Mim1, a protein required for the assembly of the TOM complex of mitochondria

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The translocase of the outer mitochondrial membrane (TOM complex) is the general entry site for newly synthesized proteins into mitochondria. This complex is essential for the formation and maintenance of mitochondria. Here, we report on the role of the integral outer membrane protein, Mim1 (mitochondrial import), in the biogenesis of mitochondria. Depletion of Mim1 abrogates assembly of the TOM complex and results in accumulation of Tom40, the principal constituent of the TOM complex, as a low-molecular-mass species. Like all mitochondrial β -barrel proteins, the precursor of Tom40 is inserted into the outer membrane by the TOB complex. Mim1 is likely to be required for a step after this TOB-complex-mediated insertion. Mim1 is a constituent of neither the TOM complex nor the TOB complex; rather, it seems to be a subunit of another, as yet unidentified, complex. We conclude that Mim1 has a vital and specific function in the assembly of the TOM complex.

Keywords: mitochondria; TOM complex; TOB complex; assembly; Mim1

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INTRODUCTION

An early step in the import of precursor proteins into mitochondria is their recognition by the translocase of the outer mitochondrial membrane (TOM complex). The TOM complex is involved in the import of virtually all mitochondrial precursor proteins. The TOM holo complex is composed of at least seven different subunits. Some components have domains that are exposed to the cytosol and function as preprotein receptors. Tom20 and Tom70 are the primary receptors, whereas the subunits Tom40, Tom22, Tom7, Tom6 and Tom5 form the TOM core complex also called general insertion pore (GIP) (Dekker *et al*, 1998; Künkele *et al*, 1998a; Ahting *et al*, 1999). Tom40 forms the protein-conducting channel of the TOM complex (Hill *et al*, 1998; Künkele *et al*, 1998b; Stan *et al*, 2000; Ahting *et al*, 2001). Although all other Tom

components are anchored to the outer membrane by single α -helical transmembrane segments, predictions and secondary structure determinations suggest that Tom40 is made up of antiparallel β -strands, which form a β -barrel in the membrane (Mannella *et al*, 1996; Hill *et al*, 1998; Ahting *et al*, 2001).

The biogenesis of the TOM complex has been studied in some detail. The precursor of Tom40 is initially recognized by the receptor components and then translocated through the import pore of the TOM complex (Rapaport & Neupert, 1999; Krimmer *et al*, 2001; Model *et al*, 2001; Rapaport, 2002). From the TOM complex, the Tom40 precursor is transferred to the TOB/SAM complex (Kozjak *et al*, 2003; Paschen *et al*, 2003; Wiedemann *et al*, 2003). The principal component of this complex is Tob55 (also named Sam50/Omp85). Tob55 is essential for viability of yeast cells and promotes insertion of Tom40 (and other β -barrel proteins) into the membrane (Kozjak *et al*, 2003; Paschen *et al*, 2003; Gentle *et al*, 2004). Other components of the TOB complex are Tob38 (also called Sam35) and Mas37. Tob38, together with Tob55, forms a functional TOB core complex. Mas37 interacts with Tob55 and plays a so far undefined role in the biogenesis of β -barrel proteins (Kozjak *et al*, 2003; Paschen *et al*, 2003; Wiedemann *et al*, 2003; Johnson & Jensen, 2004; Waizenegger *et al*, 2004). Mdm10, a protein known to be involved in maintenance of mitochondrial morphology (Sogo & Yaffe, 1994), was recently suggested to be a component of the TOB complex and to have a role in the biogenesis of the TOM complex (Meisinger *et al*, 2004).

Here, we report on the identification and characterization of a new mitochondrial outer membrane protein, Mim1. Mim1 is crucial for the biogenesis of the TOM complex and is required for a step after the Tom40 precursor interacted with the TOB complex.

