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Mitochondrial Machineries for Protein Import and Assembly

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Keywords

preprotein, protein sorting, translocase, outer membrane, inner membrane, mitochondrial architecture

Abstract

Mitochondria are essential organelles with numerous functions in cellular metabolism and homeostasis. Most of the >1,000 different mitochondrial proteins are synthesized as precursors in the cytosol and are imported into mitochondria by five transport pathways. The protein import machineries of the mitochondrial membranes and aqueous compartments reveal a remarkable variability of mechanisms for protein recognition, translocation, and sorting. The protein translocases do not operate as separate entities but are connected to each other and to machineries with functions in energetics, membrane organization, and quality control. Here, we discuss the versatility and dynamic organization of the mitochondrial protein import machineries. Elucidating the molecular mechanisms of mitochondrial protein translocation is crucial for understanding the integration of protein translocases into a large network that controls organelle biogenesis, function, and dynamics.

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INTRODUCTION

Mitochondria are of central importance for the energetics, metabolism, and regulation of eukaryotic cells. According to the endosymbiont hypothesis, mitochondria are descendants of an ancient α-proteobacterium that was engulfed by a primordial eukaryotic cell. The initial view that mitochondria are just required for energy conversion to synthesize the majority of ATP needed in cells has been strongly expanded to an integrative model of mitochondrial functions in cellular health and disease. Mitochondria are involved in numerous metabolic processes, including the biosynthesis of amino acids, lipids, heme, and Fe-S clusters, and play crucial functions in cellular signaling pathways, quality control, and programmed cell death. Defects of mitochondria lead to severe diseases of the nervous system, heart, muscles, and other tissues (1).

Mitochondria possess a characteristic architecture with an outer membrane and an inner membrane, which contains invaginations termed cristae. The cristae membranes represent the main site of oxidative phosphorylation. Outer and inner membranes separate two aqueous compartments, (*a*) the intermembrane space and (*b*) the innermost compartment, which is the matrix. Mitochondria have retained their own genome and protein synthesis machinery; however, most endosymbiont genes have been transferred to the nucleus. The mitochondrial genome codes for 13 core subunits of the respiratory chain in humans and for seven respiratory chain core subunits and one ribosomal protein in baker's yeast.

Thus, about 99% of the more than 1,000 different mitochondrial proteins are encoded by nuclear genes and are synthesized on cytosolic ribosomes. Originally, it was assumed that precursor proteins were imported into mitochondria by one major translocation pathway. The analysis of mitochondrial protein biogenesis, however, revealed an unanticipated complexity of import pathways and mechanisms. The protein import machineries are part of a large network that controls mitochondrial biogenesis, dynamics, and function under physiological and pathophysiological conditions.

OVERVIEW OF MITOCHONDRIAL PROTEIN IMPORT MACHINERIES

Mitochondrial precursor proteins are synthesized in the cytosol with targeting signals that direct the proteins to mitochondria and into the correct mitochondrial compartment. Five major protein import pathways have been identified so far, each one characterized by a different type of targeting signal (**Figure 1**).

The classical import pathway is termed the presequence pathway. The vast majority of matrix proteins and many inner-membrane proteins are synthesized with N-terminal presequences that function as targeting signals (2–4). Presequence-carrying preproteins are imported by the translocase of the outer membrane (TOM) and the presequence translocase of the inner membrane (TIM23) (5–7). The presequence translocase-associated motor (PAM) drives protein translocation into the matrix (8, 9), where the mitochondrial processing peptidase (MPP) cleaves off the presequences (10).

In the four other major protein import pathways, the precursor proteins do not carry cleavable presequences, but they possess different kinds of internal targeting signals. The TOM complex, however, functions as the main mitochondrial entry gate for both cleavable preproteins and most noncleavable precursors. In the order of discovery, the carrier pathway is the second protein import pathway of mitochondria. The precursors of the multispanning hydrophobic carrier proteins of the inner membrane are imported by TOM, the small TIM chaperones of the intermembrane space, and the carrier translocase of the inner membrane (TIM22) (11–13).

The third protein import pathway is the β-barrel pathway. The precursors of β-barrel proteins of the outer membrane use the TOM complex and small TIM chaperones, followed by insertion into the outer membrane by the sorting and assembly machinery (SAM) (14), also termed TOB (topogenesis of outer-membrane β-barrel) proteins (15).

Many intermembrane space proteins contain characteristic cysteine motifs. These proteins are imported via TOM and the mitochondrial import and assembly (MIA) machinery of the intermembrane space, representing the fourth protein import pathway (16, 17).

The fifth protein import pathway is used by a number of outer-membrane proteins with α-helical transmembrane segments. The mitochondrial import (MIM) complex promotes the efficient import of outer-membrane proteins with an N-terminal signal-anchor sequence as well as multispanning (polytopic) outer-membrane proteins (18–20).

Recent studies revealed an even higher complexity of mitochondrial protein sorting since novel import routes were identified that combine elements of different import and processing pathways. For several precursor proteins, the import machineries have not been identified so far. Thus, it is likely that additional mitochondrial protein import and assembly pathways may be discovered.

Translocase of the outer mitochondrial membrane (TOM): a multisubunit protein complex that forms the main protein entry gate of mitochondria

TIM23: the presequence translocase of the inner mitochondrial membrane, which translocates cleavable preproteins into the matrix or inner membrane

Presequence translocase-associated motor (PAM): modular ATP-dependent protein machinery that drives protein translocation into the matrix

Mitochondrial processing peptidase (MPP): a dimeric protease in the matrix that removes N-terminal presequences

Small TIM chaperones: hexameric complexes in the intermembrane space that bind hydrophobic precursor proteins

TIM22: the carrier translocase of the inner mitochondrial membrane, which mediates membrane insertion of noncleavable multispanning precursor proteins

PRESEQUENCE PATHWAY TO THE INNER MEMBRANE AND MATRIX

About 60% of all mitochondrial proteins are synthesized in the cytosol with a cleavable presequence (4). Presequences are of variable length, typically from 15 to 50 amino acid residues,

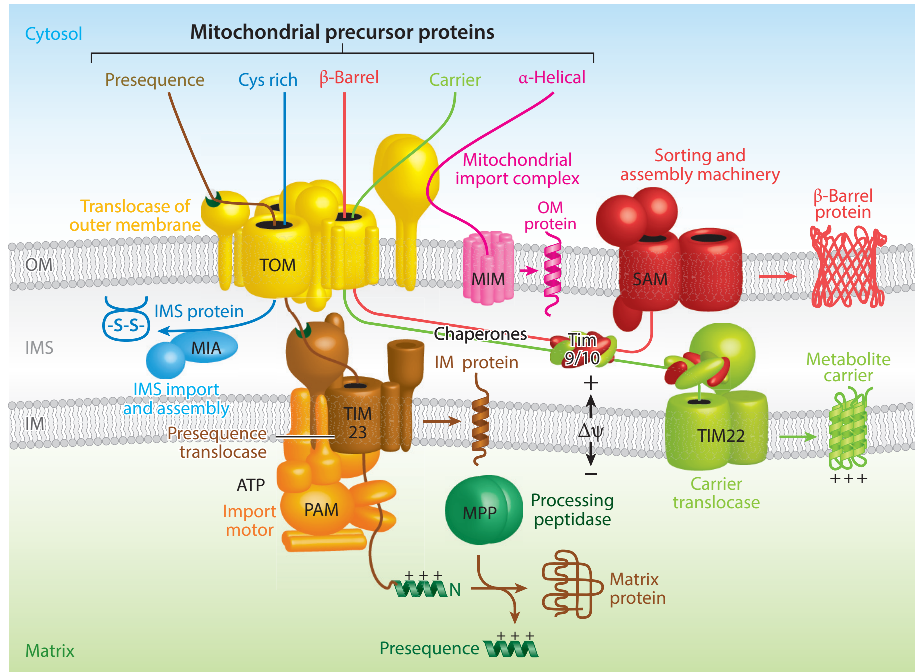

Figure 1

Overview of the five major protein import pathways of mitochondria. Presequence-carrying preproteins are imported by the translocase of the outer mitochondrial membrane (TOM) and the presequence translocase of the inner mitochondrial membrane (TIM23). Proteins with a hydrophobic sorting signal can be released into the inner membrane (IM), whereas hydrophilic proteins are imported into the matrix with the help of the presequence translocase-associated motor (PAM). The mitochondrial processing peptidase (MPP) removes the presequences. Cysteine-rich proteins of the intermembrane space (IMS) are imported by TOM and by the mitochondrial IMS import and assembly (MIA) system, which inserts disulfide bonds in the imported proteins. The precursors of β-barrel proteins are translocated through TOM to the small TIM chaperones of the IMS and are inserted into the outer membrane (OM) by the sorting and assembly machinery (SAM). The precursors of metabolite carriers of the IM are imported via TOM, small TIM chaperones, and the carrier translocase TIM22. A number of α-helical OM proteins are imported by the mitochondrial import (MIM) complex. The membrane potential (Δψ) across the IM drives protein translocation by the TIM23 and TIM22 complexes. Abbreviation: TIM9/10, hexameric Tim9 and Tim10 complex.

