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Review

Mitochondrial protein import machineries and lipids: A functional connection ☆

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ABSTRACT

Protein trafficking and translocation are essential processes in even the simplest living cells. The compartmentalisation within eukaryotic cells places a very high demand on the fidelity of protein trafficking and translocation, since a large percentage of the cell's protein complement is inserted into, or translocated across membranes. Indeed, most mitochondrial proteins are imported from the cytosol into the organelle and reach their final destination with the assistance of versatile translocation machineries. The first components involved in mitochondrial protein import were identified about 20 years ago and over the last two decades many new factors and machineries have been brought to light. However, in spite of these discoveries we still have much to explore regarding the molecular mechanisms that distinguish the different mitochondrial import pathways. In particular, an open question that requires deeper exploration is the role of lipids and lipid modifying enzymes in this process. Mitochondrial biogenesis requires the coordinated synthesis and import of both proteins and phospholipids, however, these have typically been considered as distinct research fields. Recent findings have placed phospholipids at the forefront of research dealing with mitochondrial biogenesis, in particular their role in the regulation of mitochondrial transport machineries. This article is part of a Special Issue entitled Protein translocation across or insertion into membranes.

Contents

Abbreviations: CDP-DAG, CDP-diacylglycerol; CL, cardiolipin; ER, endoplasmic reticulum; MDM, mitochondrial distribution and morphology; MIA, mitochondrial intermembrane space assembly; MLCL, monolysocardiolipin; Mmm1, maintenance of mitochondrial morphology; mtHsp70, mitochondrial heat shock protein 70; PA, phosphatidic acid; PAM, presequence translocase-associated motor; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PGP, phosphatidylglycerol phosphate; PI, phosphatidylinositol; PS, phosphatidylserine; SAM, sorting and assembly machinery; Tam41, translocator assembly and maintenance protein 41; TIM22, carrier translocase of inner membrane; TIM23, presequence translocase of inner membrane; TOM, translocase of outer membrane

☆ This article is part of a Special Issue entitled Protein translocation across or insertion into membranes.

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1. Introduction

Compartmentalisation of proteins within organelles of a eukaryotic cell serves to coordinate and separate metabolic processes. However, this poses a challenge for the cell, since it has to ensure that all nuclear-encoded and cytosolically synthesised proteins are delivered to the appropriate cellular compartment to carry out their function. The fact that mitochondria have their own genome does not liberate them from cellular control, as only a few proteins (8 and 13 polypeptides in yeast and humans, respectively) are encoded by the mitochondrial genome. Nuclear genes encode all remaining proteins,

approximately 1000 proteins in the baker's yeast *Saccharomyces cerevisiae* [1–3]. Therefore, mitochondrial biogenesis entails a coordinated effort between both genomes to ensure the simultaneous synthesis and assembly of its entire protein complement.

The presence of four mitochondrial subcompartments, the outer membrane, intermembrane space, inner membrane and matrix, can pose a challenge for successful sorting of nuclear encoded precursors, but the organelle has in place multiple machineries that operate in a collaborative manner to ensure the fidelity of this process. However, mitochondria also depend on the integration of new lipids for their biogenesis, some of which are synthesised in the organelle and others that need to be transported into the organelle from other cellular compartments. Given that phospholipids provide the environment for the assembly of translocation machineries, their influence on translocation events is likely to be significant. Indeed, recent studies have uncovered surfacing connections into the role of phospholipids on mitochondrial translocation machineries and ultimately protein import into the organelle. Herein, we will explore these two aspects. We will first describe the basic principles of the mitochondrial protein import machinery and then discuss the emerging link to phospholipids.

2. Mitochondrial protein import

The identification and characterisation of most mitochondrial translocation components have employed fungal models, in particular *S. cerevisiae*, however, the core machinery is conserved in plants and humans [1]. Indeed, multiple mitochondrial import pathways have now been well defined (Fig. 1), dramatically changing earlier perspectives that protein import into the organelle was mediated via one main pathway. These alternative import pathways adhere to some common features including: (1) specificity of substrates for precise cellular locations mediated by "organelle specific" targeting elements; (2) "receptors" on target membranes that serve as docking sites for incoming precursors; (3) translocation channels formed by membrane-embedded protein complexes; and (4) precursor translocation through narrow translocation pores typically in an unfolded or loosely folded conformation.

The vast majority of mitochondrial-destined proteins are synthesised on cytosolic ribosomes as precursor proteins. It is generally assumed that the majority of precursors are imported in a post-translational manner. However, a co-translational mode of protein translocation into mitochondria is likely for some precursors [4,5], though it is currently unknown if and which fraction of proteins are imported into mitochondria during synthesis on ribosomes and how tight the coupling between translation and translocation would be. Therefore, the typical journey of a mitochondrial precursor begins following its synthesis in the cytosol, where it must overcome the boundaries imposed by molecular crowding and find its way to the organelle. This efficient trafficking of precursors through the cytosol is promoted by both cytosolic factors [6] and organelle specific targeting elements that serve as "zip codes" to deliver the precursor to a corresponding receptor(s) on the mitochondrial surface. These zip codes also promote sorting of the precursor to the correct sub-mitochondrial location. Mitochondrial targeting elements exist as either individual or multiple units scattered along the length of the precursor and vary markedly in terms of sequence, structure and location, reflecting their role in alternative mitochondrial sorting routes. The "classical" mitochondrial targeting signal is a cleavable N-terminal positively charged sequence, termed a presequence, which directs proteins to the mitochondrial matrix, inner membrane and in a

