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/

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Figure 1. Om45 is exposed to the intermembrane space and its biogenesis involves Tom20 and Tom22.

A WT and mutant mitochondria were analysed by blue native electrophoresis and immunodetection with the indicated antisera.
B, C [³⁵S]Om45 was imported into WT and mutant mitochondria. Samples were treated with proteinase K and analysed by blue native electrophoresis (B) or SDS-PAGE (C) and autoradiography.
D WT mitochondria or mitoplasts (+ swelling) were treated with proteinase K as indicated. Proteins were analysed by SDS-PAGE and immunodetection.
E WT and Om45_{His} mitochondria or mitoplasts were incubated with anti-His antisera as indicated. Om45 complexes were detected by blue native electrophoresis and immunodetection.

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

[35 S]Om45 to a protease-protected location was similarly impaired in *tom22 Δ* and *tom20 Δ* mitochondria, but not in *tom70 Δ* mitochondria (Fig 1C; Supplementary Fig S1A).

Different views on the topology of Om45 have been discussed. Whereas all studies agree on the anchoring of Om45 in the outer membrane via the N-terminal signal-anchor domain [13,17], it has been debated if the large C-terminal domain is exposed to the cytosol [20, 21] or the intermembrane space [14,18]. We observed that endogenous Om45 was fully protected against proteinase K added to intact mitochondria, but was accessible upon opening of the outer membrane by swelling (mitoplasting) (Fig 1D). For a protease-independent topology assay, we expressed Om45 with a C-terminal His-tag and performed antibody-shift blue native electrophoresis. Anti-His antibodies bound to the Om45_{His} complex in mitoplasts, but not in intact mitochondria (Fig 1E). Om45_{His} 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 *tom6 Δ* and *tom7 Δ* 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, *tom40-25* mitochondria were impaired in import of the matrix-targeted F₁-ATPase subunit β (F₁ β), but not in import of the Tom40-independent outer membrane protein Ugo1 (Supplementary Fig S2B) [11,12]. Import and assembly of [35 S]Om45 were inhibited in *tom40-25* mitochondria, and the amount of Om45 complexes was considerably decreased (Fig 2B). Upon short-term import into mitochondria, [35 S]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). [35 S]Om45_{S19C} was incubated with mitochondria for a short import period and oxidant was added. A [35 S]-labelled crosslinking product of approximately 90 kDa was co-purified with Tom40_{HA} 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], was sufficient to transport a portion of mature cytochrome *b*₂ (Om45_{32-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-Tom20_{CD} accumulated at the mitochondrial surface (Supplementary Fig S2C and D), providing an explanation for the reported partial complementation of *tom20 Δ* yeast by expression of Om45₃₂-Tom20_{CD} [17].

To block import channels, chemical amounts of the matrix-targeted b₂(167) Δ -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 ADP/ATP 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 F₁ β (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]. *tom22-2* mutant mitochondria, which lack the intermembrane space domain [28], were impaired in import of Om45 and F₁ β (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 *tim21 Δ* 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 Om45_{32-b2}, which contains only the signal-anchor domain of Om45, was not

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 *Tom40_{HA}* 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_{S19C} was imported into WT and *Tom40_{HA}* 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-DHFR accumulated in the TOM complex also in the absence of methotrexate, indicating that the DHFR moiety was not unfolded by the Om45 import pathway in isolated mitochondria.

F Increasing amounts of recombinant *b₂(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]F₁ β were removed by treatment with proteinase K. AAC, ADP/ATP carrier.

Figure 3. Biogenesis of Om45 involves the presequence pathway.

A–D [35S]Om45 and [35S]F₁β were imported into isolated WT and mutant mitochondria as described in the legend of Fig 1B and C. [35S]Ugo1 and [35S]Tom40 were imported into WT and mutant mitochondria and analysed by blue native electrophoresis and autoradiography. *tim23-76*, *tim50-7*, *tim17-4*, *tim17-5* and the corresponding WT mitochondria were subjected to a heat shock for 15 min at 37°C prior to the import reactions at 25°C. Quantification of [35S]Om45 import into *tom22-2* mitochondria (mean ± SEM, *n* = 4); import into WT mitochondria after 20 min was set to 100% (control). Tim23, immunodetection.

E [35S]Om45-DHFR was imported into WT and mutant mitochondria in the presence of methotrexate for 5 min at 25°C. Oxa1 was imported (5 min at 25°C) after dissipation of the membrane potential. Mitochondria were subjected to a heat shock for 15 min at 37°C prior to the import reaction. Imported proteins were detected by blue native electrophoresis and autoradiography.

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 *mim1Δ* 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]Om45_{S19C} and endogenous Om14. A ³⁵S-labelled crosslinking product of

Figure 4. Assembly of Om45 involves Mim1.

- A** [^{35}S]Om45 was imported into WT and mutant mitochondria as described in the legend of Fig 1B and C. Om45 complexes in WT and *mim1* Δ mitochondria were also analysed by blue native electrophoresis and immunodetection.
- B** [^{35}S]Om45_{S19C} was imported into WT, Om14_{HA} or *mim1* Δ mitochondria for the indicated periods on ice. Samples were oxidized with 2 mM CuP and analysed by non-reducing SDS-PAGE and autoradiography.
- C** Import and oxidation of [^{35}S]Om45_{S19C} were performed as described for Fig 2D. Subsequently, samples were subjected to immunoprecipitation with the indicated antisera under denaturing conditions. Load (1%) and elution (100%) were analysed by non-reducing SDS-PAGE and autoradiography.
- D** [^{35}S]Om45 import and Om45 complexes of WT and *mim1-22* mitochondria were analysed as in (A).

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 *mim1* Δ mitochondria (Fig 4B, right panel).

Immunoprecipitation with anti-Mim1 antibodies under denaturing conditions purified [^{35}S]Om45_{S19C} crosslinking products with Mim1 (Fig 4C). The efficiency of crosslinking of [^{35}S]Om45_{S19C} 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 *mim1* Δ 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 [^{35}S]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 presequence 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 Om45_{His} and Om14_{HA} strains, the coding regions of a deca-His-tag or triple HA-tag were inserted in front of the stop codon of *OM45* or *OM14* utilizing a *HIS3*-cassette [11]. For generation of the *mim1-22* strain, point mutations were introduced into pRS424 encoding *MIM1* by Quik-Change mutagenesis (Stratagene), followed by transformation into a *mim1Δ* strain, yielding the amino acid exchanges I48A, N49A and L50A of Mim1. See Supplementary Methods for *Saccharomyces cerevisiae* strains described previously.

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.

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