RESULTS

We used a proteomic approach to characterize new proteins in the mitochondrial outer membrane from *Neurospora crassa*. A protein of a molecular mass of 14.2 kDa (NCU01101.1) that had a high sequence similarity to Mim1 (mitochondrial import) from *Saccharomyces cerevisiae* (open reading frame YOL026C) was identified. Mim1 was recently found in a high-throughput screen and was reported to be an essential protein with an undefined role in the biogenesis of mitochondria. Accumulation of the precursor of ATPase F1 β was observed in whole-cell extracts following

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depletion of Mim1 (Mnaimneh *et al*, 2004). Mim1 is highly conserved among fungi. This sequence conservation is restricted to the predicted transmembrane region (Fig 1A).

For further analysis, we investigated Mim1 in *S. cerevisiae*. We used an antibody raised against a fusion protein consisting of Mim1 and maltose-binding protein (MBP-Mim1) to study the subcellular location of Mim1. We found the latter to be located in the mitochondrial fraction (Fig 1B). The membrane topology of Mim1 was studied by protease treatment of mitochondria. When polyclonal antibody against recombinant Mim1 was used, immunoreactivity was lost when using low proteinase K (PK) concentrations. Cytochrome *b2*, which resides in the intermembrane space (IMS), was not affected by this treatment, demonstrating the intactness of the outer membrane (Fig 1C). Thus, Mim1 is an outer-membrane protein. The association of Mim1 with the outer membrane was analysed by alkaline treatment of mitochondria. Mim1 was recovered in the membrane fraction like known outer membrane proteins, Tom40 and Tom70 (Fig 1C).

To obtain more information on the topology of the protein in the outer membrane, the chromosomal copy of the *MIM1* gene

was replaced by a version encoding Mim1 with a His tag at the amino terminus (_{His}Mim1). This strain grew like the wild-type (WT) strain (Fig 1D). Mitochondria containing the His-tagged Mim1 were treated with PK. Mim1 was degraded and could no longer be immunodecorated with antibodies against the His tag (Fig 1E). Hence, the N terminus of the protein is exposed to the cytosol. We could not determine the topology of the carboxy-terminal domain of the protein, as a C-terminally tagged Mim1 had compromised function (data not shown).

To study the function of Mim1, we constructed a yeast strain in which the gene was under the control of the *GAL10* promoter. In the presence of galactose, the cells grew like WT cells. In contrast, in the presence of glucose, growth was strongly reduced after 24 h, yet did not stop completely (Fig 2A). A similar growth phenotype was reported previously (Mnaimneh *et al*, 2004). Total cell extracts were obtained from *GAL-MIM1* cells at various time periods after the shift from galactose-containing medium to glucose-containing medium. The levels of various mitochondrial proteins were analysed by immunodecoration (Fig 2B). As expected, Mim1 was not detectable after 24 h of growth on

Fig 1 | Mim1 is a mitochondrial protein integrated into the outer membrane. (A) Mim1 is conserved among fungi. Amino-acid sequences of *Saccharomyces cerevisiae* (S.c.), *Schizosaccharomyces pombe* (S.p.), *Neurospora crassa* (N.c.) and *Candida albicans* (C.a.) Mim1 are shown. Identical residues appear in white on a black background, and similar residues are in grey. The putative transmembrane segment of the *S. cerevisiae* protein is underlined. (B) Mitochondrial (M) and postmitochondrial fractions (P) were obtained from yeast cells and were subjected to SDS-PAGE and immunoblotting using antibodies against Mim1, hexokinase, a marker protein for the cytosol, and the mitochondrial protein Tom20. (C) Mitochondria were treated with proteinase K (PK) at the indicated concentrations for 15 min on ice. Samples were analysed by SDS-PAGE and immunoblotting with antibodies against Mim1, the outer membrane proteins Tom70 and Tom40 and the IMS protein Cytb2. Other samples were subjected to alkaline extraction. Untreated mitochondria (total, T), pellet (P) and a supernatant fraction (S) were analysed as above. (D) A strain containing an N-terminally His-tagged variant of Mim1 grows like WT. Cells containing His-tagged Mim1 and isogenic WT cells were tested for their ability to grow at 30 °C on YPGal medium (dilution in tenfold increments). (E) Mitochondria containing N-terminally His-tagged Mim1 were treated with PK at the indicated concentrations for 15 min on ice. Samples were analysed by SDS-PAGE and immunoblotting with antibodies against the His tag, Tom70 and ADP/ATP carrier (AAC).