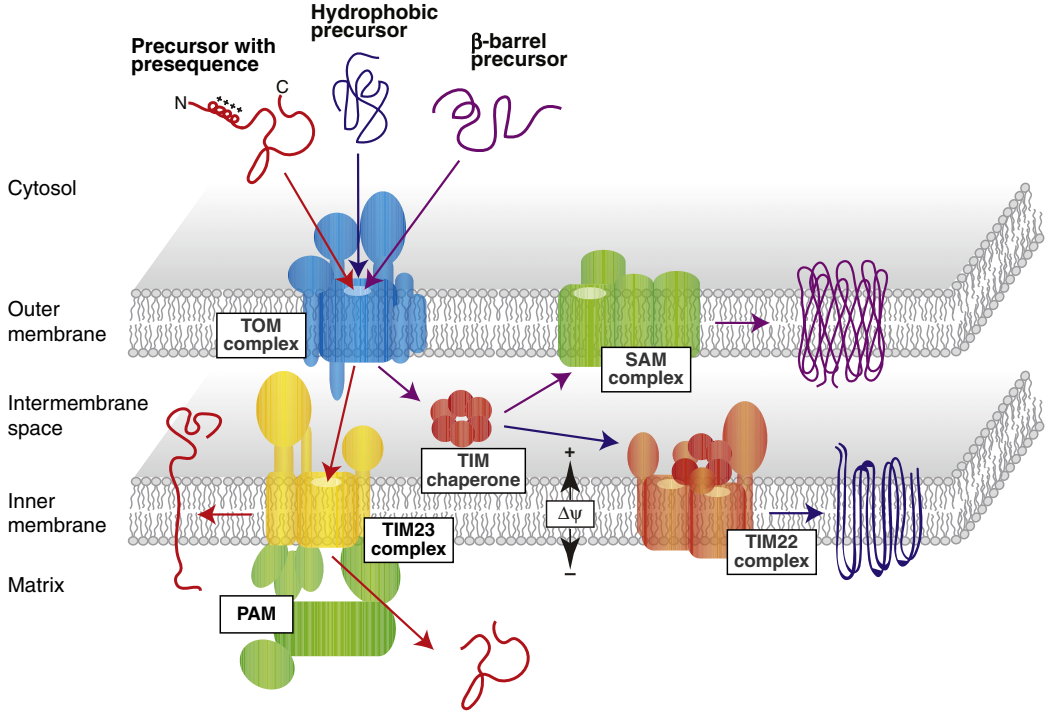

Fig. 1. The protein import machinery of the mitochondrial membranes. The greater part of all mitochondrial proteins is encoded by nuclear genes and are consequently synthesised as precursor proteins on cytosolic ribosomes. These undeveloped proteins must be targeted to and imported into mitochondria where they can acquire their functional and mature state. The translocase of the outer mitochondrial membrane (TOM complex) is the main entry gate into mitochondria and through a series of interactions with TOM complex receptors, precursors are guided to the "pore" of the complex in order to traverse the outer membrane. Upon outer membrane translocation different sorting pathways are initiated depending on the individual or multiple targeting elements contained within the precursor. Precursors possessing an N-terminal presequence are sorted to the presequence translocase of the inner membrane (TIM23 complex) in a membrane potential ($\Delta\psi$)-dependent manner. Complete translocation of precursors into the mitochondrial matrix is an ATP-driven process and requires the action of the presequence translocase-associated motor (PAM). Hydrophobic membrane proteins of the mitochondrial outer and inner membrane belonging to the β-barrel and carrier families, respectively, exploit the TIM chaperones of the intermembrane space for their passage through this aqueous environment. Following traffic through the intermembrane space, β-barrel precursors are directed to the sorting and assembly machinery (SAM complex) for outer membrane integration and carrier precursors are delivered to and inserted into the inner membrane by the $\Delta\psi$-driven carrier translocase (TIM22 complex).

few cases the mitochondrial intermembrane space. However, the greater part of mitochondrial proteins residing in the outer membrane, intermembrane space and inner membrane lack the classical presequence, but rather contain internal cryptic targeting sequences within the mature protein.

Once they have reached the mitochondria, by virtue of their "zip code," mitochondrial precursors must overcome the lipid barrier imposed by the mitochondrial outer membrane. This is accomplished by engaging with components of the Translocase of the Outer Membrane (TOM complex). The TOM complex serves a dual purpose: it supplies docking sites for incoming precursors in the form of receptors and provides a channel for precursor translocation across the outer membrane. Nearly all mitochondrial precursors are translocated through this portal and therefore the TOM complex plays an indispensable role in mitochondrial biogenesis. The TOM complex is composed of seven different subunits (Fig. 2): (i) the β-barrel subunit, Tom40, which forms the translocation pore [7,8], (ii) the central receptor Tom22, which harbours receptor function and supports the oligomeric organisation of the TOM complex [9], (iii) the three small Tom proteins, Tom5, Tom6 and Tom7, which regulate TOM assembly and stability [10–13], and (iv) the peripheral receptors, Tom20 and Tom70, which are involved in the initial recognition of precursors [14–16]. After engaging with either Tom20 or Tom70, precursors are passed to the central receptor Tom22 and are subsequently threaded into the Tom40 channel in an unfolded conformation. The components of the TOM complex are conserved from yeast to humans [17–23].

Following TOM translocation, precursors are sorted to one of at least four import pathways. According to our current knowledge, these four principle pathways appear to efficiently import and assemble the major complement of nuclear encoded mitochondrial precursors. These pathways are described below.

