The presequence pathway is involved in protein sorting to the mitochondrial outer membrane

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Abstract

The mitochondrial outer membrane contains integral α-helical and β-barrel proteins that are imported from the cytosol. The machineries importing β-barrel proteins have been identified, however, different views exist on the import of α-helical proteins. It has been reported that the biogenesis of Om45, the most abundant signal-anchored protein, does not depend on proteinaceous components, but involves direct insertion into the outer membrane. We show that import of Om45 occurs via the translocase of the outer membrane and the presequence translocase of the inner membrane. Assembly of Om45 in the outer membrane involves the MIM machinery. Om45 thus follows a new mitochondrial biogenesis pathway that uses elements of the presequence import pathway to direct a protein to the outer membrane.

Keywords intermembrane space; mitochondria; outer membrane; protein sorting; Saccharomyces cerevisiae

Subject Categories Membrane & Intracellular Transport; Protein Biosynthesis & Quality Control

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Introduction

The translocase of the outer membrane (TOM) functions as the major import site for nuclear-encoded mitochondrial preproteins. Four main import pathways have been described that involve protein translocation through the TOM complex [1–5]. Preproteins with cleavable N-terminal presequences are translocated from TOM to the presequence translocase of the inner membrane (TIM23). The precursors of non-cleavable hydrophobic carrier proteins are transferred from TOM to the small TIM chaperones of the intermembrane space and are inserted into the inner membrane by the carrier translocase (TIM22). Cysteine-rich intermembrane space proteins are imported via TOM and the mitochondrial intermembrane space assembly (MIA) machinery. The precursors of β-barrel proteins use TOM, small TIM chaperones and the sorting and assembly machinery (SAM/TOB) for insertion into the outer membrane.

Different biogenesis pathways have been reported for α-helical outer membrane proteins, including import via Tom receptors, the mitochondrial import (MIM) complex or SAM [4–12]. Importantly, however, it has been reported that several outer membrane proteins do not require proteinaceous import components, but are directly inserted into the lipid phase of the membrane, including the outer membrane protein of 45 kDa (Om45) [5,13].

Om45 is the most abundant signal-anchored protein of the outer membrane [14–16]. Signal-anchored proteins contain a single transmembrane segment at the N-terminus that functions as mitochondrial targeting signal and membrane anchor [5,13,17]. Om45 forms a complex with the outer membrane proteins porin and Om14. It has been reported that Om45 is involved in the interaction between outer and inner membrane protein complexes [18,19], thus linking the porin pore to transport proteins of the inner membrane. Studies conducted so far did not lead to the identification of proteinaceous factors required for the import of the Om45 precursor into mitochondria [7,8,13,17].

Here, we report a novel pathway of mitochondrial protein import. The precursor of Om45 embarks on the presequence pathway, including the TOM complex and the TIM23 complex, for translocation of its large C-terminal domain into the intermembrane space. Om45 is then released to the outer membrane/
intermembrane space and is assembled with the aid of the MIM machinery.

**Results and Discussion**

**Om45 is imported via the TOM complex**

We analysed the Om45 complex by blue native electrophoresis upon lysis of mitochondria with digitonin [7,8,18] and observed that the amount of the complex was diminished in mutant mitochondria lacking the receptors Tom20 and Tom22, yet not in tom70Δ mitochondria (Fig 1A). Tom20 and Tom22 play a major role in the import of presequence-carrying proteins into mitochondria, whereas the third receptor Tom70 is important for importing non-cleavable carrier proteins [2,3]. To study whether Tom20 and Tom22 were involved in the import of Om45, we incubated the [35S]-labelled precursor of Om45 with isolated mitochondria. Formation of [35S] Om45 complexes was inhibited in tom22Δ and tom20Δ mitochondria, but not in tom70Δ mitochondria (Fig 1B). Translocation of
Anti-His antibodies bound to the Om45His complex in C-terminal His-tag and performed antibody-shift blue native electrophoresis. Anti-His antibodies bound to the Om45His complex in mitoplasts, but not in intact mitochondria (Fig 1E). Om45His was properly assembled, shown by the co-purification of the two partner proteins porin and Om14 (Supplementary Fig S1B) [18]. Additionally, the His-tag or a green fluorescent protein (GFP) fused to the C-terminus of Om45 was only accessible to protease after opening of the outer membrane (Supplementary Fig S1C and D). These results demonstrate that the bulk of Om45 is exposed to the intermembrane space.

