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Review

Multiple pathways in the integration of proteins into the mitochondrial outer membrane☆

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ABSTRACT

Proteins residing in the mitochondrial outer membrane facilitate various interactions between the organelle and the rest of the eukaryotic cell. All these proteins are encoded in the nucleus and synthesized on cytosolic ribosomes. Thus, they have to bear appropriate signals that ensure both their correct import into the organelle and their ability to acquire different topologies in the lipid bilayer. None of these proteins contain a canonical cleavable N-terminal presequence. Rather, the targeting and sorting signals can be found at their termini or in internal structural elements. In this review, we summarize the current knowledge regarding the diverse molecular mechanisms by which mitochondrial outer membrane proteins are specifically targeted to the organelle and inserted into the target membrane. Recognition events in the diverse pathways and the driving force for the various stages of this process will be discussed. This article is part of a Special Issue entitled Protein translocation across or insertion into membranes.

Contents

1. Introduction

Mitochondria are found in almost all eukaryotic cells and comprise one of the major intracellular compartments. Like nucleus and chloroplasts (in plants), they harbor two membrane systems: outer and inner mitochondrial membranes. The presence of the double-membrane envelope defines four structurally and functionally different mitochondrial sub-compartments: mitochondrial outer membrane (MOM), mitochondrial inner membrane (MIM), intermembrane space (IMS) and matrix. Each of these sub-compartments contains different subsets of proteins, reflecting their individual contribution to the complete array of mitochondrial functions.

The MIM forms numerous infoldings named christae, which penetrate deep into the matrix. This membrane is rather impermeable to most of the small ions, which is important for maintaining of the electrochemical gradient across the MIM. Unlike the MIM, MOM is rather smooth with smaller overall membrane surface. The MOM is rich in pore-forming proteins, which make it permeable to small molecules (with a mass of less than 5 kDa). In addition to differences in protein content, it has been found that these two membranes differ also in their phospholipid composition [1–3].

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Mitochondria harbor between 1000 (in the budding yeast) and 1500 (in mammals) different proteins. In the former, this number reflects about 17% of the total number of predicted proteins. According to different proteomic studies, less than 5% of the whole mitochondrial proteome (about 40 different proteins) reside in the MOM of *Saccharomyces cerevisiae* and *Neurospora crassa* [4,5]. However, this group of proteins performs various functions. Some serve as enzymes that are important for different biosynthetic pathways (for example, lipids, nicotinic acid, cysteine, erythroascorbic acid). Others are involved in regulation of morphology (fusion and fission) and inheritance of mitochondria. Members of protein-import and -insertion machineries form an additional group. Besides those, proteins of the Bcl-2 family with pro- and anti-apoptotic functions (like: Bcl-2, Bcl-xL, Bid, Bim) are found to associate with mammalian MOM [6]. These proteins are involved in remodeling of mitochondrial network and controlling release of cytochrome *c* into the cytosol. Some of the pore-forming proteins in the MOM (like VDAC) play a crucial role in the regulation of mitochondria-mediated apoptosis [7]. Recently, proteins involved in communication between mitochondria and ER attracted special attention as this ER-mitochondria linkage is important for Ca^{2+} homeostasis and phospholipids biogenesis [8]. Components like the Mdm34/Mdm10/Mdm12/Mmm1 complex in yeast and Mfn2 in mammals have been proposed to mediate this connection [9,10].

Like the vast majority of mitochondrial proteins, all MOM proteins are nuclear encoded and synthesized in the cytosol. In contrast to all matrix proteins and most of the MIM and IMS proteins, which contain N-terminal cleavable mitochondrial presequences, all MOM proteins carry internal non-cleavable targeting and sorting signals [11]. These signals assure targeting to the organelle, recognition by the import machinery and insertion into the lipid bilayer where they acquire their final conformation. Depending on structure and orientation, proteins of the outer mitochondrial membrane follow different import and insertion pathways.

In this review, we summarized our current knowledge concerning the import and insertion mechanisms of MOM proteins. Most of our knowledge about the principles of mitochondrial protein import and specifically the biogenesis of the mitochondrial outer membrane proteins is based on studies with the budding yeast, *S. cerevisiae*. However, in the last decade, cultured mammalian cells have become also popular as an experimental system to study mitochondrial biogenesis.

2. Topological families of mitochondrial outer membrane proteins

According to topology, outer mitochondrial membrane proteins can be divided into several groups (Table I). Most of these proteins span the lipid bilayer with one transmembrane domain (TMD). Depending on their orientation, these single-span proteins can be classified into two groups: signal- and tail-anchored proteins, which face the IMS with either the amino- or carboxyl-terminus, respectively. These proteins typically expose the bulk of the protein to the cytosol and only very short segment faces the IMS [12]. Exceptionally, a subclass of tail-anchored proteins comprised of Tom22 and Mim1 has both a relatively long IMS domain and a cytosolic domain. Some integral MOM proteins span the bilayer with two (like Fzo1 [13]) or more helical transmembrane domains (like Ugo1 [14]). A special group of membrane proteins, β-barrel proteins, span the membrane via amphipathic β-strands, organized in a cylindrically shaped structure.

Table I
Topology categories of MOM proteins.