3. The TIM23 complex: the presequence pathway to the mitochondrial matrix and inner membrane

The classical and most well defined import pathway is taken by precursors possessing a presequence (Fig. 2). These precursors are handed over from the TOM complex to the inner membrane embedded presequence translocase, the TIM23 complex. Extensive research has provided a detailed picture of the presequence pathway, but has also provided a striking demonstration of collaboration between different multimeric machines. A sequence of interactions between the TIM23 complex and the TOM complex, as well as complexes of the inner membrane respiratory chain and the matrix exposed presequence translocase-associated motor (PAM), serve to enhance the dynamics of the machine and efficiency of precursor translocation across or into the inner membrane. Three essential inner membrane proteins constitute the core of the TIM23 complex: the channel-forming protein Tim23 [24], Tim17, which is involved in recruiting the PAM complex and lateral sorting of precursors [25], and Tim50 that exposes a large domain to the intermembrane space and adopts the role of receptor for incoming precursor proteins and is also involved in transient cooperation with the TOM complex [25–28]. Importantly the Tim50 intermembrane space domain is important for keeping the Tim23 channel closed in the absence of precursor proteins to prevent a leakage of ions across the inner membrane [24]. A fourth subunit, Tim21 also exposes a domain to the intermembrane space, which is also involved in transient interactions

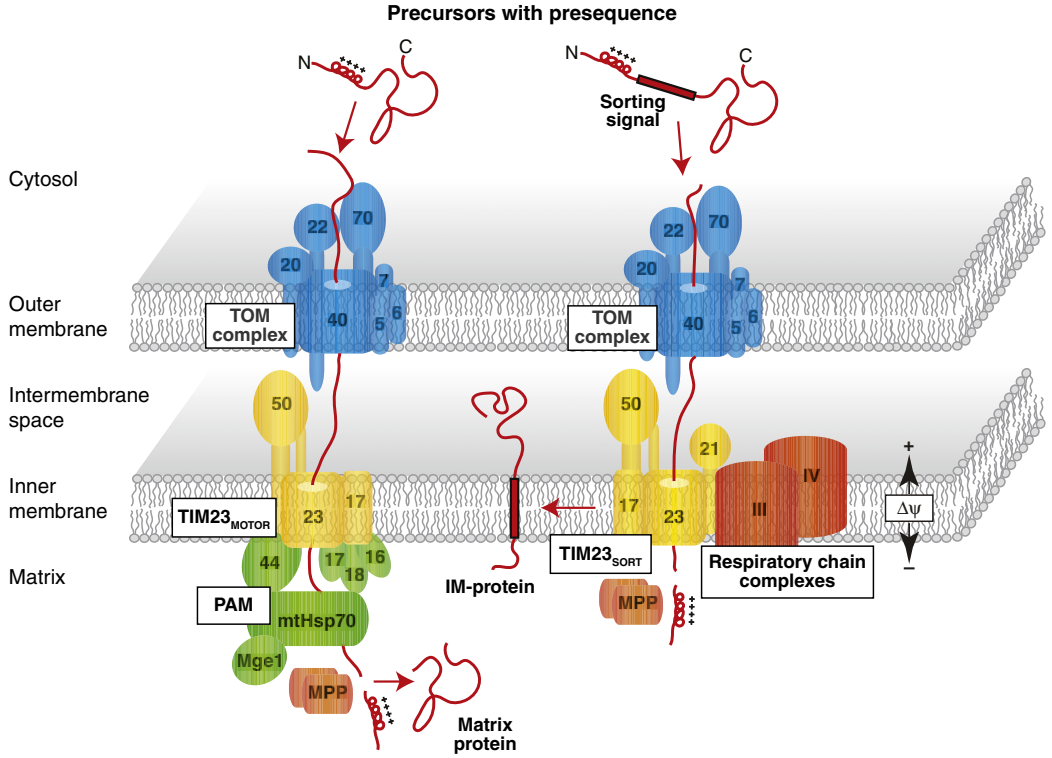

Fig. 2. The presequence pathway to matrix and inner membrane. After translocation through the TOM complex, presequence-containing precursors are directed to the TIM23 complex. The TIM23 complex can exist in two modular forms, which allow two alternative biogenesis pathways: (i) import into the mitochondrial matrix (shown on the left) and (ii) insertion into the mitochondrial inner membrane (shown on the right). Both forms of the complex contain the channel forming subunit Tim23, in addition to Tim17 and Tim50. Matrix translocation requires the membrane potential ($\Delta\psi$) in addition to the presequence translocase-associated motor (PAM). Association of PAM with TIM23 gives rise of the $TIM23_{MOTOR}$ form of the complex. PAM consists of mitochondrial Hsp70 (mtHsp70), the nucleotide exchange factor Mge1, Tim44 that links mtHsp70 to the TIM23 complex, and three additional co-chaperones Pam18, Pam16 and Pam17. Upon precursor translocation, the matrix processing peptidase (MPP) cleaves off the positively charged presequence liberating the mature protein. Conversely, precursors that contain a hydrophobic sorting signal or "stop-transfer" signal downstream of the presequence are targeted for inner membrane (IM) integration by virtue of this hydrophobic "sorting signal." Tim21 is present only in the sorting form of the TIM23 complex known as $TIM23_{SORT}$ and mediates the association of complexes III and IV of the respiratory chain with the presequence translocase. Precursor insertion into the inner membrane by the TIM23 complex occurs in a $\Delta\psi$-dependent manner.

with the TOM complex [25]. However, Tim21 also possesses a unique regulatory role and promotes the dynamic reorganisation of the TIM23 complex by promoting a transient coupling of the TIM23 complex with the respiratory chain complexes III and IV [29–31].

Precursor integration into the inner membrane is now known to only require the core components, Tim23, Tim17, Tim50 and Tim21 and this form of the presequence translocase is referred to as $TIM23_{SORT}$ (Fig. 2) [31,32]. This insertion pathway, commonly referred to as the stop transfer mechanism, is defined by the presence of a hydrophobic sorting signal behind the presequence, which arrests the translocating polypeptide in the inner membrane, causing lateral release into the lipid phase [33].