Fig 2 | Depletion of Mim1 results in reduced levels of mitochondrial proteins. (A) Downregulation of Mim1 affects cell growth. WT cells and cells expressing Mim1 under control of the *GAL10* promoter (*GAL-MIM1*) were shifted from galactose-containing medium to glucose-containing medium at time zero. (B) Depletion of Mim1 causes accumulation of mitochondrial precursor protein. Whole-cell lysates were prepared from *GAL-MIM1* cells, which were grown first at 30 °C on lactate medium containing 0.1% galactose, washed, diluted and then grown at 30 °C on lactate medium containing 0.1% glucose for the indicated time periods. Cell lysates were analysed by SDS-PAGE and immunodecoration with the indicated antibodies. P and m represent the precursor and mature forms of Hsp60, respectively.

glucose-containing medium. A severe reduction was observed for the Tom components Tom40 and Tom20. The other receptor of the TOM complex, Tom70, and the ADP/ATP carrier protein were practically unaffected. The decrease in the amounts of Tom20 and Tom40 was accompanied by accumulation of the precursor form of the matrix-destined protein Hsp60 (Fig 2B). Hence, depletion of Mim1 affects the biogenesis of the mitochondria.

To study the molecular function of Mim1, we analysed the effect of depletion of Mim1 on various mitochondrial processes. Mitochondria were isolated from *GAL-MIM1* as well as isogenic WT cells grown for 15 h in the presence of glucose. The levels of various mitochondrial proteins were determined by immunodecoration (Fig 3A). Tom20 levels were significantly reduced, whereas other components of the TOM complex such as Tom40, Tom22 and Tom5 were present at moderately reduced levels in Mim1-depleted mitochondria (Mim1 ↓) as compared with WT mitochondria. In contrast, other mitochondrial proteins, such as the outer membrane proteins Tom70, Mdm10, Porin, Tob55

Fig 3 | Mim1 is necessary for TOM complex assembly and is present in a high-molecular-mass complex. (A) Levels of various proteins in mitochondria from cells depleted of Mim1 for 15 h (Mim1 ↓), as determined by immunodecoration. (B) TOM complex in Mim1-depleted cells. Mitochondria as in (A) were lysed with 0.5% Triton X-100 (Tob55) or 1% digitonin and subjected to BNGE and immunoblotting with the indicated antibodies. Short and long exposures of the immunodecoration with antibodies against Tom40 are presented. The Tom40-containing low-molecular-mass species is indicated with an arrowhead. (C) Mitochondria from WT cells were lysed in 1.5% digitonin and subjected to size-exclusion chromatography on a Superose6 column. Fractions of 300 μl were analysed by immunoblotting for Tom22, Mim1 and Tob38. The elution peaks of marker proteins are indicated with arrows.

and OM45, the IMS component Tim13, the inner membrane protein Tim17 and components residing in the matrix (Hsp60 and aconitase), were present at roughly WT levels (Fig 3A).

The reduction of the levels of some Tom proteins raised the possibility that the biogenesis of the TOM complex was affected by depletion of Mim1, and this in turn resulted in secondary effects such as reduced protein import. To check this, the TOM complex was analysed by blue native gel electrophoresis (BNGE) and immunodecoration with antibodies against Tom40, Tom22 and Tom5. The TOM complex from Mim1 ↓ and WT cells was

indistinguishable with regard to the apparent molecular mass (Fig 3B). However, the level of assembled TOM complex was drastically reduced in the *Mim1*↓ mitochondria. After 15 h of depletion, only about 10–30% of WT level was left. In contrast, other complexes in the outer membrane like the TOB complex and complexes formed by porin were not affected (Fig 3B). In the *Mim1*↓ mitochondria, an additional Tom40-containing band of about 100 kDa apparent molecular mass was present (Figs 3B and 4A–C). This species is similar in its migration behaviour to Tom40-containing species observed in $\Delta tom6$ and $\Delta tom22$ strains, where assembly defects of the TOM complex are observed (Dekker *et al*, 1998; van Wilpe *et al*, 1999). We conclude that *Mim1* has a role in the biogenesis of the TOM complex but not of outer membrane proteins or β -barrel proteins, in general, or of other mitochondrial proteins.

