Minireview

Protein translocation pathways across the inner and outer mitochondrial membranes

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Received 29 June 2007; revised 02 May 2008

Mitochondria import different proteins that are encoded by nuclear genes and synthesized in the cytosol. Separate translocases in inner and outer mitochondrial membranes like TOM, TIM23, TOB/SAM, TIM22 complex facilitate recognition, import and intramitochondrial sorting of preproteins. Various cytosolic factors as Hsp70 and auxiliary factors assist in targeting these preproteins to their destinations. Also, different protein components in the matrix participate in this energetically driven translocation process in a reaction that depends upon membrane potential and matrix-ATP. This review summarizes the present knowledge on import and sorting of mitochondrial precursor proteins with glance on unresolved questions.

Keywords: Mitochondria, Translocation, Membrane potential, ATP hydrolysis.

Introduction

Mitochondria are not only of key importance for the bioenergetics of eukaryotic cells, but have critical functions in metabolism of various biomolecules including amino acids and lipids and also play a role in apoptosis. They are essential for cell viability, proliferation and actively participate in development and differentiation processes. Mitochondria contain about 800 proteins in yeast to 1500 different proteins in humans. Although there is a complete genetic system in the matrix, the innermost compartment of mitochondria, only ~1% of all mitochondrial proteins are encoded by organelle genome and synthesized in the matrix and rest are encoded by nuclear genes. Basic aspect of this protein sorting is the transfer of nuclearly encoded and cytoplasmically synthesized proteins both across and into the mitochondrial inner and outer membranes. Many hundreds of different mitochondrial proteins participate in these processes. In contrast, only a few protein components are encoded by mitochondrial DNA itself, synthesized on organellar ribosomes and inserted from matrix side into inner membrane.

The translocase of outer mitochondrial membrane, TOM complex represents a common entry gate for usually all the nuclear encoded mitochondrial proteins. After making their way through TOM complex, these precursor proteins are directed to at least four different import pathways: (i) Presequence pathway: imports the matrix proteins using TIM23 complex, (ii) Carrier pathway: imports hydrophobic inner membrane proteins, (iii) TOB/SAM or SAM/TOB pathway: the mitochondrial outer membrane β-barrel proteins are inserted into membrane via TOB/SAM (topogenesis of mitochondrial outer membrane β-barrel proteins also called as sorting and assembly machinery), and (iv) recently identified specific protein import machinery for small intermembrane space proteins, but little is known about this pathway.

Since mitochondria play essential roles in eukaryotic cells, knowledge regarding the protein import and biogenesis of this organelle is equally important. In this review, presequence, carrier, TOB/SAM import pathways as well as import of preproteins in intermembrane space of mitochondria are discussed.

Mitochondrial targeting signals

The targeting signals or sequences for all matrix-targeted and inner membrane (IM) proteins that contain a single transmembrane anchor, as well as some intermembrane space (IMS) proteins are typically N-terminal extensions of about 15-40 amino acids, rich in basic and hydroxylated amino acids,
which can form amphipathic \( \alpha \)-helices. However, targeting information is lost in the mature sequences of approximately 30% of mitochondrial-targeted proteins. Presequence of these precursor proteins interacts directly with the receptors of translocase of outer membrane (Tom20 and Tom22); one half of this amphipathic helix having a hydrophobic surface, is recognized by the binding groove within Tom20, whereas the other half, which is positively-charged, is recognized by the Tom22 of TOM complex.

The movement of preproteins through cytosol is associated with cytosolic factors namely chaperones and cytosolic Hsp70, an eukaryotic homologue of bacterial DnaK is one of the chaperones considered as an important factor in intracellular protein traffic. In addition, several other cytosolic factors may also be considered as targeting factors as they bind preproteins in a specific manner and deliver them to the receptor components of TOM complex. Amongst these, the best-characterized ones are mitochondrial import stimulation factor (MSF) and aryl-hydrocarbon receptor interacting protein which interacts with preproteins and Tom20. MSF has shown two activities: (i) it recognizes and forms stable complexes with mature parts of mitochondrial precursors and also facilitates depolymerization, and (ii) promotes unfolding of preproteins. Aggregated preadrenodoxin (involved in electron transfer reaction) synthesized in Escherichia coli becomes import competent in presence of MSF. Both Hsp70 and MSF mediate translocation of preproteins from cytosol to TOM complex and involvement of each depends on the nature of preprotein.