However, most presequence-containing precursors require complete translocation across the inner membrane into the matrix and this energy-dependent process is mediated by the motor PAM (Fig. 2). The central component of PAM is mitochondrial heat shock protein 70 (mtHsp70), a molecular chaperone that associates with the translocating polypeptide and exerts an import-driving activity on incoming polypeptides through ATP binding and hydrolysis. mtHsp70 acts in concert with numerous other proteins including the membrane anchor Tim44 and the nucleotide exchange factor, Mge1. In addition, three other co-chaperones are required to ensure the success of this energy-dependent event: Pam16 (Tim16), Pam17 and a homologue of the J-protein co-chaperones, Pam18 (also termed Tim14). Pam18 stimulates the ATPase activity of mtHsp70 through its J domain and Pam16, a J-related protein, forms a stable complex with Pam18 and controls its activity [34,35]. Pam17 is involved in the organisation of the TIM23-PAM interaction [36]. The motor subunits interact with several sites on the TIM23 complex, including an association of Pam18 to Tim17 and Pam17 to Tim23 [25,36–38]. The translocation sequence for presequence-containing proteins is completed by proteolytic cleavage of the presequence, which is catalysed by the mitochondrial processing peptidase (MPP), liberating the mature protein into the mitochondrial matrix or inner membrane.

To date, this highly sophisticated TIM23-PAM machine consists of ten components in yeast mitochondria: Tim23, Tim17, Tim50, Tim21, and Tim44, Pam18, Pam17, Pam16, mtHsp70 and Mge1. With the possible exception of Pam17, all of these components appear to be conserved in humans [39].

4. The TIM22 complex: the carrier pathway to the mitochondrial inner membrane

An alternative route to the inner membrane is taken by the family of metabolite carriers such as the ADP/ATP carrier (AAC), which consist of about 35 members in yeast (Fig. 3) [40]. Our knowledge of this biogenesis pathway begins in the cytosol where these largely hydrophobic precursors are maintained soluble through the action of cytosolic chaperones belonging to the Hsp70 and Hsp90 families [6]. The entire precursor–chaperone complex is delivered to the TOM complex receptor, Tom70, which possesses binding sites for both the precursor and the chaperone [6,41,42]. In an ATP-dependent step the precursor is released and transferred to the TOM complex, where it is inserted and threaded through the pore in a loop conformation [41]. Having just overcome the aqueous environment of the cytosol, the hydrophobic carrier precursor finds itself in another predicament upon outer membrane translocation, which is the aqueous environment of the intermembrane space. However, when the precursor is still in the Tom40 pore, binding of the intermembrane space Tim9–Tim10 chaperone complex avoids aggregation of precursors in this environment. Tim9 and Tim10 are involved in guiding precursors across the intermembrane space to the mitochondrial inner membrane [43–46].

Owing to the fundamental role of the carriers in mitochondrial biogenesis, the organelle has in place a specialised machinery to ensure their proper import and assembly known as the carrier translocase, or TIM22 complex. Tim22 is the major subunit of the ~300-kDa carrier translocase [47], and is the pore-forming unit of the complex (Fig. 3). The isolated TIM22 complex contains two such pores that cooperate during protein import [48]. Tim54, Tim18 and Tim12 are also members of the carrier translocase along with a fraction of the intermembrane space chaperone subunits Tim9 and Tim10 [49–52]. Tim54 exposes a large domain to the intermembrane space and is believed to provide a binding site for the Tim9–Tim10–Tim12 complex, whereas Tim18 is involved in the assembly of the TIM22 complex [49,53,54]. Precursors are handed over to the TIM22 complex to undergo insertion into the twin channel in a loop-like conformation in a step-wise manner [41,55]. Upon integration into the inner membrane in a $\Delta\psi$-dependent manner, carrier precursors can complete their biogenesis pathway and assemble into functional dimers. This last step of integration into the inner membrane remains poorly defined. Mammalian homologues of Tim22 and a number of the small Tim proteins, Tim8a, Tim8b, Tim9 and Tim10a, Tim10b and Tim13 have been reported [56–58].

In yeast Tim9, Tim10 and Tim12 play a pivotal role in the biogenesis of carrier precursors and their own biogenesis pathway has received significant amounts of attention. These proteins belong to the family of small intermembrane space proteins that contain characteristic cysteine motifs. The biogenesis of this class of precursors depends on their cysteine residues that are exploited in dithiol–disulfide exchange reactions along the MIA pathway (Mitochondrial Intermembrane space Assembly) [59]. The MIA machinery consists of two essential intermembrane space components, Mia40 [60–62] and the sulfhydryl oxidase Erv1 [63–65], and will be discussed in greater detail elsewhere in this issue.

Briefly, the translocating intermembrane space precursor engages with the receptor Mia40 via a transient intermolecular disulfide bond on the *trans*-side of the mitochondrial outer membrane [60,64] and this effectively permits entry of the precursor into the MIA pathway. The N-terminal cysteine residue of Tim9 and Tim10 is crucial to form the transient disulfide bond with Mia40 [66,67]. Erv1 cooperates with Mia40 in two ways: (i) upon oxidation of the incoming precursor, Mia40 acquires a reduced state and is subsequently reoxidized by Erv1, which permits the next round of precursor binding and import [64,68], and (ii) Erv1 forms a transient reaction intermediate, or ternary complex, with Mia40 and the precursor protein, which is thought to facilitate the transfer of multiple disulfide bonds from the sulfhydryl oxidase Erv1 to the precursor protein via Mia40 [69]. Some additional players that contribute to the MIA pathway have also been identified, and include cytochrome *c* and cytochrome *c* peroxidase, which play a role in electron flow from Erv1, and the zinc-binding protein Hot13 that promotes the reoxidation of Mia40 by Erv1 [70–73].

