Signal-anchored proteins Follow a Unique Insertion Pathway into the Outer Membrane of Mitochondria*

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Signal-anchored proteins are a class of mitochondrial outer membrane proteins that expose a hydrophilic domain to the cytosol and are anchored to the membrane by a single transmembrane domain in the N-terminal region. Like the vast majority of mitochondrial proteins, signal-anchored proteins are synthesized on cytosolic ribosomes and are subsequently imported into the organelle. We have studied the mechanisms by which precursors of these proteins are recognized by the mitochondria and are inserted into the outer membrane. The import of signal-anchored proteins was found to be independent of the known import receptors, Tom20 and Tom70, but to require the major Tom component, Tom40. In contrast to precursors destined to internal compartments of mitochondria and those of outer membrane β-barrel proteins, precursors of signal-anchored proteins appear not to be inserted via the general import pore. Taken together, we propose a novel pathway for insertion of these proteins into the outer membrane of mitochondria.

The mitochondrial outer membrane mediates numerous interactions between the metabolic and genetic systems of mitochondria and the rest of the eukaryotic cell. Proteins residing in the outer membrane include a diverse set of enzymes, components of the import machinery of the outer membrane of mitochondria (the TOM and TOB complexes), and pore-forming components (porins). In addition, the outer membrane contains proteins that control fusion, fission, morphology, and the inheritance of the organelle. In higher eukaryotes, members of the pro- and anti-apoptotic Bel-2 family also reside in the mitochondrial outer membrane. All these proteins, like the vast majority of mitochondrial proteins, are nuclear encoded and synthesized in the cytosol. They carry signals that are essential for their subsequent import into mitochondria.

The targeting of most mitochondrial preproteins is mediated by cleavable N-terminal extensions of about 12–50 amino acid residues (the presequences or matrix targeting signals). These are necessary and sufficient to direct them into the mitochondria (1, 2). In contrast, all precursor proteins of the mitochondrial outer membrane are devoid of a typical N-terminal presequence. The targeting information is rather contained in the sequence of the functional protein (3, 4).

A special class of mitochondrial outer membrane proteins comprises those containing a single transmembrane segment at their N terminus. Examples of this class are Tom20, Tom70, and OM45 (5, 6). These proteins are present in the outer membrane in an orientation, where the bulk of the polypeptide is exposed to the cytosol and only a small N-terminal segment crosses the outer membrane. They are called “signal-anchored” proteins because their transmembrane domain together with their flanking regions serve both as an intracellular sorting signal and as an anchor to the membrane (7). The important structural elements of such signal-anchored sequences were found to be a moderate hydrophobicity of the transmembrane domain and positively charged residues in its flanking regions (6, 8).

Despite this progress in the characterization of signal-anchored domains, the mechanisms by which this type of signal is deciphered at the outer membrane and the protein is inserted into the lipid bilayer are only poorly understood. One reason for this is the difficulty of differentiating between nonspecific binding of precursor proteins and physiological membrane integration in vitro import systems (4).

In this study we have established a reliable and specific assay to study the insertion of signal-anchored proteins into the outer membrane of isolated mitochondria. This new assay allowed us to obtain new insights into the mechanism of membrane insertion. Our results suggest that signal-anchored proteins are inserted into the outer membrane by a TOM complex-dependent pathway that does not require import receptors and participation of the TOM import channel.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Methods—Standard genetic techniques were used for growth and manipulation of yeast strains (9). The wild type strains BY4743 and 273-10B were employed. Transformation of yeast was carried out according to the lithium acetate method. Yeast cells were grown under aerobic conditions on YPD (1% (w/v) yeast extract, 2% (w/v) bactopeptone, 2% glucose) yeast extract, 2% (w/v) bactopeptone, 2% glucose) or on YPG (1% (w/v) yeast extract, 2% (w/v) bactopeptone, 3% glycerol) medium. The tm20 null strain YJ786 and its corresponding parental strain YJ784 were utilized (kind gift of Drs. G. Schatz and C. Koehler) (10). The tom70 deletion strain was obtained from Research Genetics (Huntsville, AL).

Biochemical Procedures—Mitochondria were isolated from yeast cells by differential centrifugation as described (11). For isolation of mitochondria from tom40-3 strain and its corresponding wild type yeast cells were grown at 25 °C. Radiolabeled precursor proteins were synthesized in rabbit reticulocyte lysate in the presence of [35S]methionine (MP Biomedicals) after in vitro transcription by SP6 polymerase from pGEM4 vectors (Promega) containing the gene of interest. Import experiments were performed in a buffer containing 250 mM sucrose, 0.25 mg/ml bovine serum albumin, 80 mM KCl, 5 mM MgCl2, 10 mM MOPS1-KOH, 2 mM NADH, 2 mM ATP, pH 7.2. Isola-

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1 The abbreviations used are: MOPS, 4-morpholinoepanesulfonic acid; IMS, intermembrane space; DHFR, dihydrofolate reductase; PK, proteinase K.
tion of the TOM core complex and its reconstitution into liposomes were performed as described before (12, 13). Blotting to polyvinylidene difluoride or nitrocellulose membranes and immunodetection were according to standard procedures, and visualization was by the ECL method (Amersham Biosciences).