**TOM or TOM40 complex**

The holo-TOM complex has a size of ~450 kDa and contains seven different proteins viz., the receptors (Tom20 and Tom70) and five additional proteins (Tom40, Tom22, Tom5, Tom6 and Tom7) make up the TOM-core complex which acts as a general insertion pore (GIP ~400 kDa) (Fig. 1). Tom20 and Tom70 of TOM complex project into cytosol and both have an N-terminal membrane anchor and hydrophilic C-terminal domain of ~17 kDa and ~65 kDa, respectively. Also, Tom22 extends an N-terminal domain of ~85 amino acid residues into cytosol and has a single transmembrane segment and a smaller C-terminal domain (~45 residues) facing the intermembrane space. Tom40 protein appears to be deeply embedded in the outer membrane of mitochondria, although it does not have many obvious contiguous hydrophobic sequence segments.

In recent years, most of the homologous components of TOM complex in mammalian system have also been identified which include: Tom20, Tom22, Tom70, Tom40, and Tom7. The yeast GIP complex resolves as a ~400 kDa species on native-PAGE, while the mammalian complex is a ~380 kDa species containing at least Tom40, Tom22 and Tom7. TOM complex plays an important role during translocation of preproteins to subsequent translocases in the import pathway.

**Role of different proteins of TOM complex**

Tom20 and Tom70 act as receptors for the precursor proteins. Tom22 has various roles including receptor functions, helps in passage of precursor proteins from the receptors to pore, and binds precursors at trans site. Tom40 forms pore-forming component of the complex and is essential for cell viability. Different mutations generated by altering 10 conserved regions of *Neurospora crassa* Tom40 protein revealed that all these affect the ability of altered Tom40 to be assembled into TOM complex in isolated mitochondria and such mitochondria have defects in importing preproteins to different subcompartments. Although the receptors of TOM complex (Tom20 and Tom22) preferentially recognize presequences, Tom70 interacts mainly with hydrophobic precursor proteins carrying internal targeting signals (carrier pathway preproteins).

Tom6 plays a major role in the stability of TOM complex and is required to promote assembly of Tom40 and Tom22, but not for their interaction during translocation. Absence of Tom7 results in more stable associations between Tom40, Tom20 and Tom22, suggesting that it plays a role opposite to that of Tom6 with respect to TOM stability. Tom7 is also involved in the trans site binding and passage of precursors to TIM23 complex. Mutants of *N. crassa* Tom proteins lacking both Tom6 and Tom7 have an extremely labile TOM complex and exhibit altered growth phenotype. Tom5 in Saccharomyces cerevisiae maintains the structure of TOM complex rather than acting in preprotein transfer, whereas single mutants lacking *N. crassa* Tom5 display no apparent TOM complex abnormalities and has minor role in maintaining TOM complex stability.

Proteins of mitochondrial inner membrane contain transmembrane \( \alpha \)-helices, like proteins present in most other membranes of eukaryotic cell. However,
outer membrane of mitochondria contains proteins anchored in membrane by multiple β-strands. Recently, it has been revealed that TOM complex is not sufficient to integrate β-barrel proteins into outer membrane and also not able to assemble its own precursor proteins into functional complexes. This led to the discovery of topogenesis of mitochondrial outer membrane β-barrel proteins (TOB) complex, also called the sorting and assembly machinery (SAM) complex (TOB/SAM complex).