Sorting and assembly machinery (SAM): an outer-membrane protein complex that inserts β-barrel proteins into the membrane

Mitochondrial intermembrane space import and assembly (MIA) machinery: a disulfide relay that promotes protein import and oxidation in the intermembrane space

although very short presequences (<10 amino acid residues) and very long ones (up to 100 amino acid residues) were observed. An important characteristic of mitochondrial presequences is the formation of an amphipathic α-helix that contains a positively charged face and a hydrophobic face (2). The elements of the amphipathic helix are specifically recognized by receptors and other import components during preprotein translocation by TOM, TIM23, and PAM.

Translocase of the Outer Mitochondrial Membrane, the Main Protein Entry Gate of Mitochondria

The TOM complex consists of three receptor proteins, the channel-forming protein Tom40, and three small Tom proteins (**Figure 2**) (5, 6, 21, 22). Tom20 forms the initial receptor for presequences and specifically recognizes the hydrophobic surface of the amphipathic helix (3).

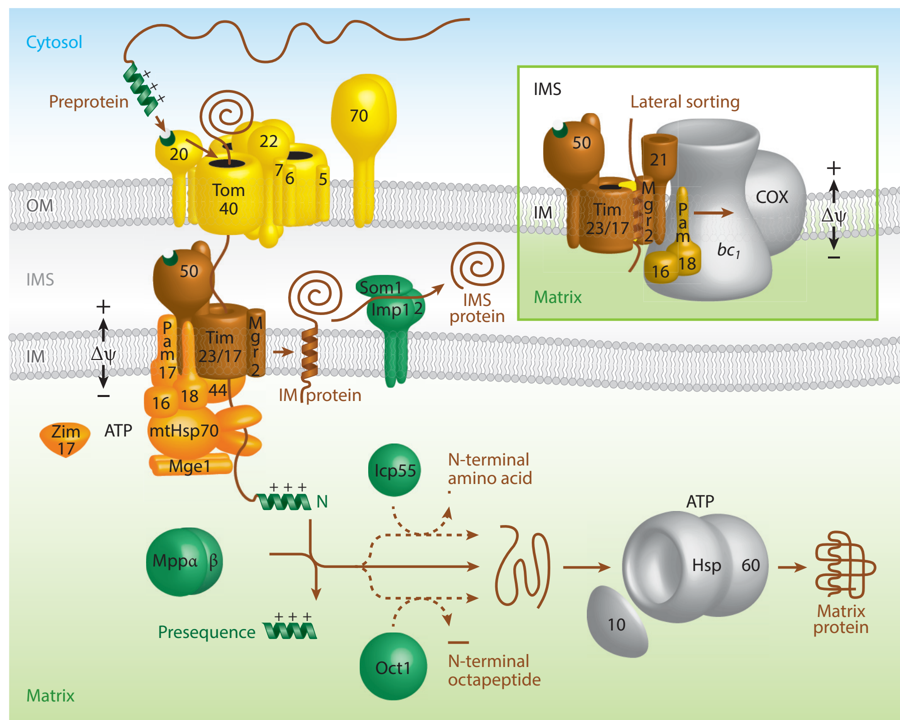

Figure 2

The presequence pathway into the mitochondrial inner membrane (IM) and matrix. The translocase of the outer membrane (TOM) consists of three receptor proteins, the channel-forming protein Tom40 and three small Tom proteins. Presequence-carrying preproteins are preferentially recognized by the receptors Tom20 and Tom22 and are translocated across the outer membrane (OM) through Tom40. The receptor Tim50 of the presequence translocase of the inner membrane (TIM23) binds the preproteins and transfers them to the channel-forming protein Tim23, which is closely associated with Tim17. The membrane potential ($\Delta\psi$) activates the TIM23 channel and drives translocation of the positively charged presequences. Protein translocation into the matrix is driven by the presequence translocase-associated motor (PAM) with the ATP-dependent mitochondrial heat-shock protein 70 (mtHsp70). Tim44 couples mtHsp70 to the TIM23 channel. The chaperone Zim17 (Hep1) prevents aggregation of mtHsp70 (184). The dimeric mitochondrial processing peptidase (MPP) removes the presequences. Additional proteases, the intermediate cleaving peptidase (Icp55) and the octapeptidyl aminopeptidase (Oct1; also termed mitochondrial intermediate peptidase, MIP), can remove destabilizing N-terminal amino acid residues from the imported proteins. The chaperonin, consisting of Hsp60 and Hsp10, promotes the folding of a number of matrix proteins. Preproteins with a hydrophobic sorting signal can be laterally released into the IM, controlled by the lateral gatekeeper Mgr2. For some preproteins, the sorting signal is removed by the inner-membrane peptidase (IMP, with the subunits Imp1, Imp2, and Som1), and the proteins are released into the intermembrane space (IMS). (*Inset*) For lateral sorting of preproteins, the TIM23 complex can associate via Tim21 with the bc_1 complex (complex III) and cytochrome *c* oxidase (COX, complex IV) of the respiratory chain, promoting the energy-dependent step of preprotein translocation. The respiratory chain complexes also serve as assembly platforms for some PAM subunits.

The presequence is handed over to the central receptor Tom22, which binds to the positively charged surface. The third receptor, Tom70, is only loosely attached to the TOM complex and plays a major role for the import of noncleavable hydrophobic precursors, such as the carrier precursors. However, the three Tom receptors possess partially overlapping substrate specificities

and can substitute for each other. Thus, when a receptor is defective, the other receptors promote import of precursor proteins, albeit with reduced efficiency (23).

Mitochondrial import (MIM): a complex that promotes the membrane insertion of signal-anchored, multispanning α-helical outer-membrane proteins

Tom40: a β-barrel protein of ~40 kDa, which forms the protein-conducting channel of the general protein import translocase of the outer mitochondrial membrane

The transmembrane channel of the TOM complex is formed by the β-barrel protein Tom40 (21). Tom40 belongs to the voltage-dependent anion channel (VDAC)–porin superfamily of β-barrel proteins, which are characterized by 19 transmembrane β-strands (24–26). The interior of the β-barrel channel contains hydrophilic and hydrophobic regions that form distinct import paths for hydrophilic precursors, such as matrix proteins, and hydrophobic precursors, such as carrier proteins (22, 27, 28). Presequences induce a rapid gating of the Tom40 channel activity (21). Tom40 is thus not simply a passive diffusion channel but specifically interacts with precursor proteins. Upon translocation through the Tom40 channel, presequences bind to the intermembrane space domain of Tom22 (29, 30). This central receptor thus provides presequence binding sites on both sides of the outer membrane.

The transmembrane segment in the middle of Tom22 is crucial for the oligomerization of the TOM complex. A full-size TOM complex contains up to three channels, each one formed by a single molecule of Tom40 (22). In the current view (**Figure 2**), a cluster of Tom22 molecules sits in the core of the TOM complex and links the Tom40 β-barrels (22). During assembly of new TOM complexes, the Tom40 molecules first form dimers in the absence of Tom22. Upon association with Tom22, the mature TOM complex is formed (14, 22). Why more than one Tom40 channel is present in the TOM complex and how the activity of the channels is coordinated are unknown.

The three small subunits, Tom5, Tom6, and Tom7, are not essential for TOM functions but are involved in the assembly and stability of the TOM complex. It has also been proposed that Tom5 may play a role in the transfer of some precursor proteins to the Tom40 channel; however, the exact role of Tom5 has not been clarified (31–33).

Preprotein Sorting at the TIM23 Presequence Translocase

Upon translocation through TOM, presequence-carrying preproteins are handed over to the TIM23 complex of the inner membrane (**Figure 2**) (34, 35). The TIM23 complex forms dynamic multisubunit machinery that recognizes preproteins and can transport them in two directions, into the inner membrane or into the matrix.

Three TIM23 subunits, Tim50, Tim23, and Tim21, expose domains to the intermembrane space. Tim50 functions as presequence receptor that binds preproteins emerging on the intermembrane space side of Tom40-Tom22 (36). Tim50 cooperates with the regulatory subunit Tim21 and the channel-forming protein Tim23 (37). Tim23 consists of an N-terminal intermembrane space domain that interacts with Tim50 and a C-terminal membrane-embedded domain that forms a channel across the inner membrane (38). In the absence of preproteins, Tim50 helps keep the Tim23 channel in a closed state. Upon binding of a presequence, the channel is activated and opened such that the preprotein can be translocated across the inner membrane (39).

Another membrane-embedded protein, Tim17, is closely connected to Tim23. The membrane domains of Tim23 and Tim17 have similarities; both consist of four α-helical transmembrane segments and contain GxxxG motifs that are crucial for the structural integrity of the TIM23 complex (40). The exact molecular function of Tim17 has not been reported, yet it is involved in regulation of the Tim23 channel and in preprotein sorting at the inner membrane (7, 41–44). A small membrane protein, Mgr2, is the most recently identified subunit of the presequence translocase. Mgr2 functions as lateral gatekeeper for preproteins that are sorted into the lipid phase of the inner membrane (45). These preproteins contain a hydrophobic sorting signal (stop-transfer sequence) in addition to the positively charged presequence (46, 47). In the current

view (**Figure 2** inset), Mgr2 binds to the sorting signal and controls its release into the inner membrane.

The membrane potential $\Delta\psi$ plays a dual role in preprotein translocation across the inner membrane. First, $\Delta\psi$ (negative on the matrix side) exerts an electrophoretic effect on the positively charged N terminus of preproteins, i.e., the presequence in most cases (48, 49). Second, $\Delta\psi$ directly activates the Tim23 channel (38). The second transmembrane segment of Tim23 undergoes a voltage-dependent conformational rearrangement (50), raising the possibility that this segment may be part of the voltage sensor of the presequence translocase (34).