Recombinant DNA Techniques—For in vitro transcription and translation of Tom20 variants, the Tom20 open reading frame was amplified by PCR from genomic yeast DNA. The resulting product was digested with BamHI and HindIII and cloned into the BamHI/HindIII sites of pGEM vector. The Tom20 variant was constructed by PCR amplification of the sequence encoding the N-terminal 38 amino acid residues of Neurospora crassa Tom70 and treatment of the DNA fragment with BamHI and subcloning it into the BamHI site of pGEM4-Tom20. The Tom20 variant was introduced into the multicopy yeast plasmid, pRS426xTPIp-URA3 (kind gift of Dr. K. Dietmeier). To improve our analysis of in vitro import experiments, an N. crassa Tom70 construct was used where a methionine residue was inserted between amino acid residues 38 and 39.

RESULTS

A major problem in analyzing the membrane insertion of N-terminally anchored signal-anchored proteins into the outer membrane is the lack of a reliable assay to control for the correct insertion into the membrane. The known N-terminally anchored signal-anchored proteins in yeast mitochondria expose only a few amino acid residues into the intermembrane space (IMS). Therefore, proteolytic cleavage of signal-anchored proteins with external protease results in the formation of protected fragments in the size range of 2–3 kDa, which are too small to be observed by SDS-PAGE. To overcome this problem, we constructed a fusion protein where the first 38 amino acid residues of Tom70 from N. crassa were fused in front of yeast Tom20 (Fig. 1A, Tom20ext). Proteolytic cleavage of this construct with external protease after insertion into the outer membrane is predicted to result in the formation of a 7–8-kDa fragment. To demonstrate that this Tom20 variant is correctly targeted to mitochondria in vivo, we used a functional complementation assay (6). Deletion of the gene encoding Tom20 in yeast results in cells that cannot grow on a non-fermentable carbon source (14). Therefore, the ability of Tom20 variants to complement the growth phenotype can be taken as a criterion for correct targeting and function in the TOM complex. Tom20ext was found to complement the growth phenotype of Δtom20, demonstrating that this variant is functional in the outer membrane (Fig. 1B).

To check whether the anticipated fragment can indeed be observed, radiolabeled Tom20ext precursor was imported into isolated mitochondria, and these were then treated with proteinase K (PK). A fragment of the expected size of 7 kDa was visible upon SDS-PAGE. This fragment was completely degraded upon solubilization of the organelle with the detergent Triton X-100, and it remained with the membrane fraction upon alkaline treatment of mitochondria (Fig. 1C). The 7-kDa fragment was not observed when either reticulocyte lysate containing the Tom20ext precursor or membrane-inserted authentic Tom20 were treated with PK. Taken together, the 7-kDa band represents a specific fragment of the membrane-inserted Tom20ext. Thus, this proteolytic assay can be employed to study the mechanism of insertion of Tom20 into the mitochondrial outer membrane.

By using the new in vitro assay, we examined the require-
Radiolabeled Tom20 from the TOM complex can be inhibited by blocking the import pore. The insertion of Tom20, as well as porin, was strongly inhibited (Fig. 3A). A pool of wild-type mitochondria was incubated with radiolabeled Tom20ext and the amount of inserted precursor was quantified by SDS-PAGE and autoradiography. Import of radiolabeled Tom20ext, however, was not reduced upon this treatment (Fig. 3C). We conclude that signal-anchored proteins do not use the general import pore or do it in a completely different way compared with other mitochondrial precursors.

Is the TOM complex involved in the import of signal-anchored proteins? We investigated the insertion of Tom20ext into mitochondria isolated from a strain harboring the temperature-sensitive allele of tom40. This strain (tom40-3) was used before to study the import of porin into the outer membrane. It did not display altered insertion and assembly of this protein which is known to use the general insertion pore of the TOM complex (15, 16). In contrast, the insertion of Tom20ext into the Tom40-3 mitochondria was strongly reduced in comparison to insertion into wild type mitochondria (Fig. 4). Thus, Tom40 does play a role in the insertion of signal-anchored proteins. This appears to be a different mechanism of signal-anchored proteins as compared with β-barrel proteins.