Mitochondria lacking protein Sam37 (formerly named Tom37) are strongly impaired in Tom40 assembly, the channel protein of outer mitochondrial membrane, whereas other known import pathways, presequence and carrier pathways are not affected. Since mitochondrial targeting signals are diverse in their nature, it is puzzling that they converge at the same import machinery for outer membrane translocation and subsequent sorting to their appropriate sub-mitochondrial compartments by different import routes.

**TOB/SAM complex**

The mitochondrial TOB/SAM complex is derived from the bacterial Omp85 complex, but to date no detailed structural analysis of a mitochondrial β-barrel protein has been achieved. The founding subunit of SAM complex is Sam37/Mas37/Tom37 (Fig. 1). For membrane integration of mitochondrial β-barrel proteins, Sam37 is required which is a subunit of SAMcore complex (~200 kDa). As compared to the integration of β-barrel proteins, simple outer membrane proteins with α-helical transmembrane segment e.g. Tom20 can be inserted into outer mitochondrial membrane with help of TOM complex alone.

The two additional subunits of SAMcore complex are Sam50/Tob55 and Sam35/Tob38. Sam50 assembles as an integral outer membrane protein with a predicted β-barrel domain, whereas Sam35 and Sam37 behave as peripheral membrane proteins that are exposed on mitochondrial surface. The central subunit of SAM complex is represented by Sam50, which in its purified form forms ring-shaped particles with a large channel. N-terminal domain of Sam50/Tob55 is exposed to the intermembrane space and represents interaction site for β-barrel precursors before insertion in SAM/TOB complex.

Sam35 and Sam37 function codependently in the biogenesis of β-barrel proteins. Sam35 is required for SAM complex to bind outer membrane substrate.
proteins and its destabilization inhibits substrate binding by Sam50, whereas Sam37 seems to assist the release of substrates from SAM complex. This SAMcore complex can further associate with a fourth subunit, the morphology component Mdm10 to form SAMholo complex. SAMcore complex is required for biogenesis of all β-barrel proteins of outer membrane, whereas Mdm10 and SAMholo complex play a selective role in β-barrel biogenesis as they promote assembly of Tom40, but not porin.

Tom7, a conserved subunit of TOM complex functions antagonistically to Mdm10 in the biogenesis of Tom40 and porin, suggesting that its role is not limited to TOM complex. Tom7 also functions in the mitochondrial protein biogenesis by a new mechanism, segregation of a sorting component, leading to a differentiation of β-barrel assembly. In yeast, assembly of Tom40 and porin in outer membrane requires Tom13 in addition to SAMcore complex.

TIM complex or TIM23 complex/translocase

TIM23 complex can be structurally and functionally sub-divided into two parts — membrane integrated translocation channel and import motor, which is located at the matrix face of channel. Although membrane integrated part of this translocase is sufficient for insertion of some preproteins into the inner membrane, but complete translocation of preproteins into matrix requires a combination of both subunits. Without motor section, preproteins cannot translocate into matrix. The components of membrane integrated part of TIM23 complex include Tim17, Tim21, Tim23 and Tim50, while import motor of translocase comprises five proteins: Tim14, Tim16, Tim44, Mge1 and mtHsp70 (mitochondrial heat-shock protein 70). An integral 23 kDa protein of yeast inner membrane, N-terminal half of Tim23 is hydrophilic in nature, whereas C-terminal half is hydrophobic. Tim17, also an integral membrane protein shares sequence similarity with the hydrophobic portion of Tim23. Tim23 also contains an additional N-terminal domain, not present in Tim17 which has a receptor function and also responds to membrane potential.

Role of different proteins of TIM23 complex

Tim17 and Tim23 form the translocation channel of TIM23 complex through which precursor proteins usually with an N-terminal cleavable presequence cross the hydrophobic barrier of inner membrane in unfolded state. Tim21 promotes coupling of the two translocator complexes (TIM23 and TOM) during translocation process. A single transmembrane domain anchors Tim21 in the mitochondrial inner membrane and it exposes its C-terminal domain into intermembrane space. The C-terminal domain of Tim21 does not to bind to any of the TIM23 components but specifically interacts with TOM complex. It has been proposed that Tim21 binds to trans site of TOM complex, thus keeping two translocases in close contact. Tim50 facilitates protein transfer from TOM40 complex to TIM23 complex.