	Topology class	Proteins
1.	Signal anchored	Tom20, Tom70, OM45, momMcr1
2.	Tail anchored	Tom5, Tom6,Tom7, Fis1, Gem1, Bcl-2 familiy (Bcl-2, Bak, Bcl-XL, Mcl1), OMP25, MAVS, MOM isoforms of cyt b5 and VAMP-1B, SLAMP1
3.	Tail anchored with an IMS domain	Tom22, Mim1
4.	Proteins with two or more helical transmembrane domains	Fzo1/Mfn1-2, Ugo1, PBR
5.	β-barrel proteins	Tom40, Tob55, Porin/VDAC, Mdm10

3. The translocase of the outer mitochondrial membrane (TOM complex)

The translocase of the outer mitochondrial membrane (TOM-complex) is embedded in the MOM and is crucial for the biogenesis of the organelle and thus for the viability of eukaryotic cells. Therefore in the following section, its components and functions are discussed in some detail. Almost all nuclear-encoded mitochondrial proteins require a functional TOM complex in order to be recognized by the organelle and to be transferred as unfolded polypeptides across the MOM. Some of the MOM proteins such as tail- and signal-anchored proteins and the peripheral protein Mas37 are the only known exceptions [15–18]. Studies with purified TOM complexes from *N. crassa* [19], *S. cerevisiae* [20] and *Arabidopsis thaliana* [21] as well as intensive studies with mammalian mitochondria [22] point to the universal basic architecture of the "mitochondrial portal". Recently, a homolog of the TOM complex was identified even in the outer mitosomal membrane of *Giardia intestinalis*, pointing to the evolutionary conservation of this important complex [23].

Fully assembled TOM translocase is a heteromolecular protein complex, named TOM-holo complex, with a molecular mass of 490–600 kDa [24,25]. The central component of the complex is Tom40, an essential β-barrel protein that forms the protein-conducting channel of the complex [26–28]. It was estimated that two or three Tom40 molecules are present per TOM complex [29]. Electron microscopy analyses and electrophysiological measurements suggested that the diameter of the import pore is about 20 Å, a space that is sufficient to accommodate two α-helices at the same time [24,27]. Many experiments support the idea that the Tom40 pore is not a passive channel, but rather acts in chaperone-like manner, by interacting with non-native proteins, preventing their aggregation and supporting partial unfolding of the substrate proteins during their translocation through the TOM [30]. Additionally, there is a large amount of electrophysiological data describing the Tom40-pore as a dynamic ion-conducting channel [27,31].

There are three components within the TOM complex which function as preprotein receptors: Tom20, Tom70 and Tom22. Each one of these proteins contains a segment exposed to the cytosol, which is responsible for the recognition of mitochondria-specific signals. In wild-type mitochondria, these proteins exhibit substrate specificity. However, if one of them is deleted the remaining receptors can complement its function. Tom20 and Tom22 possess similar substrate specificity and preferentially recognize presequence-containing proteins and different preproteins with internal targeting signals, like β-barrel proteins [32–35]. Tom70 shows specificity for the polytopic inner membrane proteins, such as metabolite carrier proteins [36,37]. In contrast to Tom20 and Tom70, which serve solely as receptors, Tom22 acts as a multifunctional protein. Unlike Tom20 and Tom70, Tom22 additionally contains relatively long segment exposed to the IMS. This segment also acts as a receptor domain, which interacts with substrate in transit through the TOM and induces its release from the pore into IMS. In addition to these functions, the transmembrane segment of Tom22 plays a crucial role in the overall stabilization of the TOM complex [38].

Three additional small proteins are part of the TOM translocase: Tom5, Tom6 and Tom7. Together with Tom40 and Tom22, they form

the so-called TOM core complex which has molecular mass of 450–500 kDa [19]. Tom5 is important for assembly of the TOM complex in the early stages of Tom40 insertion [39] and it also contributes to the maintaining of the structural stability of the complex [40]. This protein was also suggested to serve as a functional link between the import receptors and the translocation pore [41]. Tom6 and Tom7 act as antagonists in the regulation of assembly and dissociation of the TOM complex; Tom6 stabilizes the complex, probably by forming a link between Tom40 and Tom22, while Tom7 has an opposite, destabilizing role [42–45].

4. The assembly pathway of mitochondrial β-barrel proteins

β-Barrel proteins are a special topological class of MOM proteins. Unlike most of the transmembrane proteins that are anchored in the lipid bilayer via one or more membrane spanning helices, they transverse the membrane in the form of cylindrically shaped structure, built by interconnected β-strands [46]. These proteins are found in both prokaryotes and eukaryotes. In prokaryotes, β-barrel proteins are found in the outer membrane of Gram-negative bacteria. In eukaryotes, these proteins reside exclusively in the outer membrane of mitochondria and chloroplasts. Their presence in these organelles supports the endosymbiotic theory, according to which mitochondria and chloroplasts are derived from prokaryotic ancestors. Indeed, the biogenesis of these proteins in the various systems bears significant similarities [47]. The biogenesis pathway of β-barrel proteins in bacteria was recently summarized in another review [47].

In contrast to the outer membrane of Gram-negative bacteria, which predominantly contains β-barrel proteins [48], only five proteins with predicted β-barrel structure have so far been identified in the MOM of yeast (Table I)[49]. These proteins perform a variety of functions. Two of them – Tom40 and Tob55/Sam50/Omp85 – are essential for yeast viability and function as the central components of TOM and TOB complexes, respectively. Porin, which is also named voltage-dependent anion-selective channel (VDAC) in higher eukaryotes, is the most abundant MOM protein. Porin forms a diffusion pore for small molecules and has a key role in the communication between mitochondria and cytosol. An additional β-barrel protein is Mdm10, a protein with multiple functions in many cellular processes such as regulation of mitochondrial shape and segregation, assembly of β-barrel proteins, lipid biosynthesis and communication between the ER and mitochondria [10,50,51].