Whereas protein translocation into the matrix requires ATP as a second energy source to drive the import motor PAM, lateral sorting of preproteins into the inner membrane can be driven by $\Delta\psi$ itself (51). In this case, an interesting cooperation of presequence translocase and respiratory chain complexes takes place. Tim21 physically links the TIM23 complex to the respiratory chain III–IV supercomplex [bc_1 complex and cytochrome *c* oxidase (COX)] (**Figure 2** inset) (52). Tim21 thus switches between its regulatory role in TOM-TIM23 transfer (7, 37) and the recruitment of respiratory chain complexes (52–54). The TIM23-respiratory chain coupling promotes the import of sorted preproteins, particularly when the energetic activity of mitochondria is reduced. It is assumed that the electrochemical proton gradient is higher in the immediate vicinity of the proton-pumping complexes, and thus, preprotein translocation is promoted. Additionally, in human mitochondria, Tim21 is a subunit of multiple TIM23 isoforms and supports the transfer of precursor proteins from the TIM23 complex to assembly intermediates of respiratory chain complexes (55, 56). The discovery of a direct TIM23-respiratory chain connection (52) promoted a paradigm shift in our understanding of mitochondrial protein import. We now see that the protein translocases do not function as independent units but are integrated into a dynamic network of machineries that perform functions in mitochondrial biogenesis, energy conversion, membrane architecture, and quality control.

The presequences of both inner-membrane-sorted and matrix-targeted preproteins are removed by MPP, a heterodimeric peptidase located in the matrix (10). Proteolytic processing by MPP is not strictly coupled to membrane translocation of preproteins but can take place during or after transport through the TIM23 complex. The presequence peptides are subsequently degraded by the matrix peptidasome, termed presequence protease (PreP) or Cym1 (57, 58). The subunits of MPP show similarity to two peripheral subunits of the respiratory complex III, Core 1 and Core 2, and in some organisms are even identical to these matrix-exposed subunits (59). In plant mitochondria, the processing peptidase is fully integrated into respiratory complex III, and thus, the preproteins have to be cleaved by the membrane-bound peptidase (60, 61). Preprotein processing occurs independently of electron transfer by complex III, and the functional implications of the integration of plant MPP into a respiratory chain complex are open.

Some inner-membrane-sorted preproteins are cleaved twice, first by MPP and then by the inner-membrane peptidase (IMP) (62). IMP removes the hydrophobic sorting sequence, and thus, the mature protein is either released into the intermembrane space (e.g., cytochrome b_2) (**Figure 2**) or remains anchored in the inner membrane by an additional hydrophobic segment (e.g., cytochrome c_1) (46). The connection of preprotein processing to mitochondrial quality control is discussed in the sidebar titled Preprotein Processing and Quality Control.

Inner-membrane peptidase (IMP): a peptidase that removes hydrophobic sorting signal sequences from mitochondrial precursor proteins

Motor-Driven Import into the Matrix

The membrane potential drives translocation of the presequence through the TIM23 channel but is not sufficient for promoting the complete import of a protein into the matrix. The import motor PAM is crucial for the import of matrix proteins. The ATP-driven molecular chaperone,

PREPROTEIN PROCESSING AND QUALITY CONTROL

Upon removal of the presequence by MPP, some mitochondrial precursor proteins are cleaved by proteases that link protein processing to protein stability and quality control. (*a*) In the mitochondrial matrix, proteins can be degraded according to the N-end rule, depending on the N-terminal amino acid residue. When the processing by MPP has generated an N terminus with a destabilizing residue, the intermediate cleaving peptidase Icp55 (aminopeptidase APP3) removes a single residue or the octapeptidyl aminopeptidase Oct1 removes an octapeptide, thus generating N termini with a stabilizing residue (4, 177). (*b*) The inner-membrane fusion protein optic atrophy 1 (OPA1) or mitochondrial genome maintenance 1 (Mgm1) is cleaved by several proteases, leading to long and short isoforms. The proper balance between the isoforms is important for fusion and fission of mitochondria (178). The first cleavage of OPA1 or Mgm1 is performed by matrix MPP, whereas the second cleavage can be performed by different inner-membrane proteases: an ATP-dependent AAA protease or the stress-activated OMA1 peptidase in mammals (178) and the rhomboid protease Pcp1 in yeast (179). Stress conditions (OMA1) and mitochondrial ATP levels (influencing the AAA protease and the activity of the import motor for access of Mgm1 to Pcp1) can regulate the second processing and thus influence mitochondrial fragmentation, mitophagy, or even cell death (180).

mitochondrial heat-shock protein 70 (mtHsp70), forms the core of the motor (**Figure 2**) (8, 9). The motor reaction cycle involves several essential partner proteins (7, 63, 64). The peripheral membrane protein Tim44 couples mtHsp70 to the TIM23 channel (65). Tim44 is in close contact with the preprotein in transit and helps transfer it to mtHsp70, which tightly binds the unfolded polypeptide chain with its peptide-binding domain. Two membrane-bound cochaperones, the stimulatory J-protein Pam18 (also termed Tim14) and the regulatory J-like protein Pam16 (Tim16), regulate the ATP-hydrolyzing activity of mtHsp70. The nucleotide exchange factor Mge1 promotes the release of ADP from mtHsp70 to initiate a new round of the reaction cycle.

The exact mechanism of the import motor is unknown. Two models are discussed. In the Brownian ratchet (trapping) model, binding of mtHsp70 to the preprotein in transit prevents backsliding of the polypeptide chain through the import channels, and thus, by Brownian motion, the polypeptide moves inward until a another mtHsp70 molecule can bind. By ATP-dependent cycles of mtHsp70 binding and release, the preprotein is thus imported stepwise into the matrix. In the pulling model, the import motor plays a more active role. mtHsp70 is bound to Tim44 and interacts with the preprotein in transit. Conformational changes of mtHsp70 during its ATPase cycle generate an inward-directed force on the preprotein. Then another mtHsp70 binds to the polypeptide chain, pulling it further into the matrix. Arguments in favor of each model and several variations of the trapping and pulling models have been reported without leading to a final solution (8, 9, 66). We envision that mtHsp70 functions by a combination of two mechanisms, trapping and pulling, to efficiently drive protein translocation into the matrix.

Mitochondrial heat-shock protein of ~70 kDa (mtHsp70): an essential ATP-dependent chaperone of the presequence translocase-associated motor

Several observations suggest that the PAM reaction cycle involves dynamic changes not only of mtHsp70 but also of its partner proteins. The coupling protein Tim44 consists of two domains that undergo rearrangements during preprotein translocation (65). The regulatory Pam16-Pam18 module is not only associated with the motor, but it also interacts with the respiratory chain III–IV supercomplex independently of the other motor subunits (**Figure 2** inset) (53). The respiratory supercomplex may function as a platform for transfer of the Pam16-Pam18 module to the TIM23 complex during assembly of the import motor. Another regulatory protein, Pam17, is involved in the organization of the TIM23-PAM machinery, but its exact function is unknown (63, 64, 67). Taken together, TIM23, PAM, and the respiratory III–IV supercomplex form a sophisticated system for the energy-driven translocation of cleavable preproteins into the inner membrane and

matrix; substantial future work is required to understand the assembly, operation, and regulation of this translocation system.

Proteins imported into the matrix are folded to their mature, active forms. mtHsp70 plays a dual role. In the membrane-bound motor form, it drives translocation of preproteins, and in the soluble form, it exhibits typical chaperone activity to prevent misfolding and aggregation of proteins (8, 9). The J-protein Mdj1 and the nucleotide exchange factor Mge1 function as cochaperones of the soluble mtHsp70. A number of proteins are delivered to the Hsp60-Hsp10 chaperonin machinery, which forms protected folding cages for proteins (**Figure 2**) (68). Like mtHsp70, the Hsp60 machinery functions in an ATP-dependent manner.

Oxidase assembly (OXA) translocase: a protein export translocase in the mitochondrial inner membrane (homologous to prokaryotic YidC)

Oxidase Assembly Translocase–Mediated Insertion of Proteins into the Inner Membrane

The oxidase assembly (OXA) translocase functions as protein insertion machinery for the export of proteins from the matrix into the inner membrane. Oxa1, the main component, has been conserved from the prokaryotic YidC protein insertase (69). Oxa2 (Cox18) is a paralog of Oxa1 and plays only a limited role in protein export.