To investigate whether *Mim1* is a component of the TOM or TOB complexes, we analysed by size-exclusion chromatography mitochondria lysed with the mild detergent digitonin (Fig 3C). *Mim1* was present in a high-molecular-mass complex of a molecular size of about 400–450 kDa, which is distinct from that of the TOM and TOB complexes. Therefore, *Mim1* is not a Tom or Tob component.

What is the cause for the massive deficiency in assembled TOM complex in the absence of *Mim1*? Possible explanations could be a defective assembly process or decreased stability. To discriminate between these possibilities, we checked the stability of the TOM complex in *Mim1*↓ mitochondria. Although the overall amount of the TOM complex was significantly reduced in *Mim1*↓ mitochondria, no difference in the stability of the complex was seen on exposure to either 0.4% β -dodecyl maltoside or 4 M urea or on heating to 40°C. These treatments are known to destabilize the TOM complex partially (Fig 4A–C). Taken together, *Mim1* has an important role in the assembly of the TOM complex, but, once the complex is assembled, it remains stable even if *Mim1* is missing.

Next, we studied at which stage the assembly process of the TOM complex is blocked in the *Mim1*-depleted mitochondria. To avoid secondary effects due to reduced levels of Tom components, we used mitochondria depleted of *Mim1* for only 8 h. The amounts of Tom20 and Tom40 in these mitochondria are only slightly reduced as compared with WT organelles, whereas *Mim1* was below the detection level of our antibody (Fig 5A). Intermediates in the assembly of the complex were identified following *in vitro* import of Tom40. In particular, two characteristic intermediates in the assembly of Tom40 were observed (Fig 5B). Intermediate I with an apparent molecular mass of 250 kDa represents a complex of Tom40 with the TOB/SAM complex, which is thought to mediate the insertion of Tom40 from the IMS side into the outer membrane. Intermediate II (100 kDa) represents a later stage, namely when Tom40 is released from TOB/SAM into the lipid phase of the membrane. On import into isolated mitochondria, Tom40 precursor is largely halted at these steps and only a fraction of it is assembled into the TOM core complex, apparently because the reactions involved are not efficient under the *in vitro* conditions (Model *et al*, 2001; Paschen *et al*, 2003; Wiedemann *et al*, 2003). In mitochondria depleted of *Mim1*, intermediate I was formed to an extent similar to WT mitochondria, whereas intermediate II was less prominent than in WT and assembled TOM complex was almost absent (Fig 5B,C).

Fig 4 | *Mim1* is not required for the stability of the TOM complex. (A) Mitochondria isolated from either WT cells or cells depleted of *Mim1* for 15 h (*Mim1*↓) were lysed with 1% digitonin in the presence of the indicated concentrations of *n*-dodecyl β -D-maltoside (DDM). The lysates were analysed by BNGE, blotted and immunodecorated with antibodies against Tom40. (B) Mitochondria as in (A) were lysed with 1% digitonin in the presence of the indicated concentrations of urea. (C) Mitochondria as in (A) were lysed with 1% digitonin and incubated for 10 min at the indicated temperatures.

Fig 5 | Mim1 acts in a step after binding of the Tom40 precursor to the TOB complex. (A) Mitochondria isolated from WT and cells depleted of Mim1 for 8 h (Mim1 Δ) were analysed by SDS-PAGE and immunodecoration with the indicated antibodies. (B) Mitochondria as in (A) were incubated at 25 °C with radiolabelled Tom40 precursor for various time periods. Mitochondria were lysed with 1% digitonin and analysed by BNGE followed by autoradiography. P, precursor protein; I and II, assembly intermediates I and II of Tom40 and the assembled TOM complex (TOM) are shown. (C) Quantification of the bands corresponding to the various intermediates presented in (B).

We propose that Mim1 acts at a stage behind the TOB complex. Two possibilities can be assumed to explain why there is no massive accumulation of the Tom40 precursor at the TOB complex (intermediate I). First, the number of precursor binding sites at the TOB complex might be saturated under our experimental conditions. Second, although Mim1 is not an integral member of the TOB complex, in the absence of Mim1, the affinity of the TOB complex for Tom40 precursor is somewhat reduced.