We asked whether the TOM complex by itself is sufficient to promote the import of signal-anchored proteins. The TOM core complex (containing Tom40, Tom22, Tom7, Tom6, and Tom5) but lacking Tom20 and Tom70 (12) from N. crassa mitochondria was used to generate proteoliposomes (11). The precursor of Tom20ext was incubated with liposomes or proteoliposomes containing either the TOM core complex or porin. Insertion of radiolabeled Tom20ext precursor was followed by analyzing the formation of the typical 7-kDa fragment. Background levels of insertion were observed when pure lipid vesicles were used (Fig. 5A). The amount of the proteolytic fragment was increased by ~3-fold when vesicles containing the TOM core complex were used. Reconstituted porin had only a minor effect on the insertion of the protein (Fig. 5A). Similar results were obtained when the precursor of N. crassa Tom70 was used. Insertion of radiolabeled Tom70 precursor was followed by analyzing the formation of a typical fragment result-
ing from addition of external proteinase K (Fig. 5B). The relatively low enhancement factor observed with the TOM complex is most likely due to the fact that only about half of the reconstituted complexes had the correct orientation in the membrane (data not shown). Hence, only a fraction of the reconstituted TOM core complex had import activity.

**Fig. 3.** Insertion and assembly of signal-anchored proteins is not inhibited by blocking the translocation pore with import intermediates. **A,** the insertion of Tom20 is not affected by excess of matrix-targeted precursor. Isolated yeast mitochondria were incubated for 5 min at 0 °C in the presence or absence of recombinant (Recomb.) pSu9-(1–69)-DHFR. Radiolabeled precursor proteins were then added and further incubated at 25 °C for the indicated times. At the end of the import reactions, proteinase K (100 μg/ml) was added; proteins were analyzed by SDS-PAGE, and imported proteins were quantified. The insertion of Tom20ext was quantified by analyzing the formation of the 7-kDa fragment (F), whereas for pSu9-(1–69)-DHFR the protease protected mature form (m) was quantified. The precursor, intermediate, and mature forms of pSu9-(1-69)-DHFR are indicated as p, i, and m, respectively. The amount of precursor proteins imported into control mitochondria for the longest time period was set to 100%.

**B,** assembly of Tom20 into the TOM complex is not competed by excess of preprotein. Isolated *N. crassa* mitochondria were incubated for 10 min at 25 °C in the presence or absence of recombinant pSu9-(1–69)-DHFR. Radiolabeled precursor proteins were then added and further incubated at 15 °C for the indicated times. At the end of the import reactions, mitochondria were reisolated, solubilized in buffer containing 1% digitonin, and analyzed by blue native gel electrophoresis. The three main stages of import of Tom40 are indicated: I1, first intermediate; I2, second intermediate; A, assembled material (see Ref. 28). For detection of the endogenous TOM complex, antibodies against Tom22 were used. The amount of precursor proteins imported into control mitochondria for the longest time period was set to 100%. C, the insertion of OM45 is not affected by excess of matrix-targeted precursor. Isolated yeast mitochondria were incubated in the presence or absence of recombinant pSu9-(1–69)-DHFR. Radiolabeled precursor proteins were then added and further incubated at 15 °C for the indicated times. At the end of the import reactions, mitochondria were reisolated and resuspended in 0.1 M Na2CO3 for carbonate extraction. The pellets of the extraction containing the membrane-embedded proteins were analyzed by SDS-PAGE and autoradiography. p and m, precursor and mature forms of pSu9-(1–112)-DHFR, respectively. A shorter form of OM45 precursor (OM45Δ20) resulting from initiation of translation at Met-21 is indicated. This form is not insertion-competent (see Ref. 6).
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Fig. 4. Import of Tom20 is affected in tom40-3 mutant mitochondria. A, mitochondria isolated from the yeast mutant strain tom40-3 and the corresponding wild type strain (WT) were preincubated at 37 °C for 10 min. Radiolabeled precursor of Tom20ext was then added and incubated at 25 °C for the indicated times. After import the samples were treated with proteinase K, and mitochondria were reisolated. Imported proteins were analyzed by SDS-PAGE and autoradiography. B, the bands corresponding to the 7-kDa fragment (F) of Tom20ext from three experiments as described in A were quantified, and average values are presented. The amount of precursor proteins inserted into control mitochondria after incubation for 20 min was set to 100%.