Oxa1 is required for the biogenesis of a large number of inner-membrane proteins. Three different roles of Oxa1 have been observed:

1. Proteins encoded by the mitochondrial genome are exported into the inner membrane by Oxa1. Oxa1 and the protein Mba1 bind mitochondrial ribosomes and support cotranslational protein insertion into the inner membrane (**Figure 3**) (70). Numerous additional factors are involved in the biogenesis of mitochondrial-encoded proteins (reviewed in Reference 71).
2. Oxa1 mediates conservative sorting of nuclear-encoded inner-membrane proteins. The precursors of a number of inner-membrane proteins are imported by the TOM and TIM23-PAM machineries into the matrix, followed by export into the inner membrane via Oxa1 (**Figure 3**). Because the export resembles protein translocation in the prokaryotic ancestor of mitochondria, the import-export pathway is termed conservative sorting (72, 73). For a long time, it was assumed that only a small number of proteins use the conservative sorting route. A recent systematic analysis of Oxa1-deficient mitochondria, however, revealed a large number of multispanning inner-membrane proteins that are sorted by a conservative mechanism (74). Cleavable inner-membrane proteins thus can be sorted by two mechanisms: stop transfer in the TIM23 complex and lateral release or conservative sorting using TIM23-PAM and Oxa1. The previous controversial debate about which sorting route is the physiological one used by mitochondria was solved by the discovery that one and the same multispanning protein uses both mechanisms. The ABC transporter Mdl1 contains six α-helical transmembrane segments. The two middle transmembrane segments are imported into the matrix in a PAM-dependent manner and inserted into the inner membrane by Oxa1, whereas the other transmembrane segments are sorted by a stop-transfer mechanism (75). The analysis of additional multispanning Oxa1 substrates similarly revealed that a combination of stop-transfer and conservative sorting mechanisms is used; the order of the two mechanisms varies depending on the precursor protein (74, 76). Thus, we now know that single-spanning cleavable inner-membrane proteins are sorted into the inner membrane via the stop-transfer route, whereas multispanning cleavable proteins typically use a combination of stop-transfer and conservative sorting mechanisms.
3. Assembly of the carrier translocase TIM22 requires Oxa1. Surprisingly, defects of the OXA translocase impair the biogenesis of noncleavable carrier proteins, although no direct connection between the OXA translocase and carrier precursors is detectable (74, 77). However,

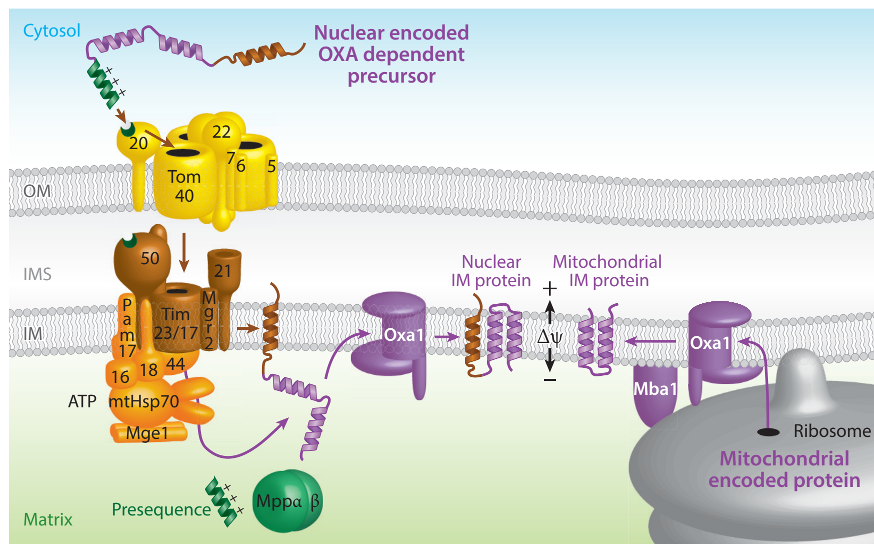

Figure 3

Role of the oxidase assembly (OXA) translocase in protein sorting. Proteins synthesized by mitochondrial ribosomes are exported into the inner membrane (IM) by the OXA translocase; the ribosome receptor Mba1 supports the cotranslational protein export. Presequence-carrying IM proteins with multiple transmembrane segments can also use the OXA translocase. The preproteins are synthesized in the cytosol, are imported by the translocase of the outer membrane (TOM) and the presequence translocase of the IM (TIM23), and are cleaved by the mitochondrial processing peptidase (MPP). The transmembrane segments are sorted by two mechanisms: Some transmembrane segments are imported into the matrix by the presequence translocase-associated motor (PAM) and are exported into the IM by the OXA translocase (conservative sorting); the other transmembrane segments are laterally released from TIM23 into the IM (by stop-transfer sorting). Abbreviation: IMS, intermembrane space.

Oxa1 is required for the biogenesis of the TIM22 complex. Two subunits, Tim18 and Sdh3, are cleavable multispanning inner-membrane proteins, which are imported by a combination of conservative sorting (the first two transmembrane segments) and stop transfer (the third transmembrane segment) (74). Oxa1 thus promotes the proper assembly of the TIM22 complex as a prerequisite for efficient import of carrier proteins.

CARRIER PATHWAY INTO INNER MEMBRANE

The mitochondrial metabolite carriers form a large family of hydrophobic proteins that contain six α-helical transmembrane segments. Most carrier proteins are located in the inner membrane and are synthesized without a cleavable presequence. Carrier precursors contain several internal targeting elements distributed over the mature primary structure (78, 79). The exact targeting mechanism has not been elucidated. It is assumed that the targeting elements cooperate in binding of a carrier precursor to the receptor Tom70 and the subsequent sorting to the inner mitochondrial membrane.

The carrier precursors are bound to cytosolic chaperones of the Hsp70 and Hsp90 classes to prevent aggregation of the hydrophobic precursors in the cytosol (**Figure 4**). The ATP-dependent chaperones directly participate in delivering the precursors to Tom70 (80, 81). The receptor possesses two distinct binding sites, one for the precursor and one for chaperones (82). Several Tom70

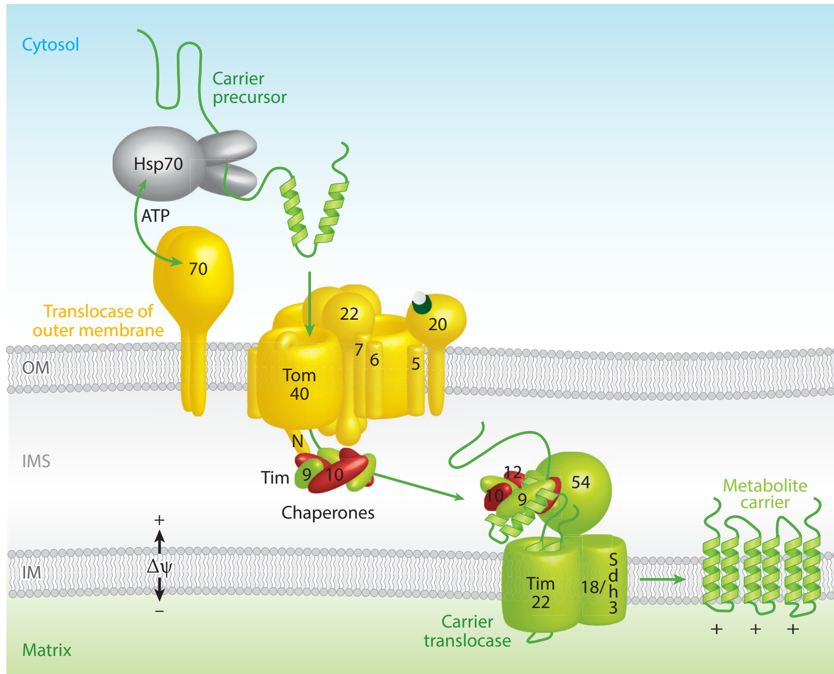

Figure 4

Carrier pathway into the inner membrane. The precursors of the hydrophobic metabolite carriers are synthesized without a cleavable presequence. The precursors are bound to cytosolic chaperones, such as the ATP-dependent heat-shock protein 70 (Hsp70). The receptor Tom70 of the translocase of the outer membrane (TOM) binds both the precursor and chaperone. The precursors are transferred to the channel Tom40 and are translocated across the outer membrane (OM) in a loop formation. The small TIM chaperones of the intermembrane space (IMS) are recruited to the OM by an N-terminal segment of the channel protein Tom40. The small TIM chaperones deliver the precursors to the carrier translocase of the inner membrane (TIM22). Tim54 contains a large IMS domain that interacts with small TIM chaperones. The membrane potential (Δψ) activates the channel protein Tim22, and the proteins are laterally released into the inner membrane (IM). Abbreviation: Sdh3, succinate dehydrogenase subunit 3.

molecules cooperate in the binding of a carrier precursor and transferring it to the central receptor Tom22, followed by insertion into the Tom40 channel (83). The role of cytosolic cofactors in mitochondrial protein import is a largely unexplored field for most import pathways. The chaperone-assisted targeting of carrier precursors to Tom70 (80–82) represents the best-studied case for defining the function of cytosolic cofactors in mitochondrial protein import.

In contrast to cleavable preproteins that are translocated in a linear manner, substrates of the carrier import pathway are inserted into the Tom40 channel in a loop formation such that both termini are still on the cytosolic side, whereas the middle portion of the precursor is passing through the channel (83, 84). The analysis of carrier translocation revealed a peculiar structural feature of Tom40. The N-terminal segment of Tom40 passes from the cytosolic side through the interior of the β-barrel channel into the intermembrane space (**Figure 4**) (22, 25). The N-terminal Tom40 segment recruits small TIM chaperones of the intermembrane space to the channel exit such that the hydrophobic precursors can be directly transferred to the chaperones.

The small TIM chaperones are heterohexameric complexes (85, 86). The main form is the soluble Tim9-Tim10 complex. A nonessential Tim8-Tim13 complex with a similar structural

Mia40: a receptor and oxidoreductase of the intermembrane space MIA system forming a disulfide relay with Erv1

organization is employed by a few precursor proteins. Another small Tim protein, Tim12, forms a Tim9-Tim10-Tim12 hexameric chaperone complex. In contrast to the soluble small TIM complexes, the Tim9-Tim10-Tim12 complex is bound to the inner membrane on the surface of the TIM22 complex (87, 88). The hydrophobic precursors are thus continuously protected from aggregation or misfolding because the chaperones directly interact with the membrane-bound translocases to prevent any release of the precursors into the aqueous phase. Hsp70 and Hsp90 deliver the precursors directly to Tom70; Tom40 recruits the small TIM chaperones, and together with Tim12 these chaperones interact with the carrier insertase TIM22.

