Minireview

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The Protein Import Machinery of Mitochondria*

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Most mitochondrial proteins are encoded by nuclear genes and then synthesized as precursors on cytosolic ribosomes after which they must be imported into the organelle. The mitochondrial membranes contain specific machineries (translocases) for recognition, translocation, and membrane insertion of precursor proteins. In recent years, important progress has been made in characterizing the molecular mechanisms and cooperation of the mitochondrial preprotein translocases. Here, we will summarize the principles of mitochondrial protein import, which are well conserved in the eukaryotic kingdom.

Protein Import Pathways into Mitochondria

Mitochondria consist of two membranes, the outer membrane and the folded inner membrane, and two aqueous compartments, the intermembrane space and the matrix (Fig. 1, *inset*). Mitochondria play crucial roles in cellular energy production and the metabolism of amino acids, iron, and lipids, as well as in apoptosis. Proteome analyses indicate that mitochondria contain about 800 (yeast) to 1,500 (human) different proteins (1–3). Although mitochondria possess a complete genetic system in the matrix, only about 1% of all mitochondrial proteins are encoded by the mitochondrial genome. The vast majority of mitochondrial proteins are synthesized as precursor proteins in the cytosol and are imported mainly by a post-translational mechanism (4–9). Cytosolic chaperones are involved in guiding the precursor proteins to receptors on the mitochondrial surface.

Mitochondrial precursor proteins can be separated into two main classes. Preproteins that are destined for the mitochondrial matrix, as well as a number of proteins of the inner membrane and intermembrane space, carry N-terminal cleavable extensions, termed presequences. These positively charged extensions function as targeting signals that interact with the mitochondrial import receptors and direct the preproteins across both outer and inner membranes (4, 8). The second class of precursor proteins, carrying various internal targeting signals, includes all outer membrane proteins along with many intermembrane space and inner membrane proteins. These precursors are synthesized without cleavable extensions, that is they have the same primary structure as the mature protein, yet their conformation typically differs from the mature protein (5, 8).

The translocase of the outer mitochondrial membrane (TOM[1] complex) represents the central entry gate for practically all nuclear-encoded mitochondrial proteins (Fig. 1). The TOM complex consists of several preprotein receptors and a general import pore. After passing through the TOM complex, the precursor proteins can follow one of three major pathways (5–9). (i) Preproteins with a presequence are transferred to the presequence translocase of the inner membrane, also termed the TIM23 complex (translocase of the inner membrane). The presequence translocase forms a channel across the inner membrane and cooperates with the matrix heat shock protein 70 (mtHsp70). The molecular chaperone mtHsp70 represents the core of the presequence translocase-associated motor (PAM), which drives the completion of protein transport into the matrix. (ii) The precursors of many hydrophobic proteins of the inner membrane, such as the metabolite carriers, follow a different pathway to the inner membrane that involves chaperone-like components of the intermembrane space and the protein insertion machinery of the inner membrane (carrier translocase, TIM22 complex). (iii) The precursors of outer membrane proteins, like the abundant protein porin, are integrated into the outer membrane by the sorting and assembly machinery (SAM complex).

Translocase of the Outer Mitochondrial Membrane

The TOM complex consists of seven different subunits that can be grouped into three categories (Fig. 2): the receptors Tom20, Tom22, and Tom70; the channel-forming protein Tom40; and three small Tom proteins, Tom5, Tom6, and Tom7 (10–12). Tom20 is the first receptor involved in recognizing the presequence of a preprotein. A typical presequence has a length of about 10–30 amino acid residues and forms an amphipathic α-helix. One half of the helix possesses a hydrophobic surface that is recognized by a binding groove within Tom20 (13), whereas the other half is positively charged and recognized by the receptor Tom22 (14). With the help of the small protein Tom5, the preprotein is then transported to the general import pore (15) formed by the essential β-barrel protein Tom40 (10). After translocation through the Tom40 pore, the presequence binds to the intermembrane space domain of the receptor Tom22 (16). Tom40 itself does not simply form a passive pore but rather interacts with the preproteins in transit. Preproteins with a cleavable presequence are thus guided across the outer membrane by a chain of binding sites, including the cytosolic receptors Tom20, Tom22, Tom5, the Tom40 translocation channel, and the intermembrane space domain of Tom22 (binding chain hypothesis) (16, 17). The other small Tom proteins, Tom6 and Tom7, do not directly interact with precursor proteins but are required for the assembly and stability of the TOM complex (18).