In addition to the three receptors, the TOM complex consists of the central channel-forming protein Tom40 and three small Tom proteins. Tom5 is closely associated with Tom40 and involved in the import of preproteins, whereas Tom6 and Tom7 play a role in the assembly and dynamics of TOM [2–5]. Mitochondria lacking Tom5 were impaired in import and assembly of Om45, whereas tom6Δ and tom7Δ mitochondria imported Om45 like wild-type mitochondria (Fig 2A).

To study whether the biogenesis of Om45 involved Tom40, we generated a conditional tom40– yeast mutant strain (Supplementary Fig S2A). As expected, tom40–Δ mitochondria were impaired in import of the matrix-targeted F1–ATPase subunit β (F1β), but not in import of the Tom40-independent outer membrane protein Ugo1 (Supplementary Fig S2B) [11,12]. Import and assembly of [35S]Om45 were inhibited in tom40–Δ mitochondria, and the amount of Om45 complexes was considerably decreased (Fig 2B). Upon short-term import into mitochondria, [35S]Om45 was co-purified with HA-tagged Tom40, whereas endogenous, mature Om45 was not co-purified (Fig 2C), indicating that Om45 interacts with the TOM complex during import. We inserted a cysteine residue into the transmembrane segment of Om45 (Supplementary Fig S2C). [35S]Om45S10C was incubated with mitochondria for a short import period and oxidized was added. A [35S]-labelled crosslinking product of approximately 90 kDa was co-purified with Tom40HA under denaturing conditions (Fig 2D), demonstrating that the Om45 precursor is in close vicinity to Tom40 during import.

To obtain additional evidence that Om45 uses the TOM complex during import, the passenger protein dihydrofolate reductase (DHFR) was fused to the C-terminus of the Om45 precursor (Fig 2E, lane 1; Supplementary Fig S2C). Upon incubation with mitochondria, the fusion protein with the folded DHFR moiety accumulated in a complex of approximately 500 kDa (Fig 2E, lanes 3 and 4). Formation of the complex was impaired in *tom* mutant mitochondria and antibodies directed against Tom5 or Tom22 shifted the complex (Fig 2E, right panels), demonstrating that Om45-DHFR was accumulated in the TOM complex. A fusion protein carrying DHFR at the N-terminus of Om45 (DHFR-Om45; Fig 2E, lane 2; Supplementary Fig S2C) was not accumulated at the TOM complex (Fig 2E, lanes 5 and 6), supporting the view that Om45 is directed into mitochondria by its N-terminal signal-anchor domain. The signal-anchor domain, located within the N-terminal 32 amino acid residues of Om45 [13,17], sufficed to transport a portion of mature cytochrome b2 (Om4532–b2) to a protease-protected location (Supplementary Fig S2C and D). Interestingly, the signal-anchor domain failed to transport the cytosolic domain of Tom20 to a protease-protected location, yet the fusion protein Om4532–Tom20CD accumulated at the mitochondrial surface (Supplementary Fig S2C and D), providing an explanation for the reported partial complementation of tom20Δ yeast by expression of Om4532–Tom20CD [17].

To block import channels, chemical amounts of the matrix-targeted b2(167)3-DHFR fusion protein were arrested in a TOM-TIM23 supercomplex in the presence of methotrexate (Fig 2F) [22,23]. Since TOM complexes are approximately 3- to 4-fold more abundant than TIM23 complexes [22,24,25], the preprotein blocks only a fraction of TOM complexes (Fig 2F, lanes 2–4) and import of Ugo1 into the outer membrane and ATP/ADP carrier into the inner membrane were not or only moderately affected (Fig 2F, right panels). Remarkably, the import of Om45 was inhibited by the arrested preprotein like the import of F1β (Fig 2F, lanes 10–12).

In summary, Om45 is exposed to the intermembrane space and import of the Om45 precursor into mitochondria involves the TOM machinery.

**Import of Om45 uses elements of the presequence pathway**

We asked which factors are required for translocation of Om45 towards the intermembrane space. The intermembrane space domain of Tom22 promotes TOM-TIM23 cooperation and is involved in import of presequence-carrying preproteins [23,26–28]. *tom*22–2 mutant mitochondria, which lack the intermembrane space domain [28], were impaired in import of Om45 and F1β (Fig 3A).