The TIM22 complex consists of the channel-forming protein Tim22, the receptor-like protein Tim54, the Tim9-Tim10-Tim12 chaperone complex, and the Tim18-Sdh3 module (**Figure 4**). Tim54 exposes a large domain into the intermembrane space and recruits the Tim9-Tim10-Tim12 complex into the translocase (87, 89). Tim54 may also bind precursor proteins, but this has not yet been directly demonstrated. Tim22 mediates the insertion of precursor proteins into the inner membrane in a $\Delta\psi$-driven manner (90). Tim22 shows similarity to Tim23 (and Tim17) of the presequence translocase, suggesting that the channel-forming proteins of both the presequence translocase and carrier translocase have been derived from a common ancient channel.

The Tim18-Sdh3 module plays a role in the assembly of the TIM22 complex. Tim18 and Sdh3 are evolutionarily related to the membrane-integral part of respiratory complex II (succinate dehydrogenase, SDH) of mitochondria and bacteria. Whereas Tim18 shows similarity to Sdh4, Sdh3 possesses a dual localization as a subunit of both the respiratory complex II and of the TIM22 complex (91). Thus, in the evolution of the TIM22 complex, the membrane part of a respiratory complex apparently served as a building block for the formation of a translocase (92). The biogenesis of Tim18 and Sdh3 involves the conservative sorting route via TOM, TIM23-PAM, and the OXA translocase, explaining the requirement of the OXA translocase for the proper assembly and function of the TIM22 complex discussed above in the section titled Oxidase Assembly Translocase–Mediated Insertion of Proteins into the Inner Membrane (74). It is unknown if the dual localization of Sdh3 leads to a functional cross talk between the respiratory chain and carrier translocase.

In addition to numerous carrier precursors, the TIM22 complex also transports other non-cleavable inner-membrane proteins that contain multiple transmembrane segments, including members of the Tim17, Tim22, and Tim23 family (84). The molecular mechanism of membrane insertion, however, has not been solved for any of the substrates. So far, neither a high-resolution structure of the TIM22 complex nor a site-specific mapping of the precursor-translocase interaction has been reported. Future studies are needed to understand how multiple transmembrane segments are translocated by TIM22 and laterally released into the inner membrane.

MITOCHONDRIAL INTERMEMBRANE SPACE IMPORT AND ASSEMBLY MACHINERY

The mitochondrial intermembrane space contains numerous proteins with characteristic cysteine motifs, such as Cx_3C and Cx_9C motifs, that form intramolecular disulfide bonds. Initially, it was assumed that these proteins are in a reduced state like the majority of proteins in the cytosol and mitochondrial matrix. The identification of the oxidoreductase Mia40 (16, 17), however, revealed that the intermembrane space contains an oxidative protein folding machinery, which catalyzes the formation of disulfide bonds in imported proteins (93, 94). The disulfide bonds promote the conformational stabilization and assembly of many intermembrane space proteins. For example, the small TIM chaperones are typical substrates of Mia40.

Mia40 cooperates with the sulfhydryl oxidase Erv1 (essential for respiration and vegetative growth) or ALR (augmenter of liver regeneration) in a disulfide relay (95, 96). A disulfide bond

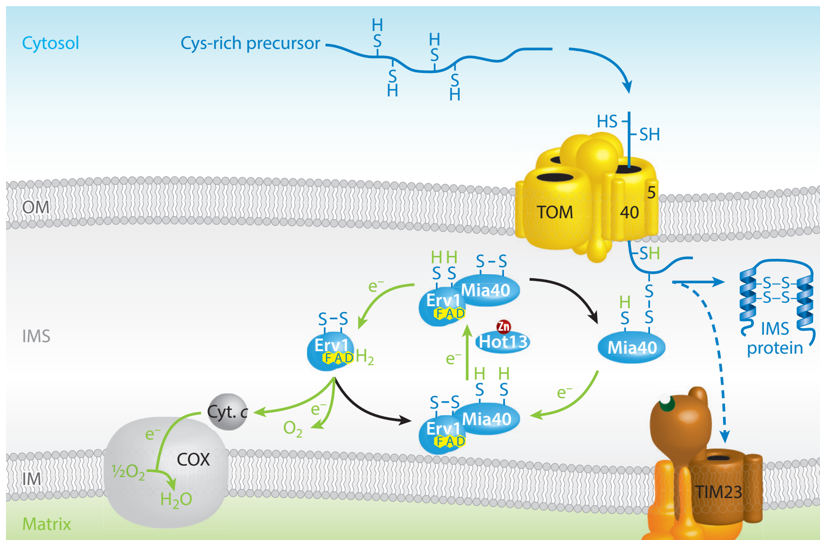

Figure 5

Mitochondrial intermembrane space import and assembly (MIA) machinery. Many intermembrane space (IMS) proteins contain characteristic cysteine motifs. The precursors are kept in a reduced and unfolded state in the cytosol and are translocated across the outer membrane (OM) via the Tom40 channel. Mia40 functions as a receptor in the IMS and binds to precursors via hydrophobic interaction and a transient intermolecular disulfide bond. The imported proteins are oxidized by the oxidoreductase activity of Mia40, leading to the formation of intramolecular disulfide bonds. Mia40 is reoxidized by the FAD-dependent sulfhydryl oxidase Erv1 (essential for respiration and vegetative growth) with assistance of the zinc-binding protein Hot13 (helper of Tim). Electrons derived from the oxidation of imported proteins flow via Mia40 to Erv1 and from here to O_2 or cytochrome *c* (Cyt. *c*) of the respiratory chain. The MIA system not only promotes the biogenesis of IMS proteins, but some inner membrane (IM) and matrix proteins are also MIA substrates. Abbreviation: COX, cytochrome *c* oxidase.

generated by Erv1 is transferred to Mia40 and then to substrate proteins, involving the formation of a transient intermolecular disulfide bond between Mia40 and a substrate during the transfer reaction (**Figure 5**). Electrons are thus transferred from the oxidized substrates via Mia40 to Erv1 and then to cytochrome *c* or molecular oxygen (97–101). This involves a redox-active cysteine pair of Mia40 that transiently interacts with substrates and is reoxidized by Erv1. The zinc-binding protein Hot13 (helper of Tim of 13 kDa) promotes the oxidation of Mia40 by keeping it in a zinc-free state (102, 103).

Mia40 not only promotes the folding of imported proteins but is also crucial for the translocation of precursor proteins across the outer membrane. The precursor proteins are kept in a reduced state in the cytosol (104) and are translocated through the Tom40 channel across the outer membrane (33). Interestingly, none of the Tom receptors was found as a requirement for the import of MIA substrates (32, 33). Instead, Mia40 functions as intermembrane space receptor that recognizes and binds the precursor proteins as soon as they emerge on the intermembrane space surface of the Tom40 channel (105–108). Precursor proteins contain a signal with hydrophobic residues and a cysteine residue, termed mitochondrial intermembrane space sorting signal or intermembrane space targeting signal (109, 110). The signal is recognized by a hydrophobic binding pocket of Mia40 (108), followed by formation of a mixed disulfide bond between the cysteine of the mitochondrial intermembrane space sorting or targeting signal and Mia40. Subsequently,

Erv1: a sulfhydryl oxidase, partner of Mia40 in the intermembrane space disulfide relay

the disulfide bond is transferred to the imported protein. The disulfide isomerase activity of Mia40 contributes to the formation of the correct (native) intramolecular disulfide bonds in substrates (111).

Sam50: a channel-forming subunit of ∼50 kDa of the sorting and assembly machinery for biogenesis of outer-membrane β-barrel proteins

Recent studies revealed that the substrate spectrum of the MIA system is larger than expected. (*a*) This involves integral subunits of the inner-membrane translocases. Tim17 and Tim22 contain an intramolecular disulfide bond exposed to the intermembrane space (43, 44, 112, 113). Mia40 interacts with the precursor of Tim22 via noncovalent interactions as well as with a transient disulfide bond (112). Formation of an intramolecular disulfide bond in Tim22 then promotes membrane integration and proper assembly of the protein. The precursor of Tim17 interacts with the hydrophobic binding pocket of Mia40 during import. However, the oxidation of Tim17 to form an intramolecular disulfide bond can be directly performed by Erv1 (43), raising the possibility that Erv1 may directly interact with a number of substrates of the MIA system. The disulfide bond in Tim17 stabilizes the TIM23 complex and promotes the gating of the TIM23 channel. (*b*) The mitochondrial ribosomal subunit Mrp10 is imported by the presequence pathway; however, it contains an unusual proline-rich N-terminal matrix-targeting signal that permits access of the precursor to Mia40 during passage through the intermembrane space (114). Oxidized Mrp10, containing two disulfide bonds, is then translocated through the TIM23 complex into the matrix; thus, the precursor polypeptide is not translocated as a linear chain but likely in a loop formation. Oxidation is not crucial for the function of Mrp10 but stabilizes the ribosomal subunit by preventing its proteolytic degradation (114). Thus, the MIA system not only imports and folds intermembrane space proteins but also contributes to the biogenesis and quality control of inner-membrane and matrix proteins.