The precursors of the hydrophobic carrier proteins of the inner membrane, *e.g.* the ADP/ATP carrier or the phosphate carrier, are initially recognized by a different receptor at the

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[1] The abbreviations used are: TOM, translocase of outer mitochondrial membrane; mtHsp70, matrix heat shock protein 70; $\Delta\psi$, membrane potential; Hsp, heat shock protein; MPP, mitochondrial processing peptidase; PAM, presequence translocase-associated motor; SAM, sorting and assembly machinery; TIM, translocase of inner mitochondrial membrane.

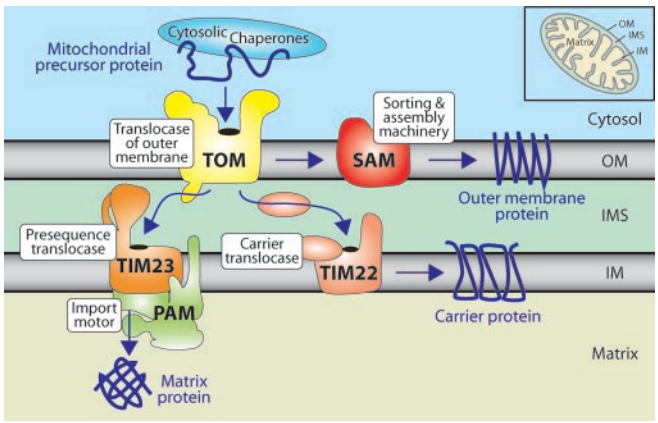

FIG. 1. **Protein import pathways into mitochondria.** Most mitochondrial proteins are synthesized in the cytosol. With the help of cytosolic chaperones, mitochondrial precursor proteins are transferred to the general entry gate of mitochondria, the TOM complex, from where they are subsequently sorted into one of the mitochondrial subcompartments. The precursors of β-barrel outer membrane proteins require the SAM complex. Preproteins destined for the matrix depend on the presequence translocase (TIM23 complex) and its associated import motor (PAM complex) for their transport across the inner mitochondrial membrane. Carrier proteins are inserted into the inner membrane with the help of the carrier translocase (TIM22 complex). *OM*, outer membrane; *IMS*, intermembrane space; *IM*, inner membrane.

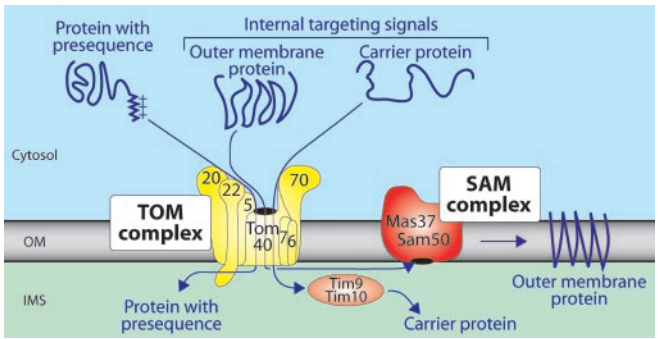

FIG. 2. **Protein translocation across and into the outer mitochondrial membrane.** Mitochondrial precursor proteins possess either an N-terminal presequence (positively charged amphipathic α-helix) or contain internal targeting signals. The presequence is recognized by the import receptors Tom20 and Tom22, after which the preprotein is transferred with the help of Tom5 to the general import pore formed by Tom40. The TOM complex contains two Tom40 pores (not shown in the figure). After presequence proteins emerge from the Tom40 channel they bind to the intermembrane space domain of Tom22 prior to their transfer to the presequence translocase of the inner membrane. Carrier proteins with internal targeting signals are initially recognized by the receptor Tom70. With the help of the other receptors, Tom20, Tom22, and Tom5, internal loops of the carrier proteins are translocated through the Tom40 channel and bind to the Tim9-Tim10 complex of the intermembrane space (*IMS*). The import of outer membrane proteins initially depends on the receptors Tom20 and Tom22. After transport through the TOM machinery, the SAM complex is required for the insertion of β-barrel proteins into the outer membrane (*OM*).