Most of our knowledge regarding the assembly pathway of β-barrel proteins comes from in vitro experiments utilizing radiolabeled Tom40 precursors and isolated mitochondria. In the insertion and assembly process, Tom40 forms different assembly intermediates, which can be analyzed by BN-PAGE and easily detected by autoradiography. Although not all import intermediates were formally demonstrated with all known β-barrel precursors, it is assumed that they all follow a common pathway (Fig. 1). After synthesis in the cytosol, β-barrel precursors are delivered to the mitochondrial surface in a process that is largely uncharacterized. Although it is assumed that cytosolic chaperones are involved in this delivery, their identity and precise role are unknown. Irrespective of the cytosolic pathway, β-barrel precursors are initially recognized on the surface of the organelle mainly by the import receptor Tom20 [15,32,52,53].

The identity of the signal within β-barrel precursors that allows the TOM complex to recognize them is currently ill defined. Efforts to identify linear sequences that can fulfill this task have failed so far [54,55]. Thus, it was proposed that the signals are contained in β-barrel-specific structural elements rather than in a conserved linear sequence [47]. Recent studies demonstrated that bacterial β-barrel proteins can be assembled as native-like oligomers into the MOM and that this insertion follows a pathway similar to that of mitochondrial β-barrel proteins [56]. Hence, one can speculate that structural elements with high β-sheet content might serve as a crucial signal for specific targeting of β-barrel proteins. Recently, a conserved sequence

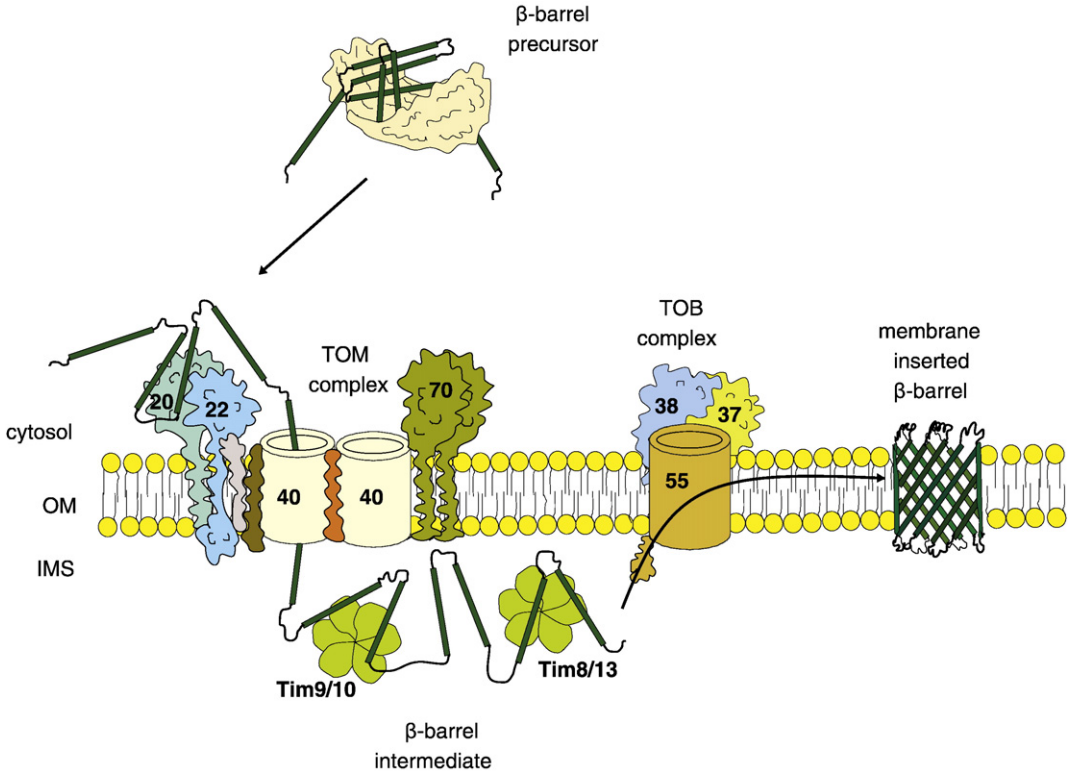

Fig. 1. Working model for the insertion of mitochondrial β-barrel proteins. Cytosolic factors deliver β-barrel precursors to the Tom receptors. Precursor proteins are then translocated through the import pore of the TOM complex and become exposed to the IMS. At this stage the β-barrel precursors bind to the small Tim chaperones. Finally, with help of the TOB complex β-barrel precursors are inserted and assembled into the MOM.

termed the β-signal that is essential for the interaction of β-barrel proteins with the TOB complex and is universal in the eukaryotic kingdom was identified. This signal is located in the last transmembrane β-strand and contains four conserved residues: a large polar residue (mostly lysine or glutamine), an invariant glycine and two large hydrophobic residues [57]. However, this signal does not seem to be required for the initial recognition by the TOM complex. Precursors with mutations in the β signal were still imported into the organelle, but their interactions with the TOB complex were altered [57].

Following the initial interaction with the TOM complex β-barrel precursors are translocated across the MOM via the import pore of the TOM complex (Fig. 1) [39,52,53,58]. Once the precursors reach the IMS they interact with the small Tim chaperones, namely with Tim9-Tim10 and Tim8-Tim13 complexes [15,59,60]. These interactions explain why rupturing the MOM (which causes the loss of small Tim chaperones) results in reduced assembly of β-barrel precursors [61]. The next step in the biogenesis is the association with the TOB complex that mediates insertion of the precursors into the outer membrane where they can achieve their final conformation. However, it should be noted that the individual steps in the import of β-barrel proteins are probably coupled and highly synchronized [15]. Supporting this assumption is the observation that a soluble intermediate of β-barrel precursor associated with the small Tims could not be identified.