5. Import and assembly in the mitochondrial outer membrane

The mitochondrial outer membrane, like that of the bacterial and chloroplast outer membrane, contains a number of proteins that adopt a β-barrel conformation. The channel forming subunit of the TOM complex, Tom40, and the abundant outer membrane porins are representative of these proteins in mitochondria. Given the conservation of β-barrel proteins and their important role in mitochondrial biology, the machinery that governs their assembly has also been maintained throughout evolution. In bacteria, the β-barrel Assembly Machinery, or BAM complex, is responsible for the biogenesis of this class of proteins [74,75]. In mitochondria, the Sorting and Assembly Machinery (SAM complex) takes on this task. The core of the SAM complex is composed of the central β-barrel component Sam50, also termed Omp85/Tob55 [76–78] and two partner proteins Sam35 (Tob38) and Sam37 [79–82], which expose domains to the cytosol. Sam50 is homologous to the central component of the BAM machinery, BamA, however, the further subunits of the BAM and SAM complexes are not related to each other.

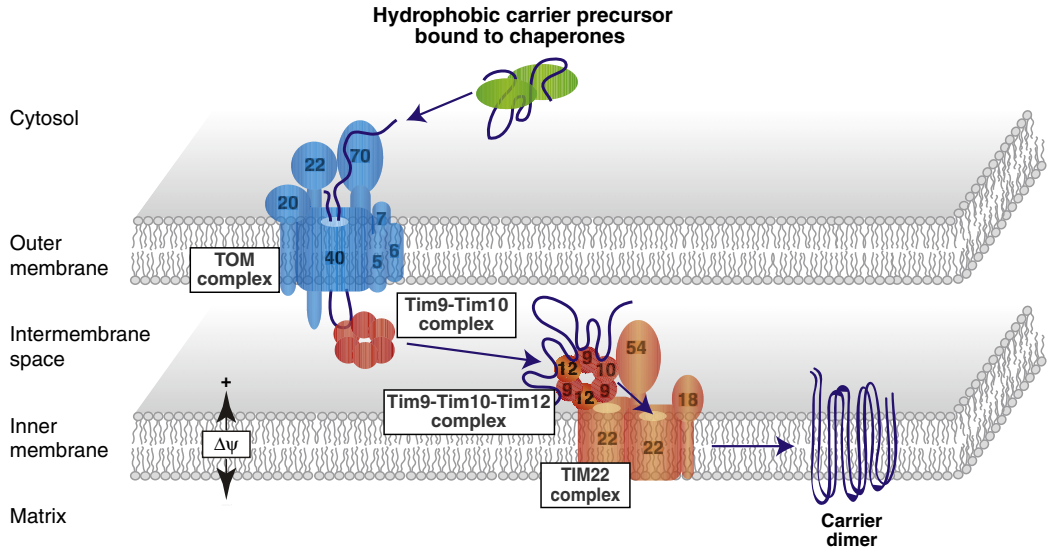

Fig. 3. The carrier import pathway to the inner membrane. Hydrophobic carrier precursors are maintained unfolded and soluble in the cytosol by molecular chaperones and are delivered to the receptor Tom70 on the mitochondrial surface. Carriers traverse the Tom40 pore in a loop conformation and are met by the Tim9–Tim10 chaperone complex on the intermembrane space side, which aids in the journey of the hydrophobic precursors through this aqueous environment. Precursors dock onto the TIM22 complex via the Tim9–Tim10–Tim12 module and are inserted into the twin-pore formed by Tim22 molecules. Tim22 inserts the precursors into the inner membrane in a membrane potential ($\Delta\psi$)-dependent manner and carriers assemble into functional dimers.

β-barrel precursors are recognised by TOM receptors and are first translocated across the outer membrane through the TOM complex into the intermembrane space [11]. In doing so, the topological position of the incoming SAM complex substrate mimics the bacterial state where the β-barrel substrate associates with BamA from the periplasmic side. Once in the intermembrane space, chaperone complexes of the small Tim proteins [83,84] facilitate the transfer of the hydrophobic β-barrel precursors to the SAM complex for membrane integration (Fig. 4). Recently, a specific sorting signal in the most C-terminal β-strand of β-barrel proteins across all eukaryotic kingdoms, termed the β-signal, was identified [85]. The β-signal initiates the insertion of β-barrel precursors into a hydrophilic, proteinaceous membrane environment by forming a complex with Sam35 and Sam50, which can accommodate protein insertion into the lipid phase [85]. Subsequent release of precursor proteins from the SAM complex is believed to be mediated through the action of Sam37 [86]. Several other outer membrane proteins have been suggested to participate in the assembly of β-barrel proteins. Mdm10, which was originally identified for its role in maintaining mitochondrial morphology, has been shown to associate with a fraction of SAM complexes to promote the assembly of Tom40 [87]. Mdm10 is also found in another outer membrane complex, termed the MDM complex, that contains the additional proteins Mmm1, Mdm12 and Mdm34 (also