Mutant mitochondria of the small TIM chaperones were only moderately affected in the import of Om45 (Supplementary Fig S3A). Taken together, the import characteristics of Om45 resemble the import of presequence-carrying preproteins, including import via Tom20/Tom22 and inhibition by a TOM-TIM23 arrested preprotein [22,23,26–28].

We thus analysed mutant mitochondria, which are impaired in subunits of the TIM23 complex [29–32]. Conditional mutants of the essential subunits Tim23, Tim50 and Tim17 were considerably impaired in the import of Om45 (Fig 3B and C). As control, import of Ugo1 and Tom40 were not inhibited or even enhanced in the mutant mitochondria (Fig 3B and C). Tim21 plays a regulatory function in the TIM23 complex, yet is dispensable for import of presequence-carrying preproteins [29]. Import of Om45 was not inhibited in *tim*21Δ mitochondria (Fig 3D). Tim50 and Tim23 have receptor functions for presequence-carrying preproteins in the intermembrane space [30–32] and promote translocation of the preproteins through the TOM complex [23,29]. Accumulation of Om45-DHFR in the TOM complex depended on these subunits of the presequence translocase in a manner comparable to the accumulation of the cleavable preprotein Oxa1 in TOM (Fig 3E) [29]. Import of Om4532–b2, which contains only the signal-anchor domain of Om45, was not

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Figure 2. The precursor of Om45 is imported by the TOM complex.

A, B [35S]Om45 was imported into WT and mutant mitochondria as described in the legend of Fig 1B and C. Om45 complexes were also analysed by blue native electrophoresis and immunodetection.

C [35S]Om45 was imported into WT and Tom40Δx4, mitochondria for 5 min followed by affinity purification via the HA-tag. [35S]Om45 was detected by SDS–PAGE and autoradiography (load 1%, elution 100%). Endogenous proteins were analysed by SDS–PAGE and immunodetection (load 4%, elution 100%).

D [35S]Om45 was imported into WT and Tom40Δx4 mitochondria for 5 min and subjected to oxidation with 2 mM Cu-phenanthroline (CuP) followed by affinity purification under denaturing conditions via the HA-tag. Oxidized (crosslinking) products were detected by non-reducing SDS–PAGE and autoradiography. Load 1%; elution 100%.

E Reticulocyte lysates containing [35S]Om45-DHFR (25% of input) or [35S]DHFR-Om45 (20% of input) (lanes 1 and 2, SDS–PAGE, arrowheads) were incubated with WT or mutant mitochondria in the presence of methotrexate. Where indicated mitoplasts were incubated with the indicated antisera for antibody-shift analysis. Samples 3–17 were analysed by blue native electrophoresis and autoradiography. [35S]Om45 was detected by SDS–PAGE and autoradiography. Load 1%; elution 100%.

F Increasing amounts of recombinant b1(167)Δ-DHFR were imported into WT mitochondria in the presence of methotrexate (for details see Supplementary Methods). Samples 1–8 were analysed by blue native electrophoresis and immunodetection. Import of the indicated radiolabelled precursor proteins was studied by SDS–PAGE or blue native electrophoresis and autoradiography. Non-imported [35S]Om45 and [35S]F1β were removed by treatment with proteinase K. AAC, ADP/ATP carrier.
impaired in tim23 and tim50 mutant mitochondria (Supplementary Fig S2E), indicating that the presequence translocase is involved in import of the large C-terminal domain of Om45 into the intermembrane space.

We conclude that the presequence translocase of the inner membrane is required for the import of Om45. In support of this conclusion, a dependence of Om45 import on the inner membrane potential is reported in a parallel study [33]. Om45 is not transported into the matrix since its biogenesis was not affected in mutant mitochondria of the mitochondrial heat shock protein 70 (Ssc1), which is strictly required for import of matrix-targeted preproteins (Supplementary Fig S3B) [1–3].