Mitochondrial Hsp70 (mtHsp70) in matrix functions as import motor to drive vectorial translocation and unfolding of precursor proteins in cooperation with its partner proteins, mitochondrial associated motor and chaperone (MMC) proteins. Tim44 provides anchor for this motor (mtHsp70), so that it can bind to the polypeptide emerging out from TIM23 channel. The cochaperone Mge1 is nucleotide exchange factor for mtHsp70 and enhances its ATPase activity. A new member of this translocator system Tam41 (translocation assembly and maintenance) has been identified from the yeast is a peripheral inner mitochondrial membrane protein facing matrix and not a constituent of TIM23 complex. It does not promote motor function of mtHsp70 but is instead involved in the maintenance of TIM23 complex integrity. Roles of Tim14 and Tim16 are discussed later.

Insertion of preproteins into TIM23 channel depends mainly on the presence of membrane potential across inner membrane which plays a dual role in import process. Firstly, it activates the channel protein itself and secondly it exerts an electrophoretic effect on positively-charged presequence, thereby driving presequence to matrix side. Although, the exact mechanism of import motor is still not understood, it is clear that several rounds of ATP-dependent binding to and release from the mtHsp70 lead to complete translocation of polypeptides into matrix of mitochondria. The components of TIM23 translocase are highly conserved throughout eukaryotic kingdom.

Pathways of preprotein translocation across and into the outer membrane

After translocation through β-barrel protein the Tom40 pore, presequence binds to intermembrane space domain of receptor Tom22. Tom40 itself
interacts with preproteins in transit. Preproteins which follow the presequence pathway have a cleavable presequence and are guided across outer membrane by a chain of binding sites, including the cytosolic receptors Tom20, Tom22, Tom5 and Tom40 which form translocation channel and intermembrane space domain of Tom22. Other small proteins Tom6 and Tom7 are required for assembly and stability of TOM complex (Fig. 1), but they do not interact with the precursor proteins.

Tom70 recognizes the precursors of hydrophobic carrier proteins of inner membrane such as the ADP/ATP carrier. These precursors possess multiple internal targeting sequences but lack presequence. The cytosolic chaperones, particularly Hsp70 and Hsp90 classes bind these hydrophobic precursors and prevent their aggregation in the cytosol. Heat-shock proteins then specifically interact with Tom70 and deliver the carrier precursors to this receptor. These heat-shock proteins are essential in mitochondrial biogenesis, as revealed in Caenorhabditis elegans which develops early aging or progeria-like phenotypes, if HSP6 (a nematode orthologue of mtHsp70) is reduced by RNA interference. Several Tom70 molecules simultaneously bind to one precursor molecule which is subsequently transferred to the import pore, Tom40.

The effective internal diameter of protein import channel in mitochondrial outer membrane appears to be between 20-26 Å during translocation. β-Barrel proteins represent a distinct class of mitochondrial outer membrane proteins, but little is known about how these newly synthesized proteins sort in eukaryotic cell, integrate into lipid bilayers and assemble into oligomeric structures. Like other outer membrane proteins, β-barrel proteins such as porin and Tom40 first translocate across TOM complex and in a manner dependent on chaperones in intermembrane space, pass on to TOB/SAM complex for their insertion into outer membrane. In the intermembrane space, small TIM complexes, essential Tim9-Tim10 complex and non-essential Tim8-Tim13 complex promote transfer of β-barrel precursors to TOB/SAM complex. These small proteins have chaperone-like functions which probably shield hydrophobic segments of the precursor proteins against aggregation in aqueous intermembrane space, keeping β-barrel precursors in a competent state for their insertion.