Why do mitochondrial β-barrel proteins follow such a complicated pathway of insertion into the MOM? One could imagine a much more simplified process of insertion where the precursors are directly inserted into the MOM from the cytosolic side, avoiding translocation through the TOM and requirement for the IMS-factors. The answer to this question lies in the evolutionary origin of mitochondria. Mitochondria evolved from an independent prokaryotic organism into an endosymbiont organelle. This process involved a gradual transfer of the endosymbiont's genes to the host's nucleus. Therefore, β-barrel precursors are synthesized in the eukaryotic cytosol. As will be discussed later, the central component (BamA) of the bacterial machinery that inserts bacterial β-barrel proteins is conserved also in mitochondria (Tob55), suggesting that the organelle kept the basic mechanism of membrane integration of β-barrel proteins. However, β-barrel precursors in bacteria approach the OM from the periplasm, i.e. from within the cell. Therefore, usage of this mechanism in eukaryotes requires that such precursors first cross the MOM in order to approach the TOB complex from the IMS side of the membrane. Since the TOM-complex evolved as a general mitochondrial portal for almost all mitochondrial proteins synthesized in the cytosol, it was easier in evolutionary terms to use the same translocase also as a portal for β-barrel proteins than to invent new import machinery.

4.1. Structure–function relationship of the TOB complex

Unlike the TOM complex, which serves as a "general entry gate" for the vast majority of nuclear encoded mitochondrial proteins, the TOB (topogenesis of outer membrane β-barrel proteins) known also as the SAM (sorting and assembly machinery) complex in the MOM is mainly dedicated to the insertion of β-barrel precursors [58,62,63]. In fungal mitochondria, this complex is composed of three proteins: Tob55/Sam50/Omp85, Tob38/Sam35/Tom38 and Mas37/Tom37/Sam37 [64–66]. Currently, the stoichiometry of these components is still unknown. In yeast cells, both Tob55 and Tob38 are essential, while Mas37 is required for growth only at higher temperatures [64,65,67,68].

The central component of the TOB complex is Tob55 [58,62,63], a protein with orthologues in all eukaryotes and with sequence homology with Toc75 of chloroplasts and bacterial BamA (YaeT/Omp85) [69,70]. The C-terminal portion of Tob55 is predicted to form a membrane-embedded β-barrel structure while its N-terminal segment, protruding into the IMS, contains one predicted polypeptide transport associated (POTRA) motif [71]. Similar to its role in bacteria [72,73], a receptor-like function of the Tob55 POTRA domain, was suggested [74]. However, a later study challenged this view and currently the function of the POTRA domain of this protein is controversial [57].

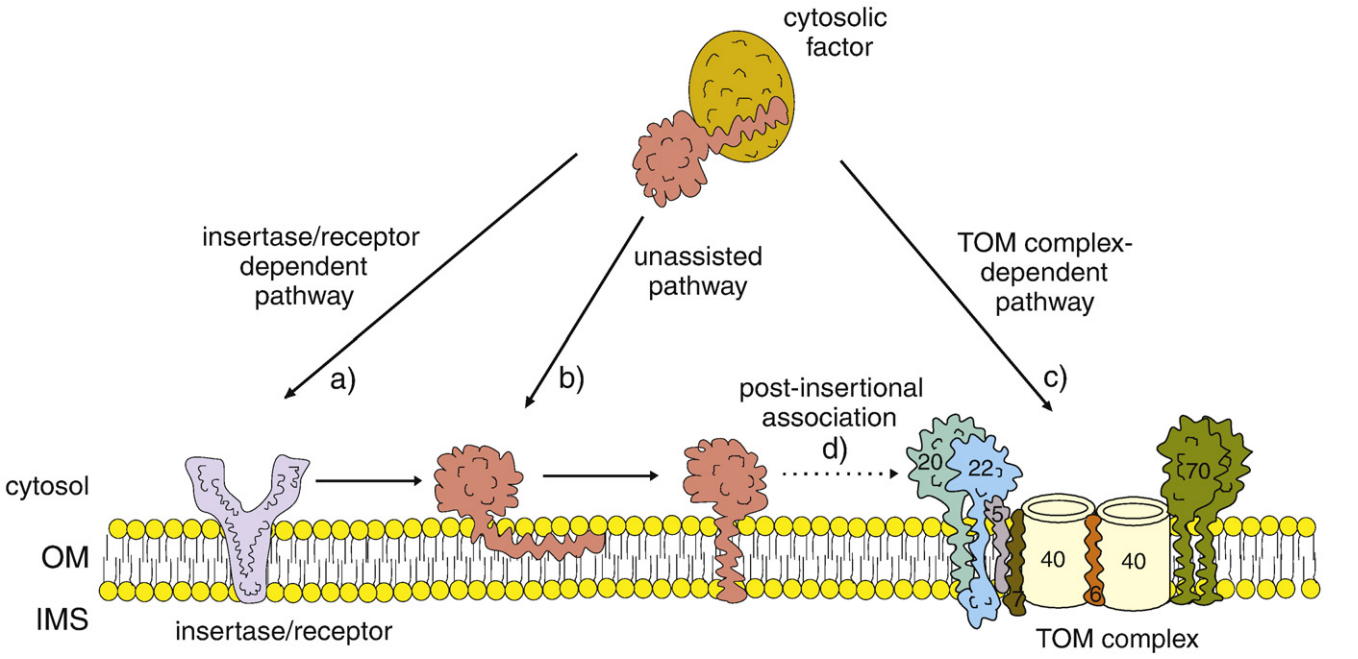

Fig. 2. Possible import pathways for signal- and tail-anchored MOM proteins. The TMD of both types of proteins is probably engaged by cytosolic factors. Further insertion can be mediated either by an insertase (a) or can happen without assistance of other proteins (b). Signal-anchored Tom components require the presence of pre-existing TOM complexes for their assembly. The TOM complex can be involved in the initial steps of the membrane integration (c) or alternatively its participation can be a post-insertional event, occurring after the initial insertion into lipid bilayer (d).

