Minireview

The Protein Import System of Mitochondria*

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Mitochondria import virtually all of their proteins from the cytoplasm. This import process faces two unique challenges; the proteins must be routed to their correct submitochondrial compartment, and those destined for the innermost compartment (the matrix) must be transported across two membranes. These challenges are met by the joint action of two distinct protein transport systems, one in the outer membrane and the other in the inner membrane. The molecular composition of these two systems is now quite well known, and we are beginning to understand how they work (1–4). Most of our information on mitochondrial protein import has come from studies in the yeast Saccharomyces cerevisiae and the mold Neurospora crassa, but it is likely that protein import into mammalian mitochondria occurs by very similar mechanisms (5, 6).

The Import Machinery: Cytosolic and Mitochondrial Components

A protein destined to be imported into mitochondria is normally synthesized with a transient N-terminal extension that functions as a targeting signal. For proteins imported into the matrix, this signal is a highly degenerate sequence of 20–30 residues capable of folding into a positively charged amphiphilic helix (Fig. 1). If this “matrix targeting” signal is fused to a non-mitochondrial protein, this protein is transported to the mitochondrial matrix. Proteins imported into other mitochondrial subcompartments usually have an additional signal downstream from the matrix targeting signal; in many instances, this “sorting signal” interrupts translocation across either the outer or the inner membrane, thereby rerouting the protein to the outer membrane, the intermembrane space, or the inner membrane. Targeting signals are usually removed once the protein has reached its correct intramitochondrial location.

Most precursor proteins that have been tested so far can be imported into isolated mitochondria post-translationally, i.e. after synthesis of the precursor chain has been completed. Import can also be post-translational in vivo (12, 13), but this has only been shown for a few precursor proteins. It is still not settled whether mitochondrial protein import in vivo is cotranslational (14, 15), but in intact cells under physiological conditions the mitochondrial surface is essentially devoid of bound cytosolic ribosomes whereas bound ribosomes are readily detected on the cytosolic surface of the endoplasmic reticulum. The weight of evidence thus favors the view that most proteins are normally imported into mitochondria post-translationally.

The eukaryotic cytoplasm contains several different molecular chaperones that bind newly synthesized precursors and prevent their aggregation or irreversible misfolding. These chaperones include members of the 70-kDa heat-shock protein (hsp70) family, which bind non-native proteins targeted to several subcellular compartments. However, at least one cytosolic chaperone appears dedicated to mitochondrial protein import; the heterodimeric protein termed mitochondrial import stimulating factor (MSF) specifically binds matrix targeting signals (16). hsp70s and MSF are both ATPases whose activity is greatly stimulated by binding of non-native proteins. Some small and hydrophilic precursors may well travel through the cytosol without assistance by a chaperone, but this fact has not been clearly established.

The Tom Complex

Mitochondrial precursors are recognized by a specific “import receptor” on the mitochondrial surface, which delivers the bound precursors to a protein import channel in the outer membrane (17, 18). Receptor and channel each consist of several non-identical subunits referred to as Tom subunits (for transport across the outer membrane (19)). The receptor consists of at least four non-identical integral membrane proteins named (after their approximate molecular masses in kDa) Tom70, Tom37, Tom22, and Tom20. Upon solubilization of mitochondria with non-denaturing detergents, the receptor readily dissociates into two subcomplexes: a Tom70-Tom37 heterodimer and a less well characterized Tom22-Tom20 complex. Genetic and cross-linking experiments show clearly that these two subcomplexes interact with each other in vivo via 34-residue-long “tetratrico-peptide” motifs, which are present in Tom70, Tom37, and Tom20 (20).

The two receptor subcomplexes recognize precursors by at least two different mechanisms. The first mechanism involves specific recognition of the basic targeting signals by acidic patches (termed “acid bristles”) in the cytosolic domains of Tom22 and Tom20 (17, 18). This recognition also operates if the precursor is bound to a cytosolic hsp70 (21). Unexpectedly, import of precursors that are bound to a cytosolic hsp70 does not require extramitochondrial ATP, even though hsp70s must normally hydrolyze ATP in order to release a bound protein. Binding of the precursor to the mitochondrial import receptor may bypass this ATP requirement.

The second recognition mechanism is mediated by the Tom70-Tom37 subcomplex and operates with precursors that are presented to mitochondria as a complex with MSF (16, 21, 22). When the MSF-precursor complex binds to the Tom70-Tom37 heterodimer, the precursor is transferred to Tom70-Tom37, the ATPase of MSF is quenched, and MSF is recycled to the cytosol. The precursor’s prescence is then transferred to Tom22-Tom20. Import of precursors via the MSF-dependent pathway thus requires interaction between the two receptor subcomplexes and ATP hydrolysis outside the mitochondria, unlike import via cytosolic hsp70. Both receptor mechanisms have so far only been demonstrated with isolated mitochondria; whether they also operate in vivo remains to be shown.

Although the two receptor subcomplexes have different functions, only Tom22 is essential for protein import. Each of the

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The abbreviation used is: MSF, mitochondrial import stimulating factor.
across the inner membrane by the electrochemical potential positively charged presequence is electrophoretically pulled through a transmembrane channel formed by subunits of the Tim complex (26, 27) or whether it moves through a phospholipid bilayer (28, 29). It is not yet clear whether the presequence moves directly through the inner membrane under non-denaturing conditions (28, 31).