mitochondrial surface, Tom70. These precursors are usually synthesized without a presequence but contain multiple internal targeting signals (19–21). Cytosolic chaperones, particularly heat shock proteins of the Hsp70 and the Hsp90 classes, bind these hydrophobic precursors and prevent their aggregation in the cytosol. The heat shock proteins then specifically interact with Tom70 and deliver the carrier precursors to this receptor (22). Several Tom70 molecules simultaneously bind to one precursor molecule, most likely to prevent misfolding of the precursor (21). The carrier precursors are subsequently transferred to the import pore Tom40. This transfer likely involves the other receptor proteins, Tom20 and Tom22, as well as Tom5.

Although the receptors Tom20 and Tom22 preferentially recognize presequences, while Tom70 mainly interacts with hydrophobic precursor proteins carrying internal targeting signals, the three mitochondrial import receptors show a partially overlapping specificity (8). Upon inactivation of one receptor, *e.g.* in yeast mutants, the other receptors can partially substitute for its function. Interestingly, the precursors of outer membrane proteins that contain internal targeting signals mainly use the receptors Tom20 and Tom22.

Because most mitochondrial precursor proteins are imported after their synthesis on ribosomes has been completed (post-translational import), they are likely guided to the mitochondria by cytosolic chaperones as described for the carrier proteins above. In addition to the classical heat shock proteins (22), additional cytosolic factors have been reported, including the mitochondrial import stimulation factor (16) and the aryl hydrocarbon receptor-interacting protein, which interacts with preproteins and Tom20 (23). In special cases, however, preproteins can also be imported into mitochondria in a co-translational manner, *i.e.* the presequence inserts into the TOM machinery while a C-terminal portion is still undergoing synthesis on the ribosome (24).

Sorting and Assembly Machinery of the Outer Membrane

Outer membrane proteins with a relatively simple topology, *e.g.* one transmembrane segment, only require the TOM machinery for insertion into the membrane. Other outer membrane proteins such as porin and Tom40, however, possess a more complicated topology with multiple β-strands (β-barrel proteins). The precursors of these proteins are first imported via the TOM complex to the intermembrane space side (18). With the help of the small Tim proteins of the intermembrane space (25, 64), the precursor proteins are then passed on to the SAM in the outer membrane (Fig. 2). The SAM complex was identified by analyzing the role of the outer membrane protein Mas37 (26). Mitochondria lacking Mas37 efficiently import presequence-carrying preproteins and carrier proteins, yet the assembly of outer membrane β-barrel proteins is strongly impaired. Recently, a second subunit of the SAM complex was identified, the essential outer membrane protein Sam50, also termed Tob55/Omp85 (27–29). Sam50 has been highly conserved throughout evolution. Its C-terminal domain shows a remarkable similarity to the bacterial outer membrane protein Omp85, which is possibly involved in protein integration into the bacterial outer membrane (30). Thus, the mechanism of insertion of β-barrel proteins into the outer membrane is likely conserved from bacteria to mitochondria. With the help of the TOM complex, the precursors of mitochondrial β-barrel proteins are translocated to the intermembrane space side (18) where they can then follow the bacteria-like pathway of export and integration into the outer membrane.

Presequence Translocase and Import Motor

The presequence translocase of the inner membrane consists of three integral and essential membrane proteins: Tim50, Tim23, and Tim17 (7, 8) (Fig. 3). After release from the TOM complex, the presequence-carrying preproteins first contact Tim50, which exposes a large domain to the intermembrane space (31–33). Tim50 then guides the preproteins to the import channel formed by Tim23 (34). Tim17 is tightly associated with Tim23 and probably influences the channel activity, although the exact role of Tim17 remains unknown. Tim23 possesses two domains: an N-terminal segment in the intermembrane space that recognizes the presequences and a channel-forming C-terminal domain (16, 34, 35). Insertion of preproteins into the Tim23 channel strictly depends on the presence of the mem-