The translocation of MIA substrates through Tom40 can also occur in the opposite direction. Several intermembrane space proteins are retro-translocated into the cytosol when their oxidative folding is impaired (115). This export is more efficient for small proteins. The exported proteins are degraded by the proteasome (116), indicating that the cytosolic quality control system can contribute to the removal of folding-defective intermembrane space proteins.

SORTING PATHWAYS OF MITOCHONDRIAL OUTER-MEMBRANE PROTEINS

The mitochondrial outer membrane possesses two types of integral membrane proteins. (*a*) β-Barrel proteins are integrated into the membrane by multiple transmembrane β-strands. (*b*) α-Helical membrane proteins are membrane-anchored by one or more α-helical transmembrane segments. β-Barrel proteins reflect the origin of mitochondria from Gram-negative bacteria, which contain a large number of β-barrel proteins in their outer membrane, whereas the numerous α-helical outer-membrane proteins of mitochondria have been introduced by the eukaryotic cell. Consistent with the different evolutionary origins of outer-membrane proteins, their precursors are not imported by one unique pathway. Recent studies revealed a large variety of protein-sorting pathways into the mitochondrial outer membrane.

Sorting and Assembly Machinery for β-Barrel Proteins

Mitochondria contain only a small set of β-barrel proteins; however, these proteins perform crucial functions and include the metabolite channel porin or VDAC, the general protein import channel Tom40, and the channel Sam50 of the SAM complex. Like all mitochondrial outer-membrane proteins, the precursors of β-barrel proteins are synthesized on cytosolic ribosomes. The precursors are imported by the TOM complex, directed by a targeting signal that

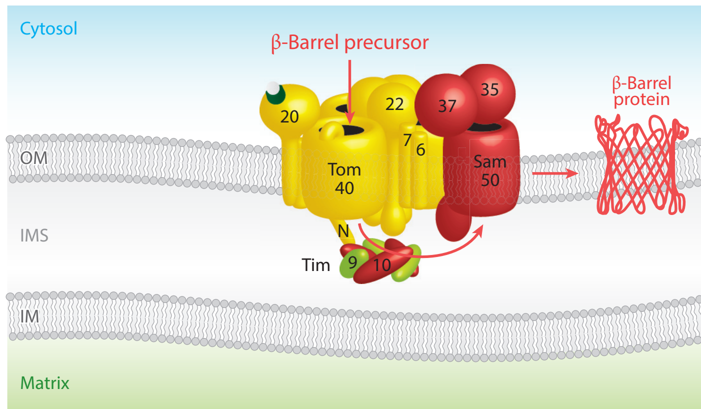

Figure 6

Biogenesis of β-barrel proteins of the outer mitochondrial membrane. The precursors of β-barrel proteins are initially imported by the translocase of the outer membrane (TOM), bind to small TIM chaperones in the intermembrane space (IMS), and are inserted into the outer membrane (OM) by the sorting and assembly machinery (SAM). The receptor Tom22 and the peripheral membrane protein Sam37 link TOM and SAM into a transient supercomplex that promotes the efficient transfer of precursor proteins. Folding of the β-barrel occurs at Sam50-Sam35, followed by release of the barrel into the lipid phase of the OM. Abbreviation: IM, inner membrane.

consists of a β-hairpin element (117). The β-hairpin contains two adjacent β-strands, typically the two most C-terminal β-strands of the precursor, and the connecting loop. Upon translocation through the Tom40 channel, the precursors are bound to the small TIM chaperones of the intermembrane space (**Figure 6**). These chaperones thus protect both the hydrophobic carrier precursors of the inner membrane and the β-barrel precursors of the outer membrane from aggregation in the aqueous intermembrane space.

Insertion of the β-barrel proteins into the outer membrane is performed by the SAM complex, which contains a membrane-integrated protein, Sam50 (Tob55), and two peripheral membrane proteins exposed to the cytosol, Sam35 and Sam37 (14, 15, 118). The central protein Sam50 has been conserved from bacteria (BamA) to mitochondria and forms a 16-stranded β-barrel channel. Although the other SAM and BAM subunits do not show any similarity, the basic mechanism of β-barrel sorting has been well conserved, as bacterial β-barrel proteins can be properly imported and assembled in the mitochondria (119–121). Two opposing models have been discussed, explaining how Sam50 and BamA promote membrane insertion of β-barrel proteins: The precursors are either inserted at the protein-lipid interface on the outer surface of BamA/Sam50, or the precursors are threaded into the BamA/Sam50 channel and are laterally released in the lipid phase (122). Recent high-resolution structures of BamA suggest a flexible interaction of the first and last β-strands, thus raising the possibility of a lateral opening of the β-barrel channel (123).

The most C-terminal β-strand of the precursor functions as a β-signal that specifically binds to Sam50-Sam35 and directs membrane insertion (124). The β-signal induces a conductance increase of the SAM channel. We propose that the β-signal induces an opening of the lateral gate of Sam50, and thus, the precursor polypeptide may be laterally released from the Sam50 channel into the membrane. An experimental demonstration of this hypothesis will require a mapping of precursor intermediates in transit through the SAM complex.

Sam50 carries an N-terminal polypeptide transport-associated (POTRA) domain located on the intermembrane space side. BamA possesses five POTRA domains that play an important role in precursor transfer from the bacterial periplasm to the membrane. A single mitochondrial POTRA domain is involved in efficient precursor transfer through SAM; however, its exact function has not been determined (125, 126).

The third SAM subunit, Sam37, directly interacts with the cytosolic receptor domain of Tom22 (25, 127). A fraction of SAM and TOM complexes is thus coupled into a large, but transient, TOM-SAM supercomplex. This supercomplex promotes the transfer of β-barrel precursors from TOM to SAM (25). Taken together, elements on both sides of the outer membrane, Tom22-Sam37 on the cytosolic side and small TIM chaperones in the intermembrane space, are involved in efficient precursor transfer to SAM (**Figure 6**). The formation of the β-barrel then occurs in association with Sam50-Sam35, followed by release of the folded β-barrel into the lipid phase.

Endoplasmic reticulum–mitochondria encounter structure (ERMES): a multisubunit protein complex that connects the endoplasmic reticulum and mitochondrial outer membrane

Mitochondrial distribution and morphology protein 10 (Mdm10): a β-barrel protein with dual localization in SAM (protein assembly) and ERMES (mitochondrial morphology)

Dual Localization of the Mitochondrial Distribution and Morphology Protein 10 in the Sorting and Assembly Machinery and Mitochondria–Endoplasmic Reticulum Junctions

The identification of a fourth SAM subunit led to a surprising connection between the SAM complex and the endoplasmic reticulum–mitochondria encounter structure (ERMES). The mitochondrial distribution and morphology protein 10 (Mdm10) is a subunit of both SAM and ERMES (**Figure 7**) (128–131).

Mdm10 belongs to the porin and Tom40 superfamily and forms a 19-stranded β-barrel (132). Mdm10 associates with the SAM core complex, forming a SAM-Mdm10 complex. This complex interacts with the precursor of Tom22. Mitochondria lacking Mdm10 are impaired in a late stage of assembly of the TOM complex, which involves an association of the precursors of Tom22 and Tom40 (129). The SAM-Mdm10 complex thus provides an assembly platform for the TOM complex. The precursor of Tom40 is folded by the β-barrel pathway via Sam50-Sam35 (25), and the precursor of the α-helical protein Tom22 uses Mdm10 for its biogenesis (129). Thus, both precursors are brought into close proximity by SAM-Mdm10, promoting an efficient assembly of the TOM complex.

The ERMES complex stably connects the endoplasmic reticulum and mitochondria (130). Mdm10 is one of the four core subunits of ERMES. Owing to its β-barrel structure, Mdm10 is stably anchored in the outer membrane. The subunits Mdm34 and Mdm12 connect it to the ER-anchored Mmm1 protein. Defects in ERMES subunits lead to an altered mitochondrial morphology and likely also affect phospholipid transfer between both organelles. The exact molecular function of Mdm10 in the ERMES complex has not been determined. It is an open question whether, in addition to its membrane anchor function, Mdm10 plays a direct role in the maintenance of mitochondrial morphology or lipid transport.

The Mdm10 pools in SAM and ERMES are not strictly separated but are in a dynamic exchange. This involves a small TOM subunit, Tom7, which shows dual localizations (133, 134). The major fraction is present in the TOM complex; however, a small fraction of Tom7 molecules bind to Mdm10. Tom7 only interacts with Mdm10, which has been released from SAM, and favors the transfer to ERMES (**Figure 7**). Tom7 thus traps SAM-free Mdm10, explaining why Tom7 plays an inhibitory role in the assembly of the TOM complex as it reduces the amount of SAM-bound Mdm10 (133–135). We suggest that Mdm10 and Tom7 are part of a regulatory network that controls assembly of the TOM complex and functionally links it to mitochondria–endoplasmic reticulum junctions.