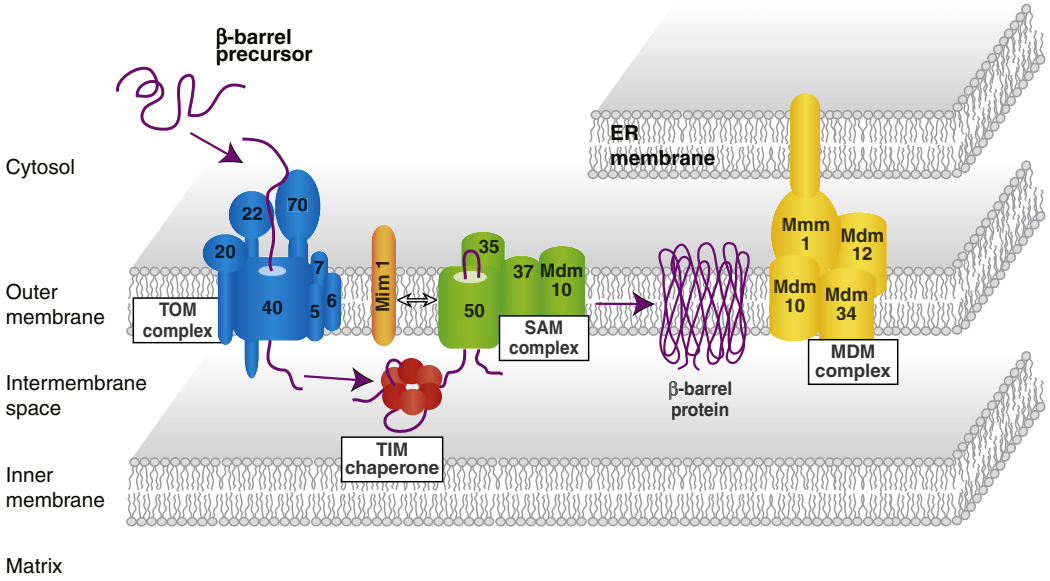

Fig. 4. β-barrel assembly in the mitochondrial outer membrane. β-barrel precursors are recognised on the mitochondrial surface by the TOM receptors and traverse the outer membrane through the Tom40 channel to the intermembrane space. The TIM chaperones of the intermembrane space engage with the hydrophobic β-barrel precursors and assist in their transfer from TOM to the Sorting and Assembly Machinery of the outer membrane (SAM complex). The SAM complex is composed of Sam50 and the two peripheral components Sam35 and Sam37 and mediates the membrane integration of β-barrel precursors. Mdm10 associates with the SAM complex and plays a role in the maturation of the Tom40 precursor. Mdm10 is also a member of the MDM complex, along with Mdm12, Mdm34 and Mmm1, which physically links the ER and mitochondrial outer membrane. β-barrel precursor maturation post-SAM has also been shown to be dependent on components of the MDM complex, namely Mdm12 and Mmm1. Mim1 likely functions as insertase for several precursor proteins that contain α-helical transmembrane segments; Mim1 can transiently interact with the SAM complex.

called Mmm2) [88,89]. Yeast mutants of different MDM subunits were shown to affect β-barrel assembly [90].

The outer membrane of mitochondria is also resident to proteins defined by the presence of single or multiple α-helical transmembrane segments. Until recently the mechanisms governing insertion of these proteins into the lipid phase have remained largely enigmatic. The precursor of Tom22, which contains a transmembrane domain in an internal segment of its amino acid sequence, is now known to utilise the SAM complex for insertion into the outer membrane [91]. Conversely, outer membrane proteins that contain N-terminal transmembrane domains, the so-called signal-anchored proteins, are inserted into the outer membrane with the assistance of Mim1, which appears to be behaving as an outer membrane insertase [92–95]. In another example, proteins with several transmembrane α-helical segments require the Tom70 receptor but no other Tom proteins [96]. Thus, current observations are pointing to remarkable variation in the machineries and mechanisms that promote the insertion of this class of proteins into the outer membrane.

6. Mitochondrial phospholipids

In addition to the dedicated protein transport machines outlined above, which permit the incorporation of the mitochondrial protein complement, the organelle is heavily dependent on the successful incorporation of phospholipids for its biogenesis. In addition to barrier functions, phospholipids permit cellular membranes to undergo processes that are essential for cellular survival, including budding, tubulation, fission and fusion. Mitochondria can synthesise several acidic phospholipids autonomously, including phosphatidic acid (PA), CDP-diacylglycerol (CDP-DAG), phosphatidylglycerol (PG), and cardiolipin [97]. In contrast, phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), and sterols are synthesised in other organelles and transported to mitochondria. Imported phosphatidylserine is rapidly decarboxylated within the mitochondrion to form phosphatidylethanolamine (PE) [97].

Cardiolipin is the signature phospholipid of the mitochondrion and has been implicated in diverse mitochondrial functions. For instance, the respiratory chain of the mitochondrial inner membrane is known to be organised in higher ordered assemblies, referred to as supercomplexes [98]. However, mitochondria isolated from a *crd1*Δ strain, which lacks the enzyme cardiolipin synthase (Crd1) and thus also cardiolipin, display reduced supercomplex stability [99], implying a crucial role for cardiolipin is the dynamic association of these molecular assemblies. The binding of cardiolipin to the inner membrane ADP/ATP carrier has been suggested to modulate the protein's activity [100,101], while the binding of cardiolipin to cytochrome *c* has been proposed to serve a regulatory role, sequestering a fraction of the protein to the inner membrane and thus limiting the soluble pool of cytochrome *c* [102].

The cardiolipin biosynthesis pathway is well delineated in *S. cerevisiae* and involves a sequence of enzymatic reactions that take place in the mitochondrial inner membrane (Fig. 5A) [103]. To begin the process, phosphatidic acid is converted to CDP-diacylglycerol. Phosphatidylglycerol phosphate (PGP) synthase (Pgs1) then catalyses PGP synthesis from CDP-DAG and glycerol-3-phosphate (G-3-P) [104]. PGP is then dephosphorylated to PG by the PGP phosphatase Gep4 [105,106]. Cardiolipin synthase (Crd1) catalyses an irreversible condensation reaction in which CDP-DAG is linked to PG via cleavage of a high energy anhydride bond to form cardiolipin [104,107–109]. Cardiolipin remodelling by deacylation then leads to the formation of monolysocardiolipin (MLCL) by the phospholipase Cld1 [110], which is then reacylated with another fatty acid [111]. The reacylation reaction is catalysed by tafazzin (Taz1) [112]. The importance of cardiolipin remodelling is highlighted by its role in human Barth Syndrome, an X-linked cardioskeletal myopathy caused by mutations in tafazzin which can affect respiratory complex assembly [113,114].

