with occasional gentle shaking, and centrifuged at $5,000 \times g$ for 10 min. The resulting precipitate was suspended in Buffer A and used as mitoplasts.

2.2. *Preparation of inverted inner membrane vesicles and right side-out inner membrane vesicles*

Preparation of inverted and right side-out vesicles was performed by a modified method based on the former reports [12,13]. Mitochondria suspended in Buffer A were disrupted by sonication. The sonicate

was adjusted to 1.5 M sucrose by adding 2.5 M sucrose containing 10 mM potassium phosphate buffer (pH 7.5) and centrifuged for 12 h at 100,000 × g. The pellet was suspended in Buffer A and used as inverted inner membrane vesicles. For preparation of right side-out inner membrane vesicles, mitochondria were first suspended in a hypotonic solution of 5 mM potassium phosphate buffer (pH 7.5) and kept for 30 min at 0°C. Subsequent procedures, including sonication, were as for preparation of inverted vesicles.

2.3. *Other procedures*

Cell-free translation programmed by rat liver RNA was performed using a rabbit reticulocyte lysate [14], and the translation reaction was terminated by the addition of cycloheximide (final concentration, 10 μg/ml). Cell-free translation products were centrifuged for 30 min at 320,000 × g to remove ribosomes and a final concentration of 0.48 M mannitol was added to the supernatant before its use.

Transport of preproRSA into membrane vesicles was analyzed by immunoprecipitation, SDS-PAGE, and fluorography as described previously [14].

3. RESULTS

3.1. *Transport of preproRSA into inverted vesicles but not right side-out vesicles*

Inverted inner membrane vesicles were incubated with cell-free translation products, and re-isolated by centrifugation. After solubilization of the vesicles with detergent, precursors of albumin were immunoprecipitated, subjected to SDS-PAGE, and detected by fluorography. Two distinct bands of preproRSA and proRSA, respectively, were found (lane 1, Fig. 1), suggesting that these two proteins were bound to or imported into the vesicles. Treatment of the vesicles with proteinase K before detergent-solubilization resulted in the disappearance of the band of preproRSA, but not of proRSA (lane 2). These results clearly indicate that preproRSA was transported into inverted vesicles and that during this transport, the prepro-form of albumin was converted to its pro-form, possibly by a peptidase in the inner membrane. This conclusion was supported by the finding that proRSA was completely digested when the vesicles were incubated with proteinase K in the presence of Triton X-100 (lane 3). In contrast, no transport of preproRSA into right side-out vesicles was

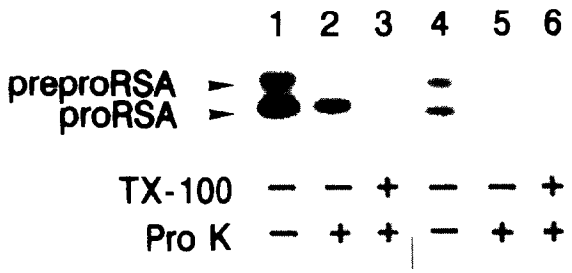

Fig. 1. Transport of preproRSA into two kinds of inner mitochondrial membrane vesicles. Inverted inner membrane vesicles and right side-out inner membrane vesicles (1.5 mg/ml each) were incubated with cell-free translation products in a final volume of 400 μl for 60 min at 32°C in the presence of cycloheximide (final concentration; 10 μg/ml). Then each reaction mixture was divided into three portions. One portion was chilled on ice, while the second and third portions were treated with 200 μg/ml proteinase K in the absence and presence of 1% Triton X-100 for 90 min at 0°C, respectively. The first and second portions were layered onto 2 ml of 0.75 M sucrose containing 5 mM potassium phosphate buffer (pH 7.5) and 150 mM potassium acetate in centrifuge tubes and centrifuged for 30 min at 320,000 × g to precipitate the vesicles. Each precipitate and the third portion were solubilized with 2% SDS and analyzed by immunoprecipitation, SDS-PAGE, and fluorography TX-100 and Pro K indicate Triton X-100 and proteinase K, respectively. Lanes 1–3, inverted vesicles; lanes 4–6, right side-out vesicles.

observed (lanes 4–6). These findings suggest that the inner membrane is equipped with transport-machinery for preproRSA from the matrix side to the intermembrane space.

3.2. *No post-translational transport of preproRSA into microsomes, mitochondria, or mitoplasts*

In order to confirm that the transport of preproRSA is a specific event in inverted vesicles of the inner mitochondrial membrane, we examined microsomes, mitochondria, and mitoplasts for their ability to transport the cell-free translation products. Microsomes bound a large amount of preproRSA (lane 1, Fig. 2), which disappeared completely after treatment with proteinase K (lane 2), indicating that preproRSA was only bound to the surface of the microsomes. These observations are