Biogenesis of Tom40, pore-forming channel of outer mitochondrial membrane involves the movement of precursors in unfolded state to SAMcore complex to form an assembly intermediate I (~250 kDa) which further assembles into mature TOM complex by passing through assembly intermediate II (~100 kDa). How this assembly intermediate II separates from SAMcore complex is under investigation. Mdm10 also associates with SAM to form a larger complex of ~350 kDa and plays a specific role in assembly of TOM complex, probably by functioning as a scaffold, where the late steps of assembly of the multi-subunits TOM complex takes place.

Transfer of preproteins from TOM to TIM 23: Translocation of preproteins across the inner mitochondrial membrane

Majority of presequence carrying proteins are imported into the matrix by cooperation of presequence translocase of inner membrane, TIM23 complex and the associated motor (presequence associated motor, PAM). Protein import into matrix requires two forms of energy – an electrochemical gradient (positive outside) across inner membrane which exerts an electrophoretic force on the positively charged N-terminal targeting sequence and hydrolysis of ATP in matrix. Ability of generating strong translocating driving force solely depends upon ATP-driven import motor and membrane potential-increases the interaction of preprotein with motor. Precursor proteins are imported with help of TOM complex and TIM23 complex into matrix (Fig. 1).

This ATP-driven import motor consists of five proteins, three essential proteins–mtHsp70, peripheral inner membrane protein Tim44 and a nucleotide exchange factor of matrix termed mitochondrial GrpE (Mge1) and two cochaperones Pam18/Tim14 and Pam16/Tim16.

Molecular mechanism of the import motor involves the following steps: (i) mtHsp70 with bound ATP interacts with Tim44 and preproteins, but with low affinity (ii) Pam18/Tim14 stimulates ATP hydrolysis which in turn leads to the ADP-bound form of mtHsp70, stabilizing its interaction with Tim44 and preproteins. (iii) Mge1, a soluble matrix protein then promotes the release of ADP-from mtHsp70. PAM, thus operates as a multi-step motor, but the exact order of events during reaction cycle of mtHsp 70 is not fully understood. TOM and TIM23 complexes do not appear to form stable super complex in the absence of precursor proteins.

TIM23 channel is also tightly regulated, so as to maintain the permeability barrier of inner membrane.
mitochondrial membrane. In absence of precursor proteins, it remains in its inactive state, but regains its active state, when precursor proteins are present and opens up for translocation of polypeptide chains. Both Tim50 and presequence act in an antagonistic manner in this process. Whereas the hydrophilic IMS domain of Tim50 promotes oligomerization and voltage-dependent closure of channel, presequences selectively override Tim50-induced closure and activate the channel, thereby crossing the channel⁴⁵. This process is very complicated under in vivo conditions and there is no clear-cut answer to the question as how energy sources of mitochondria, membrane potential and matrix ATP cooperate to drive preprotein import.

A new model for the mechanism of import motor called “entropic pulling” has been proposed that postulates generation of an active import-driving force based on thermodynamic principles and molecular geometries during translocation. According to this model, translocation force is generated by the excluded-volume constraint between mtHsp70 and membrane and requires efficient interaction of mtHsp70 and Tim44 for efficient translocation and unfolding of proteins⁴⁶. A new model for the mechanism of import motor called “entropic pulling” has been proposed that postulates generation of an active import-driving force based on thermodynamic principles and molecular geometries during translocation. According to this model, translocation force is generated by the excluded-volume constraint between mtHsp70 and membrane and requires efficient interaction of mtHsp70 and Tim44 for efficient translocation and unfolding of proteins⁴⁶.