7. Phospholipids and the inner membrane protein transport machinery

A role for cardiolipin in the import of proteins into mitochondria was first suggested when defects in protein import into mitochondria were noted when yeast cells were treated with the cardiolipin-binding compound doxorubicin [115]. Subsequent studies with the

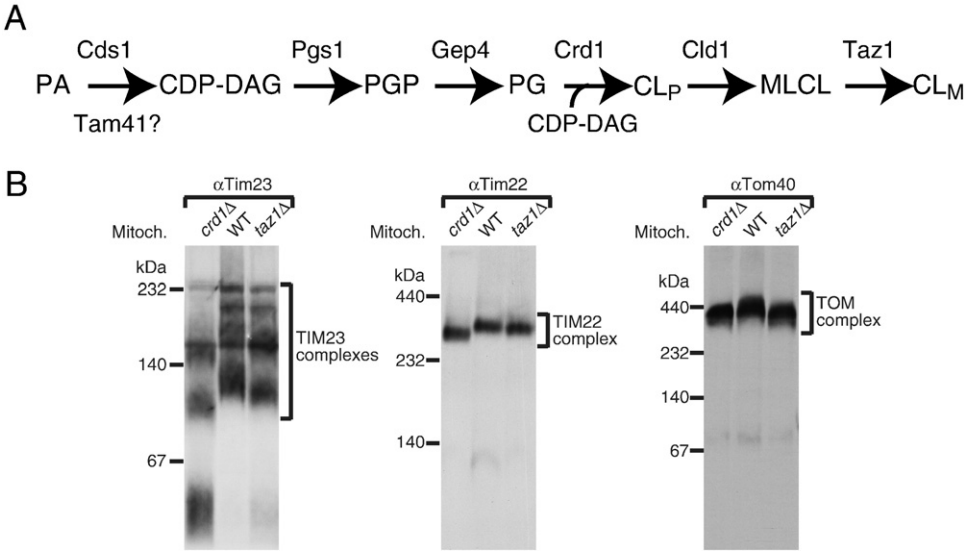

Fig. 5. Mitochondrial cardiolipin: biosynthesis and influence on protein translocases. (A) A depiction of the enzymatic reactions undertaken during the biosynthesis of cardiolipin and the enzymes that mediate the reactions in *Saccharomyces cerevisiae*. PA, phosphatidic acid; Cds1, CDP-diacylglycerol synthase; Tam41, translocator assembly and maintenance protein 41; CDP-DAG, CDP-diacylglycerol; Pgs1, PGP synthase; PGP, phosphatidylglycerol phosphate; Gep4, PGP phosphatase (genetic interactor of prohibitins 4); PG, phosphatidylglycerol; Crd1, cardiolipin synthase; CL_P, premature cardiolipin; Cld1, cardiolipin-specific deacylase; MLCL, monolysocardiolipin; Taz1, tafazzin; CL_M, mature cardiolipin. (B) Cardiolipin affects protein translocases of mitochondria. Mitochondrial protein complexes were separated by a native gel system (blue native electrophoresis) and detected by Western blotting (α, antibody against). In mutant mitochondria either lacking cardiolipin (*crd1*Δ lacks cardiolipin synthase) or containing premature cardiolipin and monolysocardiolipin (*taz1*Δ lacks tafazzin), the mobility of TIM and TOM complexes is altered [119,133].

*crd1*Δ mutant indicated that the absence of cardiolipin leads to a reduced membrane potential (Δψ) and consequently decreased protein import [116]. Furthermore, reconstitution of the $TIM23_{SORT}$ complex indicated that membrane integration of mitochondrial presequence-containing precursors is most efficient when the presequence translocase is reconstituted in cardiolipin-containing membranes [32].

More recently a role of phospholipids in protein import was uncovered with the clarification of the function of Tam41. Tam41 (translocator assembly and maintenance protein 41) was identified and initially described as an additional inner membrane translocator component, with a role in the import of presequence-containing proteins via assembly and maintenance of the TIM23 complex [117,118]. However, on closer examination of a *tam41*Δ mutant a striking phenotypic similarity to that of the cardiolipin-deficient *crd1*Δ mutant was noted, including alterations in various inner membrane complexes, such as protein translocases, assembled AAC and respiratory chain supercomplexes [119] (shown for the TIM23 and TIM22 complexes of *crd1*Δ mutant mitochondria in Fig. 5B). These common phenotypes between the *tam41*Δ and *crd1*Δ mutants suggested a possible role of Tam41 in cardiolipin regulation. This prompted an analysis of the phospholipid content from mitochondria isolated from *tam41*Δ cells. Indeed, a complete loss of cardiolipin and its precursor PG was apparent in *tam41*Δ cells, however an accumulation of PA was evident, suggesting that Tam41 regulation in the biosynthetic pathway of cardiolipin may occur at the level of CDP-DAG synthase (Fig. 5A) [119]. Therefore, it appears that Tam41 has a role in the biosynthesis of PG and cardiolipin and that the import defects observed in *tam41*Δ cells are a consequence of these lacking phospholipids. Tam41, therefore, affects protein import into mitochondria by regulating phospholipid composition. Consistent with these findings two intermembrane space proteins Ups1 and Ups2/Gep1 have been found to antagonistically regulate cardiolipin levels in mitochondria [120,121]. As a likely consequence of the altered cardiolipin levels, loss of Ups1 and Ups2 was also shown to affect protein import and the integrity of the TIM23 translocase [121].

The emerging data suggest the structural integrity of the inner membrane and the functionality of proteins within this membrane is likely to be largely influenced by the lipid environment and ultimately a dynamic interplay between both lipids and proteins. In line with this concept, an intimate relationship between the biosynthesis of both cardiolipin and PE and the mitochondrial prohibitins was recently reported [120]. Prohibitins form an evolutionary conserved family of membrane proteins found in multiple cellular compartments, including the mitochondrial inner membrane, where they can function as protein or lipid scaffolds to maintain mitochondrial integrity [122]. Osman and colleagues identified 35 genes or GEPs (genetic interactors of prohibitins) that were required for the survival of prohibitin deficient yeast cells using a synthetic genetic array analysis [120]. Amongst these were *PSD1* and *CRD1*, the genes coding for PE and cardiolipin synthesising enzymes, respectively, demonstrating a new dimension to mitochondrial phospholipid regulation and intimately linking the process to the mitochondrial prohibitins.