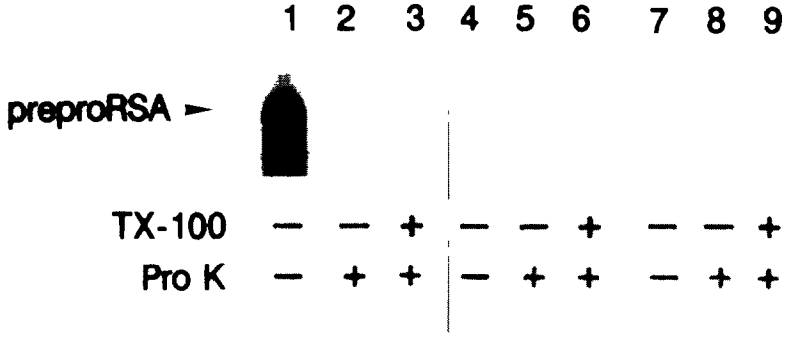

Fig. 2. Incompetence of microsomes, mitochondria, and mitoplasts for import of preproRSA. Microsomes, mitochondria, and mitoplasts (1.5 mg/ml each) were individually incubated with cell-free translation products in a final volume of 400 μl for 60 min at 32°C in the presence of cycloheximide (10 μg/ml). Then, each reaction mixture was divided into three portions. Subsequent procedures were as described in the legend for Fig. 1. Lanes 1–3, microsomes; lanes 4–6, mitochondria; lanes 7–9, mitoplasts.

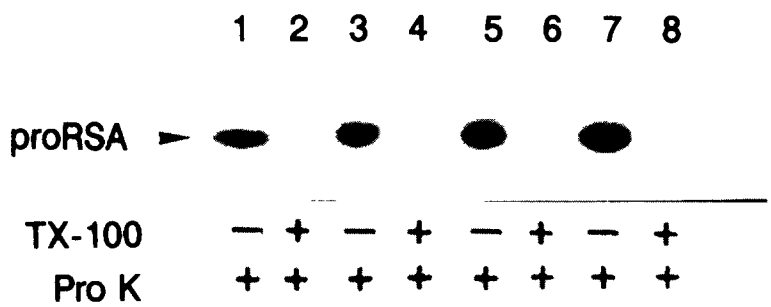

Fig. 3. Membrane-potential independent transport of preproRSA into inverted vesicles. Cell-free translation products were individually incubated with inverted vesicles that had been pretreated for 20 min at 0°C with one of the reagents listed below. Subsequent procedures were as described in the legend for Fig. 1. Lanes 1 and 2, untreated; lanes 3 and 4, treated with 20 μM carbonyl cyanide *m*-chlorophenylhydrazone; lanes 5 and 6, treated with a mixture of 20 μM oligomycin and 10 μM antimycin; lanes 7 and 8, treated with 2 μM valinomycin.

consistent with the established view that protein translocation into microsomes of eukaryotic cells is co-translational, but not post-translational. No transport of preproRSA into mitochondria was detected (lanes 4–6). Mitoplasts also did not import preproRSA (lanes 7–9), as expected from the results with right side-out vesicles.

The precursor of ornithine aminotransferase, a mitochondrial matrix enzyme, which has a cleavable presequence at its N-terminus, was not imported into inverted vesicles (data not shown), but it was efficiently imported into mitochondria, consistent with our previous result [15].

These findings indicate that post-translational transport of preproRSA is specific to inverted inner membrane vesicles, and suggest that the transport machinery accepts only precursor proteins with N-terminal sequences similar to that of the presequence of preproRSA.

3.3. *Requirement of ATP, but not a membrane potential for transport of preproRSA*

Energy from both a membrane potential and ATP outside mitochondria are commonly required for transport of mitochondrial precursor proteins into the matrix. Therefore, we examined the effects of some reagents that destroy the membrane potential such as carbonyl cyanide *m*-chlorophenylhydrazone, a mixture of oligomycin and antimycin, and valinomycin. These reagents did not inhibit, but rather stimulated transport of preproRSA (Fig. 3). We confirmed that these reagents disrupted the membrane potential, since they completely blocked import of pre-ornithine aminotransferase into mitochondria under similar experimental conditions (data not shown). Therefore, a membrane potential is apparently not necessary for transport of preproRSA into inverted vesicles.

To test requirement for ATP, we depleted ATP by treatment with apyrase, which hydrolyzes ATP to ADP and AMP. This treatment resulted in complete inhibition of transport of preproRSA into the inverted vesicles (lane 3, Fig. 4). Moreover, subsequent addition of ATP to the reaction mixture restored the import of preproRSA and a considerable amount of proRSA was again detected as seen in lane 5. These observations clearly indicate that the translocation reaction requires ATP outside the vesicles.