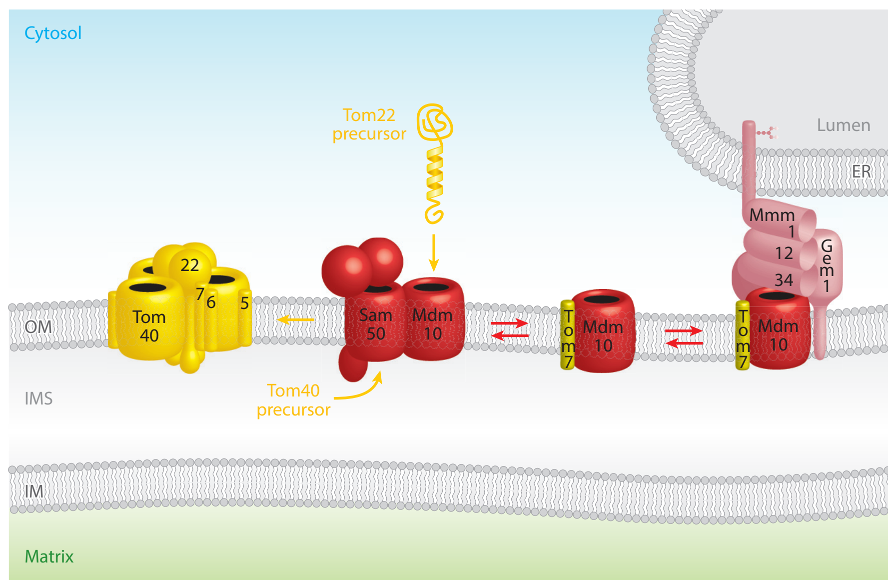

Figure 7

The dual role of mitochondrial distribution and morphology protein 10 (Mdm10) in protein assembly and organelle contact sites. Mdm10 associates with the sorting and assembly machinery (SAM) of the outer membrane (OM) and promotes the biogenesis of the precursor of the receptor Tom22. Because the precursor of the channel protein Tom40 is folded by SAM, the SAM-Mdm10 complex provides an assembly platform for the TOM complex. Mdm10 is also present in the endoplasmic reticulum (ER)-mitochondria encounter structure (ERMES). Mdm10 is anchored in the OM, and Mdm34 and Mdm12 link it to Mmm1 (maintenance of mitochondrial morphology 1), which is anchored in the ER membrane. Gem1 is a regulatory GTPase associated with ERMES. Tom7 has a dual localization: It is a subunit of the TOM complex and a binding partner of Mdm10. Tom7 binds to Mdm10 released from SAM and thus favors the association with ERMES. Abbreviation: IMS, intermembrane space.

Different Pathways for Insertion of α-Helical Proteins into the Outer Membrane

The biogenesis of α-helical outer-membrane proteins is only understood in part (136, 137). Three main protein classes can be distinguished: signal-anchored proteins, tail-anchored proteins, and polytopic (multispanning) outer-membrane proteins (**Figure 8**). Signal-anchored proteins and tail-anchored proteins contain an α-helical transmembrane segment at the N terminus and C terminus, respectively. Together with flanking positively charged amino acid residues, these transmembrane segments can function as both membrane anchors and targeting signals. Polytopic proteins contain multiple transmembrane segments that may contain targeting information; however, the exact targeting signals of these proteins are largely unknown.

The MIM complex functions as a protein insertase for signal-anchored and polytopic outer-membrane proteins. The MIM complex consists of multiple copies of Mim1 and ~1 copy of Mim2; both are small single-spanning outer-membrane proteins (**Figure 8**) (18–20, 138). The molecular mechanism of the MIM complex has not been elucidated, yet it promotes the efficient membrane insertion of many signal-anchored proteins and polytopic proteins. In case of polytopic proteins, the receptor Tom70 cooperates with the MIM complex. Tom70 recognizes the precursors and transfers them to the MIM insertase (139, 140). The MIM complex is not strictly essential for

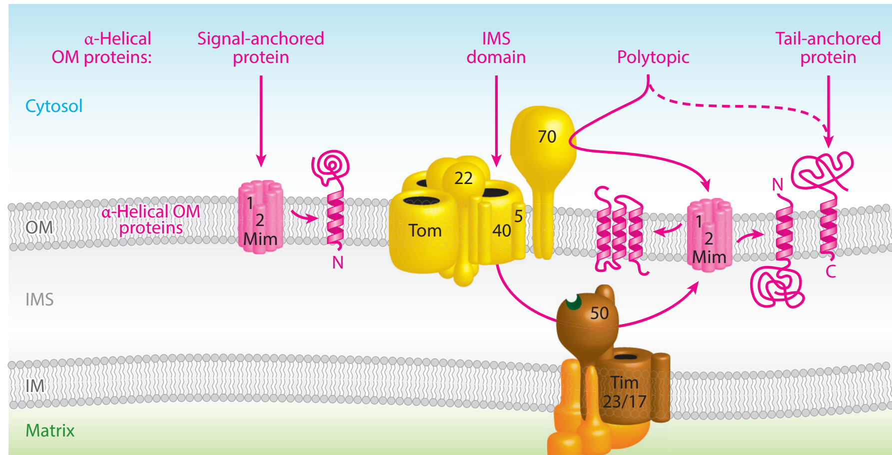

Figure 8

Multiple import pathways for integral α-helical proteins of the mitochondrial outer membrane. The precursors of proteins with an N-terminal signal anchor sequence are typically inserted into the outer membrane (OM) by the mitochondrial import (MIM) complex. The precursors of polytopic (multispanning) OM proteins can be imported via the receptor Tom70 and the MIM complex. Some polytopic proteins as well as tail-anchored OM proteins may be inserted into the OM in a lipid-assisted manner, possibly independent of specific proteinaceous machinery. However, several unexpected import routes have been observed, such as the import of a signal-anchored protein with a large intermembrane space (IMS) domain via the translocase of the outer membrane (TOM), the presequence translocase of the inner membrane (TIM23), and OM insertion by the MIM complex. Abbreviation: IM, inner membrane.

membrane insertion of some polytopic proteins. Vögtle et al. (141) showed that the precursor of Ugo1, a component of the mitochondrial fusion machinery, is inserted into protein-free liposomes. The membranes have to contain the nonbilayer phospholipid phosphatidic acid in order to promote the insertion of Ugo1. *In organello*, however, the import of Ugo1 is strongly promoted by Tom70 and by the MIM complex (139, 140), suggesting that both the proteinaceous machinery and the proper lipid composition are required for an optimal membrane insertion of the protein.

For the majority of tail-anchored outer-membrane proteins, no proteinaceous machinery has been identified, and their import is assisted by the lipid composition of the membrane (**Figure 8**) (142–144). The precursors are inserted into membranes with a low-ergosterol content. The low-sterol content of the mitochondrial outer membrane in comparison to other cell organelles may ensure the proper targeting of tail-anchored proteins to mitochondria.

The number of distinct import routes into the outer membrane is likely considerably higher. Several individual examples revealed a surprising variety of transport routes. (*a*) Mim1 and Tom22 contain a single transmembrane segment in the middle of the protein, and their C termini are located in the intermembrane space. The import routes of both precursors employ TOM receptors and the SAM (TOB) complex (145–147), yet differences were observed for the initial targeting and final step of membrane insertion. The precursor of Mim1 interacts with the Hsp40-type cochaperone Djp1 and the chaperone Hsp70 in the cytosol (147). The chaperones prevent aggregation and guide the precursor to the receptor Tom70, resembling the principle of chaperone-guided transfer of hydrophobic carrier precursors (80, 81). In the case of Tom22, the final step of membrane insertion requires the β-barrel protein Mdm10 as discussed above in the section titled Dual Localization of the Mitochondrial Distribution and Morphology Protein 10 in the

Sorting and Assembly Machinery and Mitochondria–Endoplasmic Reticulum Junctions (**Figure 7**). (*b*) The precursors of the outer-membrane proteins Om45 and Mcp3 are imported by an unexpected route. They first follow the presequence pathway, which includes the TOM and TIM23 complexes; then escape from the TIM23 complex; and are exported into the outer membrane with the help of the MIM complex (148–150). The sorting signals, however, differ between both proteins. The precursor of Mcp3 contains an N-terminal presequence, including a stop-transfer signal for lateral release into the inner membrane. Here, IMP removes the N-terminal signal, and the protein is transferred to the MIM complex (150). Om45 does not contain a cleavable targeting signal, and its N terminus is finally anchored in the outer membrane. The exact signal for the intramitochondrial sorting of Om45 has not been determined, yet the presequence translocase is required for translocation of the large intermembrane space domain of Om45 into mitochondria (**Figure 8**) (148, 149). (*c*) Finally, it is conceivable that the Tom40 channel may be opened laterally to release proteins into the outer membrane. So far, this transport route has been demonstrated with a fusion protein (151). It will be interesting to see if native outer-membrane proteins also use a Tom40-release route.

Mitochondrial contact site and cristae organizing system (MICOS): machinery for maintenance of inner-membrane crista junctions and formation of contact sites

Mic10: the smallest MICOS subunit; assembles into oligomers that are crucial for formation of crista junctions of the inner membrane

Mic60: the largest MICOS subunit; forms contact sites with the outer membrane and promotes import of MIA substrates and β-barrel precursors

MITOCHONDRIAL MEMBRANE ARCHITECTURE AND PROTEIN BIOGENESIS

The mitochondrial contact site and cristae organizing system (MICOS) is a large multisubunit protein complex enriched at crista junctions of the inner membrane (106, 152–154). Crista junctions are tubular openings that connect the invaginations of the inner membrane (cristae) with the inner-boundary membrane that is adjacent to the outer membrane. The MICOS subunits are termed Mic10 to Mic60 according to their approximate molecular mass in kilodaltons (**Figure 9**).

MICOS plays two major roles. First, it is required for the maintenance of crista junctions and the proper architecture of the inner membrane. Second, it forms contact sites with the outer membrane, which supports the import of precursor proteins. The TOM complex and the SAM complex are major interaction partners of MICOS. The largest MICOS subunit, Mic60 (mitofilin), is crucial for forming contact sites with the outer membrane (155–158). In addition, Mic60 transiently interacts with Mia40 and is thus in contact with three protein transport machineries (106).