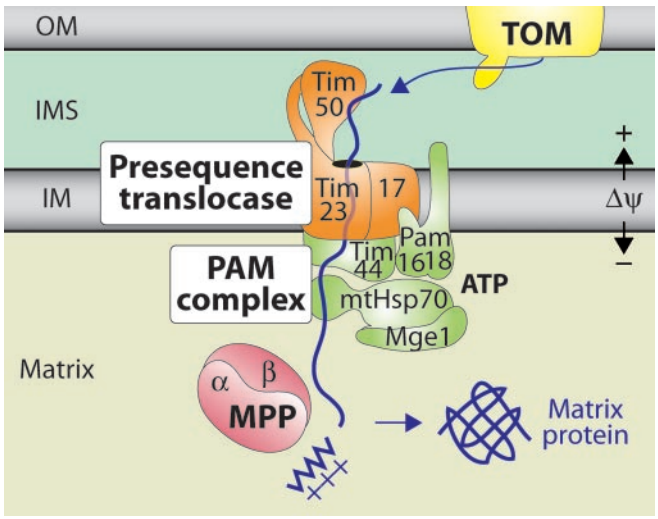

FIG. 3. **Import of presequence proteins into the mitochondrial matrix.** After passing through the channel of the TOM complex, presequence-carrying preproteins are recognized by an intermembrane space (*IMS*) domain of Tom22. From here, they are brought into contact with Tim50, which then assists in guiding them to the presequence translocase (TIM23 complex). Their subsequent insertion into the channel, formed by Tim23, requires the membrane potential ($\Delta\psi$) across the inner membrane (*IM*). A further driving force is provided by the PAM in the matrix. The central component of this motor, mtHsp70, is transiently anchored at the translocase by Tim44 and requires assistance from Pam18, Pam16, and Mge1 for promotion of the reaction cycle. The presequences of the precursor proteins are cleaved off by the MPP in the matrix. *OM*, outer membrane.

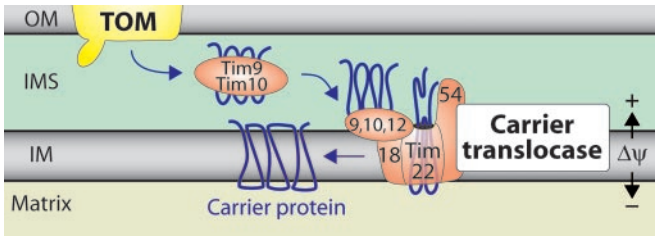

FIG. 4. **Insertion of carrier proteins into the inner mitochondrial membrane.** The Tim9-Tim10 complex of the intermembrane space (*IMS*) promotes the translocation of carrier proteins across the outer membrane (*OM*) and then aids in guiding them across the intermembrane space to the carrier translocase (TIM22 complex) of the inner membrane (*IM*). The carrier proteins dock onto the carrier translocase with additional assistance from Tim12. Their subsequent $\Delta\psi$-dependent insertion into the inner membrane is facilitated through the channel formed by Tim22. The carrier translocase contains two pores formed by Tim22 (twin-pore translocase; not shown in the figure).

brane potential $\Delta\psi$ across the inner membrane. The membrane potential plays a dual role in the import process. $\Delta\psi$ activates the channel protein itself as well as exerting an electrophoretic effect on the positively charged presequences, thereby driving the presequences to the matrix side (34–37).

Some preproteins possess a presequence and a hydrophobic stop transfer signal. These proteins are arrested in the inner membrane by the hydrophobic signal and are then laterally released into the lipid phase of the membrane (38). Such proteins can be inserted into the inner membrane just by the $\Delta\psi$-driven presequence translocase without requiring the ATP-dependent import motor PAM (39).