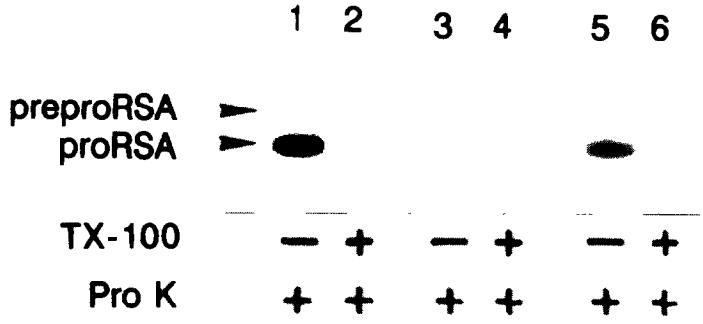

Fig. 4. Effect of ATP depletion on the transport reaction. The cell-free translation products with or without apyrase treatment (0.2 U/ml) for 30 min at 25°C were used for transport-reactions. In experiments on ATP supplementation, 1 μmol of ATP was added at 15 min intervals from the beginning to the end of transport-reactions carried out for 60 min at 32°C. Other reaction conditions and experimental procedures were as described for Fig. 1. Lanes 1 and 2, untreated translation products; lanes 3 and 4, translation products treated with apyrase; lanes 5 and 6, translation products treated with apyrase, and then supplemented with ATP.

4. DISCUSSION

Two models have been proposed in sorting of the intermembrane space proteins, as already mentioned. In the stop-transfer model, not any translocation machinery present in the inner membrane are required. However, we showed the first evidence that the inner membrane of mitochondria has transport machinery for preproRSA from the matrix side to the intermembrane space. So it is highly possible that the intermembrane space proteins are transported by the conservative pathway. Because of similarities of the presequences of preproRSA and the intermediate-sized form of precursor proteins destined for the intermembrane space, preproRSA may be transported by the transport machinery that might have originated from a prokaryotic ancestor and functions naturally to re-export the intermediate-sized form.

We found that during transport of preproRSA into the inverted vesicles, its signal sequence was cleaved. Possibly a protease present in the inner membrane, named inner membrane protease I [16,17], with similar properties to those of the leader peptidase in *E. coli*, functions as a processing enzyme.

We found that ATP is essential for transport of preproRSA into the inverted vesicles, although it is not clear which step requires ATP. Probably the inner membrane may have some factor like SecA which is necessary for translocation of proteins across the bacterial membrane consuming ATP [18–21]. Interestingly, however, transport of preproRSA into inverted inner membrane vesicles did not require a membrane potential.

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REFERENCES

[1] van Loon, A.P.G.M., Brändli, A.W. and Schatz, G. (1986) Cell 44, 801–812.
[2] van Loon, A.P.G.M., Brändli, A.W., Pesold-Hurt, B., Blank, D. and Schatz, G. (1987) EMBO J. 6, 2433–2439.
[3] Hartl, F.-U., Ostermann, J., Guiard, B. and Neupert, W. (1987) Cell 51, 1027–1037.
[4] Stuart, R.A., Nicholson, D.W., Wienhues, U. and Neupert, W. (1990) J. Biol. Chem. 265, 20210–20219.
[5] Hartl, F.-U. and Neupert, W. (1990) Science 247, 930–938.
[6] Sequi-Real, B., Stuart, R.A. and Neupert, W. (1992) FEBS Lett. 313, 2–7.
[7] Jensen, R.E., Schmidt, S. and Mark, R.J. (1992) Mol. Cell. Biol. 12, 4677–4686.
[8] van Loon, A.P.G.M. and Schatz, G. (1987) EMBO J. 6, 2441–2448.
[9] Nguyen, M., Bell, A.W. and Shore, G.C. (1988) J. Cell Biol. 106, 1499–1505.
[10] Glick, B.S., Brandt, A., Cunningham, K , Müller, S., Hallberg, R.L. and Schatz, G. (1992) Cell 69, 809–822.
[11] Ono, H. and Ito, A. (1984) J. Biochem. 95, 345–352.
[12] Malviya, A.N., Parsa, B., Yodaiken, R.E. and Elliott, W.B. (1968) Biochim. Biophys. Acta 162, 195–209.
[13] Kagawa, Y. (1984) in: Methods of Cell Fractionation (in Japanese) (Mohri, H. and Kagawa, Y., eds) Lectures on Experimental Biology, vol. 6, pp. 141–152, Maruzen, Tokyo.
[14] Ono, H., Yoshimura, N., Sato, M. and Tuboi, S. (1985) J. Biol. Chem. 260, 3402–3407
[15] Ono, H. and Tuboi, S. (1986) Eur. J. Biochem. 155, 543–549.
[16] Behrens, M., Michaelis, G. and Pratje, E. (1991) Mol. Gen. Genet. 228, 167–176.
[17] Schneider, A., Behrens, M., Scherer, P., Pratje, E., Michaelis, G. and Schatz, G. (1991) EMBO J. 10, 247–254.
[18] Kawasaki, H., Matsuyama, S., Sasaki, S., Akita, M. and Mizushima, S. (1989) FEBS Lett. 242, 431–434.
[19] Cunningham, K., Lill, R., Crooke, E., Rice, M., Moore, K., Wickner, W. and Oliver, D. (1989) EMBO J. 8, 955–959.
[20] Lill, R., Cunningham, K., Brundage, L.A., Ito, K., Oliver, D. and Wickner, W. (1989) EMBO J. 8, 961–966.
[21] Akita, M., Sasaki, S., Matsuyama, S. and Mizushima, S. (1990) J. Biol. Chem. 265, 8164–8169.