Protein insertion machinery of the inner membrane (TIM22 complex)

Substrate proteins for TIM22-mediated translocation contain multiple hydrophobic segments that can be inserted into inner membrane. Import of ADP-ATP carrier (AAC) protein of inner mitochondrial membrane has been characterized⁷⁹. This 300 kDa complex consists of a pore-forming subunit Tim22 along with Tim18 and Tim54 that play unknown function and small Tim proteins Tim9, Tim10 and Tim12⁸⁰. The general import of inner membrane proteins is summarized here (Fig. 1). After recognition by Tom70 and translocation by TOM pore across outer membrane as a loop, these precursor proteins are bound by Tim9-Tim10 (70 kDa) complex⁸¹. Non-essential Tim proteins (Tim8-Tim13 complex) also support the transfer of selected inner membrane proteins, whereas Tim9–Tim10 complex docks precursor proteins to the TIM22 complex. These Tim proteins contain the “twin CX₃C” motif that is required for formation of 70 kDa complex⁸². Function of this motif is not fully known, but it seems to be important for Zn²⁺ coordination in these small Tim proteins. It has also been suggested that cysteine present in this motif helps to form juxtapositional intramolecular disulfide bonds⁸³. These proteins can bind zinc but insertion of intramolecular disulfide bonds is requisite for assembly of complex⁸⁴. An intermembrane space protein Hot13 is also required for efficient assembly of these Tim proteins into TIM complexes. Thus, small TIM complexes have a specialized assembly and the local redox state of these complexes mediate translocation of inner membrane proteins⁸⁵.

Precursor is then translocated to the core of insertion machinery, channel forming protein Tim22⁵³ which also shares homology with Tim23. Indeed, like presequence translocase, Tim22 channel is also activated by membrane potential, but no ATP-driven machinery is required in this process. Membrane potential is sufficient for both initial insertion of precursor polypeptide into translocase and for completion of transport i.e. the lateral release of protein into inner membrane⁷.

Translocation of proteins to intermembrane space (IMS)

Precursors destined for intermembrane space as cytochromes b₂ and c₁ contain a bipartite targeting sequence and N-terminal directs precursors to TIM23 complex. After cleavage by matrix processing peptidase (MPP), precursor arrests at the “stop transfer” domain in translocon⁵⁴. The inner membrane protease (IMP) consisting of complex Imp1/Imp2 and facing intermembrane space mediates a second cleavage, thereby arresting cytochrome b₂ in intermembrane space⁵⁵. This translocation also requires hydrolysis of matrix ATP and electrochemical membrane potential Δψ. For proteins lacking N-terminal and not requiring electrochemical membrane potential for their translocation thereby bypassing TIM23, their import mechanisms depends upon – energy gain of protein around the cofactor and association of a protein with high affinity binding site such as another protein⁵⁶.

Polynucleotide phosphorylase (PNPase), which is an exoribonuclease as well as poly(A) polymerase that regulates RNA levels is also localized in the intermembrane space and not in cytosol or matrix as considered earlier⁵⁷. Import of PNPase depends on Δψ and MPP cleavage of presequence. Translocation of PNPase into intermembrane requires i-AAA protease Yme1 which has a chaperone-like activity⁵⁶. This new Yme1 in protein translocation gives an idea of the diverse protein translocation pathways of intermembrane space which needs investigation.
Conclusions and Perspective

During recent years, important insights have been revealed into the machineries involved in targeting and sorting of proteins to mitochondria. However, in spite of rapid progress in our understanding of the mechanisms of protein import into mitochondria, many questions still remain to be answered. For example, general import pathways have been revealed on the basis of detailed analysis of import of various proteins, which depends upon one or two of translocators TOM, TIM23 and TIM22 complexes during translocation. But there may be more specific pathways for several mitochondrial proteins, including those synthesized inside mitochondria that remain to be elucidated. More than 30 proteins have been identified as translocator components, indicating that pathways of import and sorting are much more complex than previously envisaged. Identification and characterization of SAM/TOB pathway led to several surprising twists in the analysis of organellar biogenesis. Important areas for future studies will be: the characterization of internal targeting and sorting signals of precursor proteins, structural analysis of membrane integrated transport complexes and import machinery mechanism.

References


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