Fig. 5. Insertion of Tom20ext and Tom70 into proteoliposomes containing the TOM core complex. Radiolabeled precursors of Tom20ext (A) and N. crassa Tom70 (B) were incubated with either liposomes without reconstituted proteins or proteoliposomes containing either reconstituted porin or the reconstituted TOM core complex. After import, samples were halved. One half was left untreated, and the other was treated with PK (500 μg/ml). The proteoliposomes were reisolated (40 min, 36,700 × g), resuspended in sample buffer, and subjected to SDS-PAGE and autoradiography. The bands corresponding to the proteolytic fragments were quantified, and the insertion efficiency into liposomes was set to 1.

DISCUSSION

What are the mechanisms and components by which signal-anchored proteins are inserted into the outer membrane of mitochondria? The investigation of this problem has been hindered by the lack of available assays by which insertion of such precursor proteins can be reliably analyzed. In previous studies resistance toward alkaline extraction was taken as a criterion for correct insertion of the proteins into the outer membrane. This method, however, is hampered by the inability to discriminate between nonspecific binding and physiological membrane integration of precursor proteins (4). Therefore, we have developed a reliable assay to measure insertion, namely formation of a defined proteolytic fragment in intact mitochondria. This fragment contains the inserted transmembrane anchor and the intermembrane space segment which are not accessible to externally added protease.

Our results suggest that the known import receptors Tom20 and Tom70 as well as domains of other proteins exposed on the surface of mitochondria are not required for proper insertion. These results are in line with previous observations where the mitochondrial targeting of Tom20 and Tom70 was found to be independent of protease-accessible surface receptors and not to be affected by addition of antibodies against Tom20 and Tom70 (19–21). Furthermore, transformation of yeast cells lacking the chromosomal copy of TOM20 with a yeast expression vector encoding Tom20 rescued the growth phenotype of the Δtom20 cells (6). Thus, pre-existing Tom20 molecules are not required for the import of newly synthesized Tom20 precursors.

It appears that signal-anchored proteins are recognized either directly by the membrane-embedded components of the TOM core complex or by a yet unidentified outer membrane protein. The first possibility appears more likely, as we found that the purified TOM core complex can facilitate the insertion of Tom20 into proteoliposomes. Moreover, our results do support an involvement of Tom40 in the insertion process. This notion is in line with a previous report (19) where antibodies against the pore protein Tom40 inhibited the membrane integration of newly synthesized Tom20. Still, additional factors may assist the insertion process.

Two different scenarios seem possible for the steps following the initial recognition by the TOM complex (Fig. 6). In the first, the signal-anchored domain is inserted into the lumen of the translocation pore formed by Tom40, after which the β-barrel structure opens and the protein is laterally released into the lipid core of the membrane (Fig. 6, pathway b). Such an opening of a β-barrel structure, however, is thermodynamically unfavorable (22). We favor a model where the precursor protein is inserted at the interface between the TOM core complex and the lipid phase of the membrane (Fig. 6, pathway a). In such a mechanism some parts of the Tom40 molecule, with the poten-
tial involvement of the membrane-embedded segments of other components of the TOM core complex, mediate the insertion of signal-anchored proteins, whereas other segments are forming the import channel. A recent report on the involvement of Tom40 in sorting of various precursor proteins would support such a model. A Tom40 variant that is ineffective in transfer of presequence-containing preproteins, but can support normal insertion of outer membrane proteins, was characterized (23). In addition, cytochrome c, which resides in the mitochondrial IMS, was also suggested to be imported by a TOM complex-dependent pathway that does not use the general import pore (24). Hence, it appears that some mitochondrial precursors can be imported by the TOM complex in a process that is distinct from that followed by most mitochondrial precursors.

This hypothesis is in line with a recent report on the insertion of outer membrane proteins in chloroplasts. The insertion of a model outer membrane protein, OEP14, was found to be mediated by the main component of the TOC complex, Toc75 (25). Furthermore, similar to our proposal for the mitochondrial system, Tu et al. (25) suggested that two different sites on Toc75 may be involved in binding to either signal sequence-containing precursors or to precursors of outer membrane proteins. Thus, the capacity of the pore-forming component of the translocases of endosymbiont organelles to mediate distinct ways of import into either internal compartments or the outer membrane seems to be an evolutionary conserved function.

The question of whether a lateral opening of the TOM channel allows precursors of outer membrane proteins to be inserted into the lipid core of the membrane is still unresolved (22, 26). Recent findings suggest that β-barrel precursors are crossing the outer membrane into the IMS via the general import pore of the TOM complex before the TOB complex mediates their insertion into the outer membrane (18, 26, 27). Hence, a lateral opening of the TOM channel appears not to occur during insertion of β-barrel proteins. Our present results suggest that the import of signal-anchored proteins does not occur by a lateral opening of the general import pore of the TOM complex. It is not excluded, however, that in certain cases the dynamics of the TOM complex does allow such a reaction.

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