8. Phospholipids and the outer membrane protein transport machinery

Contrary to the inner membrane, the mitochondrial outer membrane has been proposed to be more fluid [123]. These differences in membrane properties may be crucial when assigning specific functions to this membrane. We now have a number of observations that point to an intimate link between phospholipids and the mitochondrial outer membrane import machinery.

Sam37 (formerly Mas37), a member of the outer membrane SAM complex was originally discovered through a genetic screen for genes controlling mitochondrial phospholipid metabolism [124], however, a direct role for Sam37 in lipid metabolism could not be established at this time. In a similar light, the bacterial counterpart of Sam50 (Omp85/BamA) has also been implicated in phospholipid transfer [125]. As mentioned earlier the SAM complex in collaboration with the outer membrane MDM complex, consisting of Mdm10 (also a component of the SAM complex), Mdm12, Mdm34 and Mmm1 [87–90], facilitates the biogenesis of outer membrane β-barrel precursors. Interestingly, a recent study indicated that the MDM complex is involved in efficient inter-organellar phospholipid exchange [126]. In yeast, PS is known to be synthesised in the endoplasmic reticulum (ER) and a subdomain of this organelle known as the mitochondria-associated membrane (MAM) [127]. Morphological evidence indicates that transfer of PS into mitochondria occurs at zones of apposition between the MAM and the mitochondrial outer membrane. In the elegant study by Kornmann et al. [126], a genetic screen utilising a fusion protein that induced artificial contact sites between both organelles was employed. By screening for yeast mutants that were unable to grow in the absence of this linker the authors identified mutations in one candidate, *MDM12*, leading to the characterisation of the MDM complex as a structure forming ER-mitochondria junctions. The N-terminus of Mmm1 is integrated into the ER membrane in the MAM region and thus the MDM complex connects mitochondrial outer membrane and ER (Fig. 4). The MDM complex, also termed ERMES for ER-mitochondria encounter structure, may directly or indirectly facilitate phospholipid transfer between both organelles [126]. The locality of the MDM complex at MAM and the involvement of the complex in lipid transport could provide an explanation for part of the severe mitochondrial morphological defects observed with loss of the MDM proteins (formation of condensed giant mitochondria in the mutants in contrast to the tubular morphology of mitochondria in wild-type cells). Mutants of the SAM complex display similar morphological defects as MDM mutants [87,90], suggesting that the processes of protein assembly, ER-mitochondria connection and maintenance of mitochondrial morphology are closely connected.

It has also been suggested that the defined lipid composition of the mitochondrial outer membrane contributes and regulates the specific insertion of the mitochondrial C-tail anchored protein Fis1 [128]. The mitochondrial outer membrane is reported to have the lowest ergosterol/phospholipid ratio from all the cellular membranes that are exposed to the cytosol [129,130] and this could be the basis of a mechanism that secures the specific localisation of specific mitochondrial outer membrane proteins. In favour of this notion, both Fis1 and a further mitochondrial tail-anchored protein Mff have been shown to have a dual location at both mitochondria and peroxisomes [131,132]. Peroxisomal membranes, like the mitochondrial outer membrane, contain a low ergosterol content, whereas membranes of the secretory pathway have a much higher content [129]. Indeed, increasing ergosterol levels was shown to decrease the insertion capacity of Fis1 into lipid vesicles [128], suggesting a direct contribution of lipids to mitochondrial import events.

More recently, a role of cardiolipin in the biogenesis of outer membrane proteins was brought to light [133]. The presence of cardiolipin in the outer membrane has long been a controversial topic and different views on the topic have been reported [134,135]. A quantitative determination using highly pure outer membrane vesicles from yeast revealed that the outer membrane contains about one quarter of the cardiolipin concentration of total mitochondrial membranes [133]. Interestingly, yeast double mutants that were affected in both an outer membrane protein translocase (TOM or SAM) and cardiolipin metabolism (lacking either the enzyme Crd1 or Taz1) showed synthetic growth defects [133]. These results indicate a genetic interaction of cardiolipin mutants with components of the outer membrane protein import machinery. Cardiolipin mutants affected the mobility of TOM and SAM complexes on native gel systems, providing biochemical evidence that cardiolipin is connected to outer membrane translocases (shown for the TOM complex in Fig. 5B). The assembly of β-barrel precursors and some

α-helical precursors was shown to be delayed in mitochondria isolated from yeast cardiolipin mutants, suggesting a new regulatory role of cardiolipin in the biogenesis of outer membrane proteins [133]. Similarly, human mitochondria isolated from a Barth syndrome patient, who was defective in the tafazzin (Taz1) protein that is required for the remodelling of cardiolipin, were impaired in the assembly of the β-barrel precursor of porin (VDAC1) [133]. These observations suggest a more specific role of cardiolipin for protein sorting and influencing the activity of mitochondrial transport machineries of the outer membrane and raise the possibility that defects in outer membrane protein biogenesis may contribute to the mitochondrial defects observed in Barth syndrome patients.

9. Concluding remarks and future perspectives

We still have much to explore regarding the molecular dynamics governing mitochondrial biogenesis at both the protein and phospholipid level. However, recent findings have given us a significant leap forward in our understanding of these processes, in particular the emerging link between phospholipids and the biogenesis of mitochondrial precursor proteins. However, we are clearly just at a starting point and many exciting questions await exploration. For instance: (i) What is the cellular complement of proteins involved in the interorganelle and intraorganelle transport of lipids? (ii) How intimately linked are the protein and lipid transport machineries? (iii) What is the direct role, if any, of lipids and lipid modifying enzymes in protein translocation events? The answers to these and other questions are likely to intrigue both protein and lipid biochemists alike for many years and will foster future and exciting collaborations.

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