The mechanism by which Tob55 mediates insertion and/or subsequent folding of precursor proteins into the lipid bilayer is still not clear. One model is based on the fact that Tob55 can form pores in the lipid bilayer, which could serve as an Anfinsen-like cage, ensuring a protected environment for the folding of β-barrel precursors. This role of Tob55 would be similar to those described for chaperonins like GroEL. After this stage, folded proteins could be released from the pore into the lipid bilayer, either by lateral opening the pore or by first getting out of the pore and then re-inserting into the membrane. However, in the lateral opening scenario the walls of the pore should be formed by several Tob55 molecules, because lateral opening of the β-barrel structure would be thermodynamically unfavorable.

The second essential component of the TOB complex is Tob38 that together with Tob55 forms the so-called TOB-core complex [65]. Both termini of Tob38 are exposed to the cytosol, while most of the protein was proposed to be embedded in a proteinaceous environment of the TOB complex [57]. Depletion or mutations in Tob38 result in reduced steady-state levels of β-barrel proteins and strong decline in the detected amounts of Tob55 and Mas37 [64,65,68]. The precise function of this protein in β-barrel biogenesis is currently unresolved. A partial insight comes from studies suggesting that Tob38 in cooperation with Tob55 plays a role in the precursor binding to the TOB complex [15,75]. A later study reported that actually Tob38 and not Tob55 recognizes a β-signal peptide at the C terminus of β-barrel precursors [57]. Although the homology between yeast Tob38 and mammalian Metaxin-2 is rather limited it was proposed that Metaxin-2 serves as functional parallel of Tob38 in mammalian cells [76].

Mas37, the first member of TOB complex to be discovered, is not embedded into the MOM but is rather associated to it at the cytosolic surface [39,67]. Deletion of *MAS37* strongly blocks insertion and assembly of Tom40 and results in reduced levels of assembled TOM complex [39]. Hence, deletion of *MAS37* causes many pleiotropic effects such as reduction in import of non-β-barrel proteins [67] and altered mitochondrial morphology [50]. Indeed, stabilization of the TOM complex in *mas37Δ* strain by overexpressing *TOM6* can partially suppress these phenotypes [77]. Based on several lines of evidence, it was suggested that Mas37 acts downstream of Tob38 and helps releasing substrate proteins from the TOB complex into the lipid bilayer [75,77]. The mammalian homolog of Mas37, Metaxin-1 forms a complex with Metaxin-2 and plays an important role in β-barrel protein biogenesis [76].

The TOB complex containing Tob55, Tob38 and Mas37 associates in a dynamic manner with a fourth protein, Mdm10. This latter protein was reported to form a complex together with Mdm12 and Mmm1 which are involved in regulation of mitochondrial distribution and morphology [78]. Recently, it was also reported that Mdm10, together with Mmm1, Mdm12 and Mdm34, builds a molecular tether between ER and mitochondria [10]. Thus, it appears that Mdm10 and its interacting partners may have more than one function. The picture seems to be even more complicated since Mdm10, Mmm1 and Mdm12 were also suggested to be involved in the assembly process of β-barrel precursor proteins. Mdm10 was initially reported to mediate the assembly of the precursor of Tom40 but not of other β-barrel precursors and to be part of an enlarged form of TOB complex, named TOB-holo complex [50]. A recent study suggests that rather than being involved in a late step in a Tom40-specific route, Mdm10 regulates the timing of release of unassembled Tom40 from the TOB complex. This function of Mdm10 is performed in cooperation with Tom7 [79]. In *N. crassa* it seems that Mdm10 is important also for the biogenesis of other β-barrel proteins like porin [66]. Interestingly, the absence of Mdm12 and Mmm1 in both yeast and *N. crassa* resulted in reduced biogenesis of β-barrel proteins [66,80]. The mechanism behind this observation is not clear as interactions of these proteins with β-barrel precursors or with the TOB complex have not been reported.

It was recently reported that Mmm1 is actually anchored to the ER membrane and together with Mdm10, Mdm12 and Mdm34 it form a mitochondria-ER tethering complex Mutants in this complex caused altered lipid compositions in mitochondrial membranes. [10]. Thus, we speculate that the major function of Mmm1 and Mdm12 is the tethering function and the import and morphology defects are secondary to the primary changes in the lipid distribution. Recently, alterations in mitochondrial membrane protein function as the result of changes in lipid composition were reported. For example, changes in cardiolipin levels have been shown to affect the assembly and activity of the TOM and TOB complexes [81]. Altered lipid composition can also cause changes in mitochondrial morphology. Collectively, additional work is required to define the precise function and mechanisms of action of Mdm10, Mdm12 and Mmm1 and to distinguish between primary and secondary phenotypic effects observed in these mutants.

4.2. Driving force for the transport of β-barrel precursors through the TOM pore

An interesting and unresolved question is: what is the driving force that pulls β-barrel precursors through the TOM pore in the direction of the IMS? ATP-hydrolysis is not required and it appears that the membrane potential across the inner membrane, which could provide an electrophoretic force, is not involved [53]. Thus, it appears that the driving force arises from an array of binding sites with increasing affinities. Precursors of β-barrel proteins are recognized at the surface of mitochondria by TOM receptors, specifically by the cytosolic domain of Tom20. This could be the first binding site, responsible for selection of the proteins from the cytosol. At this stage, one part of the precursor protein is bound to the receptor, while the rest of the protein can freely move. Since this movement is random, the rest of the protein is shifted in and out of the import pore till at a certain moment the protein makes contact with another binding site, located deeper in the pore, which has higher affinity for the precursors. This interaction could result in the further moving the protein, deeper into the translocation channel. The inner walls of Tom40 pore, as well as part of the protein which faces the IMS, posses series of hydrophobic binding sites with high affinity for non-native proteins. It was demonstrated that Tom40, as some chaperones, has the potential to strongly suppress aggregation of denatured proteins [30]. Thereby, it was proposed that favorable binding of protein in transit to the walls of the TOM pore contributes not only to pulling the proteins through the channel, but also to their unfolding during translocation.