Mim1-dependent assembly of Om45

Mim1 has been shown to be involved in the import and assembly of several α-helical outer membrane proteins [8–12]. Mitochondria lacking Mim1 were moderately affected in import and membrane integration of [35S]Om45 (Fig 4A; Supplementary Fig S4A). Formation of the assembled Om45 complex was strongly impaired in mimiΔ mitochondria, both with imported [35S]Om45 and endogenous Om45 analysed by Western blotting (Fig 4A). To directly monitor assembly of Om45 with its outer membrane partner protein Om14, we studied the formation of a crosslinking product between [35S]Om45S19C and endogenous Om14. A 35S-labelled crosslinking product of...
approximately 58 kDa was quantitatively shifted in Om14 HA mitochondria (Fig 4B, left panel), demonstrating that it consists of Om45 and Om14. Formation of the Om45-Om14 crosslinking product was strongly inhibited in mim1Δ mitochondria (Fig 4B, right panel).

Immunoprecipitation with anti-Mim1 antibodies under denaturing conditions purified [35S]Om45S19C crosslinking products with Mim1 (Fig 4C). The efficiency of crosslinking of [35S]Om45S19C to Mim1 was considerably lower than that to Om14, consistent with the view that Mim1 interacts transiently with the Om45 precursor during its biogenesis and is not part of the mature Om45 complex (Supplementary Fig S1B).

The levels of the TOM complex and particularly Tom20 are moderately reduced in mim1Δ mitochondria [8,10]. To minimize indirect effects on the Om45 pathway, we generated mim1 mutant strains. mim1-22 mitochondria contained wild-type levels of TOM subunits, including Tom20 (Supplementary Fig S4B). As expected for a mim1 mutant, mim1-22 mitochondria were inhibited in assembly of the Mim1-substrate Ugo1, but not in import of the presequence-carrying preprotein Su9-DHFR (Supplementary Fig S4C). mim1-22 mitochondria transported [35S]Om45 to a protease-protected location, but were strongly impaired in formation of the Om45 complex (Fig 4D). These findings indicate that Mim1 acts after TOM and TIM23 in the biogenesis of Om45 since mutant mitochondria defective in TOM and TIM23 subunits are inhibited in the import of Om45 to a protease-protected location.

**Concluding remarks**

Our findings reveal a new pathway for protein import into the mitochondrial outer membrane that combines part of the presequence...
import pathway with assembly via the MIM machinery. We propose the following model. The precursor of Om45 is recognized by the receptors Tom20 and Tom22 and is translocated across the outer membrane with the help of Tom40/Tom5. Further import of the precursor involves the intermembrane space domain of Tom22 and the TIM23 complex. The consequence translocase promotes translocation of the large hydrophilic C-terminal domain of Om45 into the intermembrane space. Subsequent assembly of Om45 with Om14 and porin requires the MIM complex of the outer membrane. Thus, protein import machineries, which have been known to direct preproteins to different mitochondrial membranes, can be functionally connected into a dynamic and flexible network to form a new import route.

Materials and Methods

Yeast strains

The conditional mutants tom40-25, tim50-7 and ssc1-42 and the corresponding YPH 499 wild-type (WT) strains were generated by plasmid shuffling [11]; pFL39 plasmids encoding the mutant alleles were generated by error-prone PCR. To generate Om45HA and Om14HA strains, the coding regions of a deca-His-tag or triple HA-tag were inserted in front of the stop codons of

detailed information see Supplementary Methods).

8

tag were inserted in front of the stop codon of

50

Om45, non-imported precursors were removed by addition of

at -80°C [11]. After import of Om45, non-imported precursors were removed by addition of

°C [11]. In vitro transcription/translation and import reactions were performed according to Becker et al [11]. After import of Om45, non-imported precursors were removed by addition of 50 μg/ml proteinase K for 15 min on ice. Samples were analysed by SDS–PAGE or blue native electrophoresis and autoradiography (for detailed information see Supplementary Methods).

Isolation of mitochondria and in vitro protein import

Mitochondria were isolated by differential centrifugation and stored at -80°C [11]. In vitro transcription/translation and import reactions were performed according to Becker et al [11]. After import of Om45, non-imported precursors were removed by addition of 50 μg/ml proteinase K for 15 min on ice. Samples were analysed by SDS–PAGE or blue native electrophoresis and autoradiography (for detailed information see Supplementary Methods).

Supplementary information for this article is available online:
http://embor.embopress.org

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Author contributions

LSW, LO, MHS, LE, RI, LB, JQ, BG and TB performed experiments and analysed data together with MvdL, NW and NP; NP and TB designed the study and wrote the manuscript with advice from LSW, MvdL and NW.

Conflict of interest

The authors declare that they have no conflict of interest.

References