The majority of presequence-carrying preproteins, however, are imported into the matrix by a cooperation of the presequence translocase and the associated motor PAM (Fig. 3). It has been assumed for more than a decade that the ATP-driven import motor consists of three essential proteins: the polypeptide chain-binding chaperone mtHsp70; the peripheral inner membrane protein Tim44, which binds mtHsp70 and directs it to the TIM23 complex; and the nucleotide exchange factor of the matrix, termed mitochondrial GrpE (Mge1) (40–42). Recent studies, however, have led to the identification of two more membrane-bound essential co-chaperones, Pam18 (Tim14) and Pam16 (Tim16). Pam18 belongs to the J-protein family of co-chaperones and stimulates the ATPase activity of mtHsp70 (43–45). Pam16 is involved in the recruitment of Pam18 to the TIM23 complex (46, 47). The function of mtHsp70 in holding preproteins upon their exit from the import channel is thus coordinated by three membrane-bound co-chaperones: Tim44, Pam18, and Pam16. The molecular mechanism of the import motor probably involves both passive trapping and active pulling of the preprotein (40, 41). When bound to ATP, mtHsp70 interacts with Tim44 and preproteins with a relatively low affinity. The hydrolysis of ATP stimulated by Pam18 leads to the ADP-bound form of mtHsp70 and thus stabilizes its interaction with Tim44 and preproteins. The soluble matrix protein Mge1 then promotes the release of ADP from mtHsp70. Thus, the PAM operates as a multistep motor, although the exact order of events driving the reaction cycle of mtHsp70, including binding and release of preproteins, is not fully understood.

Independently from PAM involvement, the mitochondrial processing peptidase (MPP) removes the presequences from the preproteins. MPP is a metallopeptidase made up by two essential subunits (48). In the case of matrix proteins, further molecular chaperones, in particular Hsp60 in cooperation with Hsp10, promote the folding of the imported proteins to their active conformation (49, 50).

Protein Insertion Machinery of the Inner Membrane

Most multispanning inner membrane proteins are synthesized without a presequence. After recognition by Tom70 and translocation by the TOM pore across the outer membrane, these precursor proteins are bound by a complex of small Tim proteins in the intermembrane space, the Tim9-Tim10 complex (5, 6, 51) (Fig. 4). The Tim9-Tim10 complex probably exerts chaperone-like activities that prevent aggregation of the hydrophobic precursor proteins in the aqueous intermembrane space (52, 53). Besides the essential Tim9-Tim10 complex, the homologous yet non-essential Tim8-Tim13 complex also supports the transfer of selected inner membrane proteins (54, 55). The Tim9-Tim10 complex delivers the precursor proteins to the membrane-integrated insertion machinery of the inner membrane by directly docking to it. This insertion machinery is termed the TIM22 complex or, according to its major class of substrates, the carrier translocase. The peripheral membrane protein Tim12 serves as the docking site for the Tim9-Tim10 complex. The precursor is then translocated to the core of the insertion machinery, the channel-forming protein Tim22 (56, 57). Interestingly, Tim22 shares homology with Tim23. Indeed, like the presequence translocase, the Tim22 channel is also activated by the membrane potential (57). However, in the case of the carrier translocase, the $\Delta\psi$ is the only known external energy source required, while an ATP-driven machinery is not involved. The $\Delta\psi$ is required for both the initial insertion of the precursor polypeptide into the translocase and for the completion of transport, *i.e.* the lateral release of the protein into the lipid phase of the inner membrane (58). The functions of two further subunits of the carrier translocase, the membrane proteins Tim18 and Tim54, are not yet defined.

The mitochondrial inner membrane possesses a further protein insertion machinery for proteins that are exported from the matrix into the inner membrane (for review see Refs. 59 and 60). Several subunits of this export machinery have been identified, including Oxa1, which is homologous to the bacterial YidC (61, 62). Substrates of this machinery are hydrophobic

proteins that are encoded by the mitochondrial genome and a few nuclear-encoded proteins that are directed by a presequence into the matrix where they then follow an export pathway conserved from bacteria.

Concluding Remarks

The mitochondrial machinery for protein import and assembly has proven to be much more complex than anticipated. Both outer and inner membranes of mitochondria contain multisubunit protein complexes that function at distinct stages of precursor recognition, translocation, or assembly. Important topics to be addressed in future studies will be the characterization of internal targeting and sorting signals of precursor proteins, the structural analysis of the membrane-integrated transport complexes, and the mechanisms of regulation of the import machinery. It is likely that mitochondria hold more surprises for us, including new components of the import machinery, along with unexpected sorting and assembly routes of precursor proteins. As well, mitochondria play a critical role in programmed cell death (apoptosis) (63). It is therefore tempting to speculate that the channels formed by the protein import machineries, in particular the TOM and SAM complexes, may also play a role in apoptosis.

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