Finally, the precursor protein transiting through the TOM pore faces the IMS where the small Tim chaperones bind it. Interaction with these chaperones would be strong enough to pull the precursors further through the TOM pore and to prevent back sliding of the β-barrel precursor, thus assuring a net vectorial movement. The small Tim proteins could play an additional role in guiding the precursors from the TOM to the TOB complex. A transient ternary complex of the small Tims with substrate and TOB elements can be assumed but has not been observed so far. Binding the proteins to the TOB complex with very high affinity completes the pulling process. The TOB complex functions probably as a scaffold for the final irreversible insertion into the lipid bilayer. It can be assumed that formation of the β-barrel structure within the membrane provides a thermodynamic gain due to the favorable interactions of the β-strands among themselves and with the lipids. This final gain assures the unidirectionality of the whole pathway.

5. Signal-anchored proteins

The mitochondrial outer membrane contains three types of single-span proteins: signal-anchored, tail-anchored and those with a central single TMD. Signal-anchored proteins owe their name to a short portion of their N-terminus that serves as both a mitochondrial

targeting signal and an anchor to the mitochondrial outer membrane [82]. The large remaining part of the protein is exposed to the cytosol. According to current knowledge, this group comprises the TOM receptors Tom20 and Tom70 and two additional proteins: OM45 and the outer membrane isoform of Mcr1 (Table I). OM45 was found only in yeast and its function stays unresolved to date [83]. Mcr1 functions as mitochondrial NADH-cytochrome *b5* reductase, which exists in yeast in two isoforms, one located in MOM (momMcr1) and the other, shorter one, in the IMS [84].

The targeting signal of these proteins consists of the TMD and positively charged flanking regions [82]. In vivo functional complementation assays in yeast cells have shown that positively charged residues in both flanking regions are not essential for mitochondrial targeting and membrane insertion. However, if these residues were mutated to negatively charged residues, targeting and insertion of signal-anchored proteins was reduced [85]. Unlike yeast, in mammalian cells these specific flanking regions are necessary for mitochondrial targeting [86,87]. The key structural feature of the signal-sequence is modest average hydrophobicity of the TMD. Introducing a more hydrophobic TMD within the sequence of Tom20 without any change in the flanking region resulted in incorrect targeting and in growth retardation [85].

Import of signal-anchored proteins does not require any of the known import components. First, it was observed that mitochondrial targeting of Tom20 and Tom70 is not affected by addition of antibodies against Tom20 or Tom70 [88,89]. Moreover, it was also shown that removing protease-accessible import receptors does not affect in vitro import of radiolabeled Tom20, Tom70 or momMcr1 protein. Similarly, membrane insertion of these proteins appears to be independent of the import pore formed by the TOM core complex as blocking it does not affect the in vitro insertion of Tom20, OM45 or momMcr1 [17,88,90].

The membrane insertion of Tom20, a subunit of the TOM complex, seems to follow a unique pathway. Although its initial membrane insertion is independent of the import pore, it was suggested that Tom40 is required for the correct topology and assembly of newly synthesized Tom20 molecules [90]. Another unique feature of Tom20 biogenesis is its dependence on the outer membrane protein mitochondrial import 1 (Mim1). Mim1 was first identified as a protein required for mitochondrial import [91]. Although the precise molecular mechanism of its action is poorly understood, later studies in yeast cells have clearly shown that the protein is involved in the biogenesis of the TOM complex [68,92]. Deletion of *MIM1* leads to severe reduction in steady-state levels of Tom20 and to moderate reduction in the levels of other Tom components. It was observed that mitochondria lacking Mim1 are impaired in import and assembly of Tom20 [93–95] and Tom70 [93], while import of OM45 [93]and momMcr1 [17] is not affected.

It looks as if signal-anchored proteins do not share one common mechanism of targeting and insertion (Fig. 2). One can speculate that OM45 and momMcr1 follow a still uncharacterized import pathway, while Tom receptors follow another one. It was proposed that Mim1 promotes insertion of Tom20 and Tom70 into the MOM and their final integration into the TOM core complex. Since Tom receptors are a part of the TOM complex, it is reasonable to assume that they, unlike OM45 and momMcr1, require functional TOM core complex for their assembly. On the other hand, the request for Mim1 and Tom40 in the biogenesis route of Tom receptors does not have to exclude a universal mechanism of insertion of signal-anchored proteins into the MOM. A more likely scenario would be that all signal-anchored proteins follow initially a similar pathway of insertion. Currently, one cannot exclude that in vivo such a pathway involves an insertion of the N-terminal segment while the rest of the protein is still synthesized by mitochondrial-associated ribosome. The capacity of mitochondria to insert these precursors in vitro in a post-translational manner suggests that a co-translational mode of membrane integration is possible but not compulsory. In a post-translational scenario the hydrophobic N-terminal segment is probably prevented from undesired hydrophobic interaction by its interaction with a cytosolic chaperone. A dedicated chaperone with a binding specificity for this kind of segments was not identified yet. Apparently the initial insertion of the hydrophobic segment into the membrane does not require the TOM complex or any other insertase. The driving force for this step is probably the thermodynamic gain upon the formation of favored hydrophobic interactions between the TMD and phospholipids molecules surrounding it. Upon their insertion into the lipid bilayer, OM45 and momMcr1 are already in their final topological state, while Tom20 and Tom70 have to follow additional steps of assembly into the TOM complex (Fig. 2). The observation that signal-anchor domains from different proteins are functionally interchangeable supports this proposal of a general common initial insertion step [90].