Analysis of protein import in *micos* mutant mitochondria revealed a role of Mic60 in promoting the efficiency of two import pathways. (*a*) Mic60 stimulates the transfer of β-barrel precursors from TOM to SAM (156). The molecular mechanism has not been determined, yet Mic60 promotes the early stage of precursor translocation through TOM to the intermembrane space, indicating that the Mic60-TOM interaction is crucial. The functional role of the MICOS-SAM interaction, which is particularly stable in mammalian mitochondria (155), is unknown. (*b*) Mic60 stimulates the efficient import of precursors via the MIA pathway. The MIA substrates are often small proteins, and the close proximity of the receptor Mia40 to the exit site of the TOM channel promotes a rapid recognition and import of the precursors. Because Mic60 binds to Mia40 and the TOM complex, it recruits Mia40 to the TOM channel and thus enhances the efficiency of the MIA import pathway (106).

Mic19 is a close protein partner of Mic60 and plays a regulatory role in MICOS. The MIA pathway is required for efficient import of the precursor of Mic19 into the mitochondria (159), revealing an interesting cross connection. Mic60 stimulates the MIA pathway, and MIA promotes the import of a regulatory partner of Mic60.

We propose that TOM, SAM, MIA, and MICOS are crucial players of a large network that controls mitochondrial biogenesis and architecture. ER-mitochondria junctions, such as the ERMES

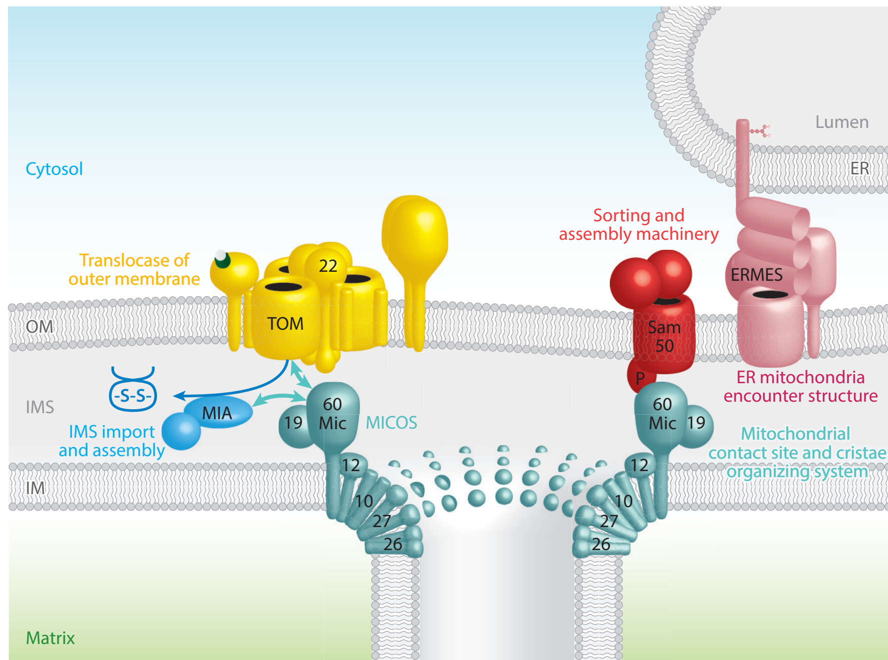

Figure 9

The mitochondrial contact site and cristae organizing system (MICOS) interacts with protein translocases. MICOS consists of two core subunits, Mic10 and Mic60. Mic10 forms large oligomers that are the basis of the inner membrane (IM) crista junctions and interacts with Mic12, Mic26, and Mic27 in a subcomplex. Mic60 forms contact sites with the outer membrane (OM) and interacts with the regulatory protein Mic19 in a subcomplex. Mic60 possesses a large intermembrane space (IMS) domain that interacts with the translocase of the outer membrane (TOM), the sorting and assembly machinery (SAM), and the mitochondrial IMS import and assembly (MIA) machinery. Mic60 thus promotes the import of MIA substrates into the IMS (as shown in the figure: Mic60 helps position Mia40 close to the TOM channel) and the import of β-barrel precursors. MICOS interacts with SAM via the polypeptide transport-associated (POTRA) domain of Sam50 (indicated by P in the figure) (156). The endoplasmic reticulum (ER)-mitochondria encounter structure (ERMES) is linked to SAM and MICOS via functional and genetic interactions. The machineries shown in this figure form a central part of an ER-mitochondria organizing network, which controls mitochondrial biogenesis and morphology.

complex, are also part of this network (**Figure 9**). ERMES is linked to SAM via the dual localization of Mdm10 (**Figure 7**) (129–131), and ERMES subunits show strong genetic interactions with MICOS subunits (152). We term this network the ER-mitochondria organizing network (160).

PERSPECTIVES

The field of mitochondrial protein biogenesis has experienced rapid development in recent years. Fifteen years ago, two protein import pathways were known, the presequence pathway and the carrier pathway, and it was assumed that the field had reached a level of saturation. However, subsequent years brought an unexpected boost with the discovery of a large number of import

MITOCHONDRIAL PROTEIN IMPORT MACHINERY AS REGULATORY HUB

The mitochondrial protein import machinery is intensively regulated and functions as sensor for the fitness of mitochondria (181). (*a*) TOM is regulated by cytosolic kinases (171, 174, 182). Casein kinase 2 promotes TOM biogenesis, cyclin-dependent kinase links TOM biogenesis to the cell cycle, and protein kinase A and casein kinase 1 regulate TOM in dependence on the metabolic state. (*b*) The activating transcription factor associated with stress-1 (ATFS-1) possesses targeting signals for the mitochondria and nucleus. In healthy mitochondria, ATFS-1 is translocated into the matrix and degraded. Upon disturbance of the mitochondrial protein import machinery (defective translocases, decreased membrane-potential), nonimported ATFS-1 molecules are transported into the nucleus and induce a mitochondrial stress response (172). (*c*) The PTEN-induced putative kinase 1 (PINK1), which is linked to Parkinson's disease, is partially translocated into mitochondria by the presequence pathway and cleaved in the inner membrane by the presenilin-associated rhomboid-like protease (PARL), followed by reverse translocation into the cytosol and degradation by the proteasome (173). When defects of mitochondria lead to a strong decrease of the membrane-potential, nonprocessed PINK1 accumulates at TOM and recruits the ubiquitin ligase Parkin to mitochondria. Upon phosphorylation of Parkin and ubiquitin, a multistep process leads to the removal of damaged mitochondria by autophagy (183).

components and three more major import pathways: the SAM, MIA, and MIM pathways. Importantly, there is intensive cross talk and exchange between the protein import pathways, leading to the formation of new import routes. The integration of the protein import machineries into regulatory networks that control mitochondrial energetics, architecture, and dynamics is covered in the sidebar titled Mitochondrial Protein Import Machinery as Regulatory Hub.

We expect that additional import pathways and mechanisms will be discovered. So far, most studies were performed with fungi and mammals, which belong to the same supergroup of eukaryotes (Opisthokonta). The analyses of mitochondrial proteins in other supergroups performed so far provided important novel findings (60, 61, 161, 162) and have great potential for understanding the central mechanisms and machineries of mitochondria and their development in evolution.

Important open questions concern the early steps of mRNA and precursor transfer to mitochondria. Evidence for both posttranslational and cotranslational translocation mechanisms has been presented; however, we only have a limited understanding of how precursor proteins are selected for either co- or posttranslational targeting and of whether and how a coupling between cytosolic ribosomes and the MIM is achieved (163–165). The actual mechanism of membrane translocation of precursor proteins is only understood in part. Site-specific mapping of precursors in transit and fluorescent mapping provided important information (22, 166, 167). However, the lack of high-resolution structures of the protein-conducting transmembrane channels is an important limitation. The roles of lipids in the function of protein translocases and protein membrane insertion are emerging fields (141, 144, 168–170). The regulation of protein import under different metabolic and stress conditions and its influence on cellular quality control and proteostatic responses (171–176) are exciting topics that provide important insights into the integration of mitochondrial biogenesis with cellular physiology and pathophysiology.

FUTURE ISSUES

1. Which cytosolic factors bind to mitochondrial precursor proteins in posttranslational import, and what are their functions in precursor targeting and chaperoning?

2. What are the mechanisms of cotranslational protein targeting, and which characteristics of precursor proteins and their mRNAs are responsible for separating co- and posttranslational targeting to mitochondria?
3. Determination of the structures of protein import channels without and with precursor proteins represents a major challenge of mitochondrial research.
4. What are the exact internal targeting and sorting signals in noncleavable membrane proteins, and how are they recognized by receptors and sorting components?
5. Do additional protein transport machineries exist in the mitochondrial membranes or aqueous compartments?
6. How are precursor proteins transferred from the inner-boundary membrane to the cristae membranes through crista junctions, and how are these transfers controlled by the metabolic state and architecture of mitochondria?
7. Which roles do lipids of the mitochondrial outer and inner membranes play in membrane translocations and insertions of precursor proteins?
8. The role of the mitochondrial protein import machinery as a regulatory hub will represent a central topic for understanding the integration of mitochondria in cellular homeostasis and their influence on cellular functions in health and disease.

DISCLOSURE STATEMENT

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