DISCUSSION

We have identified Mim1 as a component with a vital role in the biogenesis of the TOM complex. Our data show that a primary function of Mim1 is to facilitate assembly of the TOM complex. The biogenesis of other outer membrane complexes containing β -barrel proteins, such as TOB/SAM and porin complexes, was not affected by the lack of Mim1. The steps in the assembly of the TOM complex requiring Mim1 are located after the TOB-complex-dependent insertion of Tom40 into the outer membrane. It remains to be determined whether the release of Tom40 from the TOB complex or its oligomerization is controlled by Mim1 or another step in the pathway, such as assembly of the small Tom proteins or Tom22. Previous experiments have demonstrated a tendency of a Tom40 subcomplex obtained by stripping of the other subunits from the isolated TOM complex to aggregate (Ahting *et al*, 2001).

Mim1 is not a constituent of the TOM complex but rather seems to act catalytically. How Mim1, as a monotopic integral membrane protein, is doing this is presently unclear. A remarkable feature of Mim1 is the rather unusual pattern of sequence conservation. Among various related fungi, the conservation in

the hydrophilic sequences flanking the transmembrane segment is surprisingly low. At the same time, there is a strong sequence conservation of the transmembrane region. This is an unusual pattern, as in most membrane proteins, transmembrane regions are conserved to a low degree. This could suggest a specific and vital function of the transmembrane segment in the function of the protein. Mim1 could, for instance, shield membrane integral segments of incompletely assembled Tom40 subunits and thereby prevent aggregation and ensuing proteolytic degradation. Another function of Mim1 might be to help unassembled or partly assembled Tom40 to maintain competence for assembly with the small Tom proteins and with Tom22.

A further detailed dissection of the assembly pathway of the TOM complex will be necessary to clarify the precise function of Mim1. In particular, early steps in the release of Tom40 from the TOB complex have to be characterized, and the formation of Tom40 oligomers as well as their interactions with the other components of the TOM complex have to be unravelled.

During submission of the first version of this study, characterization of the protein product of *YOL026C* was reported and it was named Tom13 (Ishikawa *et al*, 2004). We prefer to remain, however, with the original name, Mim1, as there is no indication that this protein is a subunit of the TOM complex.

METHODS

Yeast strains and cell growth. Standard genetic techniques were used for growth and manipulation of yeast strains (Sherman *et al*, 1986). The WT strain YPH499 was used. The *GAL10-MIM1* strain was constructed by replacing 105 bp upstream of the *MIM1* reading frame with a *GAL10*-promoter-containing cassette in YPH499. For depletion of Mim1, *GAL-MIM1* cultures were shifted

from lactate medium containing 0.1% galactose to lactate medium containing 0.1% glucose. PCR-mediated gene manipulation was used to replace the chromosomal copy of *YOL026C* by a gene expressing a 3 × haemagglutinin tag after the coding sequence of Mim1 (Lafontaine & Tollervey, 1996), or to introduce a His tag at the N-terminus of the protein.

Biochemical procedures. Isolation of mitochondria, subcellular fractionations and *in vitro* import experiments were performed as described (Daum *et al*, 1982; Waizenegger *et al*, 2003). Blotting to polyvinylidene difluoride or nitrocellulose membranes and immunodecoration were according to standard procedures, and visualization was by the ECL method (Amersham Biosciences, Piscataway, NJ, USA). Radiolabelled precursor proteins were synthesized in rabbit reticulocyte lysate in the presence of [³⁵S]methionine (Amersham) after *in vitro* transcription by SP6 polymerase from pGEM4 vectors containing the gene of interest. BNGE was carried out as reported previously (Schägger *et al*, 1994; Rapaport & Neupert, 1999).

Antibodies against Mim1. The DNA sequence encoding Mim1 was cloned into the pMAL vector (New England Biolabs, Beverly, MA, USA) for expression of the recombinant MBP–Mim1 protein in *Escherichia coli* MH1 cells. The protein was purified by using an amylose column. Antibodies against Mim1 were raised in rabbits.

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