6. Tail-anchored proteins

Tail-anchored (TA) proteins have a similar topology to signal-anchored proteins. The difference lies in their opposite orientation in the membrane. They are composed of three domains: a helical transmembrane domain, very short C-terminal segment protruding into the IMS and the largest, N-terminally positioned domain exposed to the cytosol [96]. Because of their topology, these proteins have to be inserted into the lipid bilayer by post-translational mechanism. In addition to mitochondria, direct insertion of newly synthesized tail-anchored proteins occurs also into the membranes of endoplasmatic reticulum (ER), peroxisomes and into the outer membrane of chloroplast [96]. The insertion of these proteins into the ER membrane is dealt with in another article in this issue (Borgese and Fasana). TA proteins of the MOM exhibit wide functional diversity. Small proteins of the TOM translocase (Tom5, Tom6 and Tom7) are members of this class of proteins as well as some of the regulators of mitochondrial morphology, such as Fis1 and Gem1. Among the many mammalian proteins that belong to this class are apoptotic regulators like the proapoptotic proteins Bak and Bax and the anti-apoptotic modulators Bcl-XL and Mcl-1 [96].

Tail-anchor segments of various mitochondrial TA proteins do not contain consensus amino-acid sequence. Rather, tail-anchored proteins possess a mitochondrial targeting and sorting signal very similar to the one of signal-anchored proteins. It is composed of the transmembrane segment, with moderate hydrophobicity and length, flanked by positively charged residues [97]. Several studies demonstrated that these positively charged stretches adjacent to the TMD play an important role in the mitochondrial targeting of these proteins. It has been shown that these basic residues downstream to the TMD of the mitochondrial isoform of cytochrome b5 or VAMP-1B are crucial for the mitochondrial localization [98,99]. It is currently not clear whether these positively charged residues are required for interaction with proteinaceous element or with the negatively charged head groups of the phospholipid molecules. The short length of the TMD is also one of the structural features of tail-anchor signals relevant for their mitochondrial targeting. For example, the length of the TMD of mitochondria-targeted isoform VAMP-1B is shorter by four amino-acids in comparison to ER-targeted isoform, VAMP-1A [98]. Similarly, elongation of TMD of Tom5 by three or more valine residues leads to mistargeting of Tom5 mainly to the ER [100]. Considering the report that the MOM is thinner than the ER membrane [2], one can speculate that a shorter hydrophobic stretch of mitochondrial TA proteins provides a better hydrophobic match with the mitochondrial membrane.

Many reports suggest that moderate hydrophobicity of a tail-anchor sequence is one of the crucial factors determining mitochondrial targeting of these proteins [96]. Recently, targeting of SLAMP1 (sarcolema membrane associated protein) that exists in two isoforms with two different tail-anchoring signals (TA1 and TA2) was

investigated. The protein with TA1 is localized in the ER whereas the isoform containing TA2 is distributed in both the ER and the MOM [101]. It was proposed that overall hydrophobic profile of TA domain dictates the targeting of SLAMP1 to the corresponding membrane. It remains to be elucidated why an increased hydrophobicity results in mis-localization of TA proteins to the ER.

The targeting specificity to mitochondria is most likely generated (at least for some proteins) by some still unknown cytosolic factors. These factors should be able to specifically bind newly synthesized mitochondria-destined TA proteins, determining in that way the pool of TA proteins, which will be imported into MOM. Such a mechanism was demonstrated for peroxisomal TA proteins where the cytosolic chaperone Pex19 fulfills such a role by binding TA proteins and delivering them to the peroxisomes and thus preventing mistargeting to the mitochondria [102]. In the case of ER-destined TA proteins, the cytosolic proteins Hsp40 and Hsc70 [23,103] and multicomponent TRC complex (termed GET in yeast) [104,105] were identified as factors responsible for delivering TA proteins from the cytosol to the ER-membrane. It should be stressed that such a cytosolic factor specific for mitochondrial TA proteins has not been identified yet.

Despite the considerable knowledge regarding the structural features of the mitochondrial-targeting signal of TA proteins, little is known about the molecular mechanism by which these proteins are recognized on the mitochondrial surface and inserted into the MOM. Research so far indicates that different mitochondrial TA proteins follow different biogenesis pathway. Small Tom proteins follow an import pathway involving the TOM complex. Unlike Tom5 which requires Tom40 but not Tom receptors for the insertion into MOM [100], Tom6 and Tom7 of *N. crassa* need both protease-protected mitochondrial receptors and the TOM core complex [43]. Since these proteins are a part of the TOM complex, they could follow a special pathway of insertion and assembly which could require pre-existing Tom elements for the initial membrane-insertion step or only for the subsequent assembly into the TOM complex. An interesting twist to the discussion provides a recent study reporting that assembly of Tom5, Tom6 and Tom7 is dependent on Mas37 but not on Tob55 and Tob38. The authors indicated that Mas37 was required at a stage downstream of the membrane integration [106]. Of note, abolishing the function of the TOB complex results in hampered biogenesis of the TOM complex. Thus, it is not clear whether Mas37 directly interacts with the small Toms or whether it influences their biogenesis in an indirect manner.