The Tim Complex

Once a precursor has moved across the outer membrane, its positively charged presequence is electrophoretically pulled across the inner membrane by the electrochemical potential ($\Delta \phi$, positive outside) across that membrane (25). It is not yet clear whether the presequence moves directly through the phospholipid bilayer (26, 27) or whether it moves through a transmembrane channel formed by subunits of the Tim complex. The Tim complex (for transport across the inner membrane) is an assembly of about half a dozen proteins that form two subcomplexes. One subcomplex is represented by at least five proteins (Tim33, Tim23, Tim17, Tim14, and Tim11), most or all of them firmly embedded within the inner membrane; this subcomplex appears to form a protein-conducting channel across the inner membrane (28). The second subcomplex is represented by the peripheral membrane protein Tim44 and the chaperones mhs70 and mGrpE. mhs70 binds simultaneously to Tim44 on the matrix side of the inner membrane and to mGrpE (30–32); this subcomplex appears to function as an ATP-driven “import motor” that pulls the precursor chain across the Tim channel into the matrix space (4). Interaction between the Tim subcomplexes seems to be dynamic as they readily dissociate from each other upon solubilization of the inner membrane under non-denaturing conditions (28, 31).

The ATP-driven Import Motor

How does the Tim complex use the energy of ATP hydrolysis to pull a polypeptide chain across the inner membrane? One model proposes that the Tim44-associated mhs70 binds the precursor chain that emerges on the matrix side of the Tim channel. As the chain can oscillate in the channel, binding on the matrix side prevents back-oscillation and, by a series of binding events, converts a random oscillation into a directed movement. This “Brownian ratchet” model (34, 35) appeals by its simplicity but runs into difficulty when trying to explain the import of precursors with tightly folded domains. Since mitochondria cannot import precursors in their native conformation (36), the import rate of folded precursors according to the Brownian ratchet model is determined by spontaneous unfolding outside the mitochondria. However, mitochondria can rapidly import precursors (such as cytochrome $b_2$) whose spontaneous unfolding would require many hours. This discrepancy has led to the “translocation motor” model (37). According to this model, the Tim44-associated mhs70 binds the precursor chain and then actively pulls a segment of the bound precursor chain into the matrix. This pull is proposed to result from a conformational change of mhs70, which increases the distance between the peptide-binding domain of mhs70 and the Tim channel, resembling the power stroke of myosin in muscle contraction. Such a force-generating pull, unlike the passive capture invoked by the “Brownian ratchet” model, could not only move the polypeptide across the membrane but also force unfolding of the precursor outside the mitochondria (Fig. 2). It is not yet possible to decide between these models, but they need not be mutually exclusive (38).

Role of the Tim Complex in Protein Sorting

The protein transport systems present in different biological membranes not only transport proteins across the membrane but also “decide” whether a protein is destined to remain stuck in the membrane as an integral membrane protein. This sorting event is usually triggered by a hydrophobic stretch in the transported protein. When such a “stop-transfer” sequence is recognized by the protein translocation channel, translocation is interrupted and the protein is allowed to exit the channel laterally (4).

The same process appears to operate in the Tim complex as the mitochondrial inner membrane contains many integral proteins. In addition, however, the Tim complex recognizes the sorting sequence of several proteins that are targeted to the intermembrane space. These proteins, which include the cytochromes $b_2$ and $c_1$, are made with long presequences that include an N-terminal, highly charged matrix targeting sequence followed by a more hydrophobic sorting sequence. If the sorting sequence is deleted, or altered by mutation, the attached protein is mistargeted to the matrix (39). We have...
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Protein Folding in the Matrix

As a protein can only be imported into mitochondria while in a non-native conformation, it must fold in the matrix. Initially it was suggested (40) that imported proteins generally fold with the help of hsp60 and cpn10, mitochondrial homologs of the bacterial chaperones GroEL and GroES. However, recent work on protein import into the matrix of isolated yeast mitochondria (9) has shown that different proteins fold with the aid of different chaperones; some interact indeed with hsp60/cpn10, but many others (perhaps even the majority) can fold rapidly without the aid of hsp60/cpn10. For some, folding appears to be aided by a matrix-localized complex containing mhs70, Mdj1 (a mitochondrial DnaJ homolog), and mCprE. Still other imported proteins additionally require the assistance of Cpr3, a mitochondrial proline isomerase (10, 11). Since different proteins differ widely in their structure and folding pathways, it is perhaps not surprising that their folding in vivo is mediated by different chaperones.

Evolutionary Origin of the Mitochondrial Protein Import System

Most subunits of the mitochondrial protein import system do not exhibit any significant sequence similarity with other known protein. The only exceptions are the chaperones, the matrix-localized proline isomerase, and Tom40, whose predicted transmembrane topology is similar to that of pore-forming “porins” of bacterial outer membranes (41). The unique molecular features of the mitochondrial protein import system are puzzling and make it difficult to guess how this system evolved. Most likely, it arose from metabolite uptake systems in the prokaryotic ancestor of mitochondria, which was remodeled to import the protein products of those genes that had moved from the evolving endosymbiont into the host nucleus.

Today, mitochondria perform so many functions that they cannot be lost from a eukaryotic cell. Since protein import is a key process of mitochondrial biogenesis, any protein essential for mitochondrial protein import should be essential for cell viability even under conditions where respiration and oxidative phosphorylation are not required. This prediction has proved to be correct (33). This fact has been useful as a heuristic principle for finding yeast mutants defective in mitochondrial protein import (3, 29) and underscores the fundamental importance of mitochondria for the life of a eukaryotic cell.

Similarity to Other Protein Transport Systems

The mitochondrial protein import system shares several key features with the protein export systems of bacteria or the endoplasmic reticulum (4). All three systems involve one or more cytosolic recognition steps, a receptor system on the cytosolic face of the target membrane, a hetero-oligomeric protein transport channel, and a protein translocation motor, which is powered by the hydrolysis of nucleoside triphosphate. Less is known about the protein import system of chloroplasts, but this system seems to work by similar principles (42). These systems seemed quite different when they were first discovered, but advances in our molecular understanding have allowed us to perceive their common features.

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