The involvement of the TOM complex in the membrane insertion of single-span proteins which are not subunits of the TOM complex is under debate. An example is provided by the confusing reports concerning Bax integration into the MOM. Whereas some reports suggest the involvement of components of the TOM complex in this process [107,108], other studies argue against such involvement [109,110]. Similarly, the targeting of Bcl-2 into yeast mitochondria was reported to require Tom20 [111] while a previous report proposed the process to be independent of exposed receptors [112]. Two recent studies conducted in mammalian and yeast cells failed to identify any of the known import components at the MOM as crucial for the membrane integration of TA proteins [16,18]. Although the existence of such membrane-embedded protein(s) cannot be excluded, it was suggested that rather than a membrane-embedded protein the specific lipid composition of the MOM, such as low ergosterol content, could ensure specific targeting and kinetic advantage for the import of mitochondrial TA proteins into the MOM [16]. One of the reasonable scenarios is that the targeting and insertion of mitochondrial TA proteins could differ from one group of proteins to another and that it occurs via diverse pathways (Fig. 2).

In all cases, the energetic aspects of the process are not fully understood. As with the other MOM proteins, an ATP-driven step and a requirement for membrane potential across the inner membrane were not suggested. Although TA proteins usually contain a relatively long hydrophilic segment, the presence of a hydrophobic α-helical segment makes them thermodynamically unstable in the aqueous phase and prone for aggregation. Hence, the cell has to prevent this aggregation and keep them in an import-competent conformation until the TA precursor encounters the appropriate membrane. Since the targeting signal in the case of TA proteins is synthesized as the last element of the protein, co-translational membrane integration is very unlikely. Thus, one can suppose that a cytosolic factor, either a dedicated protein or a general chaperone, would protect the hydrophobic segment from facing water and from premature aggregation. We propose that the function of this putative cytosolic protein is to help in the specific targeting and to enhance the efficiency of the membrane insertion process. Once the precursor of the TA protein encounters the MOM it has to overcome the thermodynamic barrier to insertion of the hydrophobic segment due to the polar head groups of the lipids [113]. Hence, membrane structures with high curvature and/or increased unordered organization can provide a favored site for the spontaneous partitioning of the TA segment into the lipid bilayer. Favorable interactions among the hydrophobic lipid acyl chains and the non-polar amino acids can be formed upon insertion of the anchor peptide and thus are able to prevent retrograde movement out of the membrane.

7. Multi-span proteins

Multi-span proteins comprise a relatively small class of MOM proteins. They are inserted into the lipid bilayer via multiple transmembrane segments that are interconnected by small loops. Some of them, like Fzo1 in yeast (Mfn1/2 in mammalians), can cross the membrane twice, exposing N- and C-terminal domains towards the cytosol [13,114]. Ugo1 in yeast and human peripheral benzodiazepine receptor (PBR) are additional multi-span MOM proteins [14,115]. Using different truncation mutants of Ugo1 and Mfn2, the domain that includes the predicted TMDs was shown to harbor the information necessary for mitochondrial targeting, although additional targeting signals in other regions of the protein are not excluded [14,114]. Experiments with Mfn2 indicate similarities between polytopic and tail-anchored proteins, in terms of motifs and mechanism, responsible for their insertion into MOM. Sequence analyses revealed that Mfn2 contains numerous basic residues along its C-terminal domain. Replacement of this segment by a stretch of hydrophobic amino-acids lead to mistargeting of Mfn2 to the ER [114].

The idea that import pathways of TA and multi-span proteins overlap (at least partially) is supported by import competition assays: import of PBR was strongly inhibited by an excess amount of the TA protein, Bak [115]. However, in contrast to TA proteins biogenesis where import receptors are probably not essential for the process, it appears that these receptors do play a role in the membrane integration of multi-span proteins. A study with Fzo1 showed that this protein requires protease-sensitive import receptor(s) for its insertion into the MOM [116]. Later investigations revealed that import of PBR and Mfn2 requires interaction with Tom70 but is not dependent on other TOM components. In addition to Tom70 both proteins appear to require an unknown IMS component for efficient integration [115]. The receptor dependency of the multi-span proteins suggests that the overlap with the TA pathway occurs probably after initial docking to Tom70. A possible scenario would include some yet unidentified cytosolic factor(s), which would help in targeting the multi-span precursor proteins in an import-competent form to Tom70. Supporting this notion is the function of Tom70 as a docking element for chaperone-associated precursor proteins of the IM carrier proteins [117]. After recognition by Tom70 on the mitochondrial surface, the proteins would be delivered, in ATP-dependent manner, to the following step, which would probably involve still unknown membrane insertion machinery. The requirement of such machinery

is postulated since it is hard to envisage how proteins with several TMDs can be inserted spontaneously. One can speculate that the IMS component(s) that is (are) required for optimal insertion of multi-span proteins is the small Tim chaperones as these chaperones are known to bind hydrophobic multi-span inner membrane proteins [118]. Peptide scan analysis revealed that *N. crassa* Tim9/10 complex binds preferentially peptides that span both the TMD and their linking loops [119]. Hence, IMS chaperones might help in the integration of MOM multi-span proteins by binding to and stabilizing IMS-exposed segments.

8. Concluding remarks and future challenges

Although considerable progress has been made in revealing the mechanisms responsible for targeting and insertion of mitochondrial outer membrane proteins, future studies are necessary to help us address the many open questions. In particular, little is known regarding the molecular mechanism of insertion of multi-span proteins. Efforts are also required to identify possible cytosolic factors that recognize signal- and tail-anchored proteins and target them to the MOM. Although these two classes of proteins harbor similar mitochondrial targeting signals, it is still unclear whether they also share the same import pathway. Furthermore, it is still unclear if a dedicated insertase is required for their partitioning into the membrane. Although the components involved in the biogenesis of β-barrel proteins have been identified, details of insertion mechanism and correct folding of precursors have not been deciphered yet. Only recently we have gained some insight into the importance of lipids in membrane integration of proteins. This topic and the effect that post-translational modifications might have on biogenesis of MOM proteins are promising directions for future research in this field.

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