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Biogenesis of the Mitochondrial TOM Complex

*Mim1 PROMOTES INSERTION AND ASSEMBLY OF SIGNAL-ANCHORED RECEPTORS**

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The translocase of the outer membrane (TOM complex) is the central entry gate for nuclear-encoded mitochondrial precursor proteins. All Tom proteins are also encoded by nuclear genes and synthesized as precursors in the cytosol. The channel-forming β-barrel protein Tom40 is targeted to mitochondria via Tom receptors and inserted into the outer membrane by the sorting and assembly machinery (SAM complex). A further outer membrane protein, Mim1, plays a less defined role in assembly of Tom40 into the TOM complex. The three receptors Tom20, Tom22, and Tom70 are anchored in the outer membrane by a single transmembrane α-helix, located at the N terminus in the case of Tom20 and Tom70 (signal-anchored) or in the C-terminal portion in the case of Tom22 (tail-anchored). Insertion of the precursor of Tom22 into the outer membrane requires pre-existing Tom receptors while the import pathway of the precursors of Tom20 and Tom70 is only poorly understood. We report that Mim1 is required for efficient membrane insertion and assembly of Tom20 and Tom70, but not Tom22. We show that Mim1 associates with SAM_{core} components to a large SAM complex, explaining its role in late steps of the assembly pathway of Tom40. We conclude that Mim1 is not only required for biogenesis of the β-barrel protein Tom40 but also for membrane insertion and assembly of signal-anchored Tom receptors. Thus, Mim1 plays an important role in the efficient assembly of the mitochondrial TOM complex.

The essential biochemical function of mitochondria depends on the uptake of cytosolic-synthesized precursor proteins. The vast majority of precursor proteins are imported by the preprotein translocase of the outer mitochondrial membrane (TOM[4] complex). Subsequently the precursor proteins are sorted to the different mitochondrial subcompartments, the outer and inner membranes, the intermembrane space and the matrix (1–7). The TOM complex is composed of seven different subunits. Tom40 forms the protein-conducting channel across the outer membrane (8–10). The three receptors Tom20, Tom22, and Tom70 expose domains on the cytosolic side of the outer membrane, recognize the precursor proteins and direct them to the Tom40 channel (11). In addition, three small Tom proteins, Tom5, Tom6, and Tom7, are associated with the Tom40 core of the complex (12–14). Tom40 forms a transmembrane β-barrel, while all other Tom components are embedded in the membrane via a single transmembrane α-helix (9–10, 15). The α-helical membrane anchor is localized in the C-terminal portion of Tom22 and the small Tom proteins and thus those proteins belong to the tail-anchored proteins (15–19). Tom20 and Tom70 are integrated into the outer membrane by an N-terminal α-helix, which together with flanking regions is important for both intracellular targeting and membrane anchoring, and thus these proteins are called signal-anchored proteins (20–23).

All Tom proteins are synthesized as precursor proteins on cytosolic ribosomes and imported into mitochondria. The import pathway of the precursor of the channel-forming protein Tom40 has been studied in detail. The β-barrel precursor is recognized by TOM receptors and translocated across the outer membrane by a pre-existing Tom40 channel (24–26). On the intermembrane space side, chaperone complexes formed by small Tim proteins transfer the precursor of Tom40 to the sorting and assembly machinery (SAM complex) that promotes insertion of the precursor into the outer membrane (25, 27–28). The core of the SAM complex is composed of the central component Sam50 (Omp85/Tob55) and its partner proteins Sam35 (Tom38/Tob38) and Sam37 (25, 29–34). An additional protein complex, containing Mdm12 and Mmm1, is required after the SAM complex to promote late steps of β-barrel assembly (35). The described pathway via TOM, small Tim proteins, SAM and Mdm12/Mmm1 forms the general β-barrel assembly pathway of mitochondria that is used by all β-barrel precursors analyzed, including the most abundant outer membrane protein, porin (VDAC). Two further outer membrane proteins, Mdm10 and Mim1, were reported to be selectively required for the bio-

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[4] The abbreviations used are: TOM, translocase of outer membrane; Mim1, mitochondrial import protein; SAM, sorting and assembly machinery; TIM, translocase of inner membrane; WT, wild type; HA, hemagglutinin; MOPS, 4-morpholinepropanesulfonic acid.

genesis of Tom40 at a post-SAM stage. Mdm10 associates with the SAM_{core} complex as well as the Mdm12/Mmm1 complex and promotes the assembly of Tom40 with the precursor of Tom22 toward formation of the mature TOM complex (36–38). The 13-kDa protein Mim1 was initially identified in a high-throughput analysis of yeast mutants (39). Subsequent analysis revealed that Mim1 was located in the mitochondrial outer membrane and involved in the assembly pathway of Tom40 but not porin (32, 40). Different views were reported on the localization of Mim1 in complexes and the Tom40 assembly stage that required Mim1. Mim1 was also termed Tom13 (32). As Mim1 is not a subunit of the TOM complex, the standard name according to the *Saccharomyces* genome data base (SGD) is Mim1 (41).

The biogenesis of Tom receptors is only understood in part. While several components required for the import of tail-anchored Tom22 have been defined, little is known about the import of signal-anchored Tom20 and Tom70. Targeting of the precursor of Tom22 requires the receptors Tom20 and Tom70 (42, 43). Subsequently Mdm10 promotes the assembly of Tom22 with Tom40 to form the mature TOM complex (36). In contrast, the import of the precursors of Tom20 and Tom70 does not require Tom receptors (23, 44–46). Only an involvement of Tom40 in the biogenesis pathway has been shown, however, the pore formed by Tom40 is not needed for the import of Tom20 and Tom70 (23, 44). Moreover, neither the SAM_{core} complex nor Mdm10 are required for the biogenesis of signal-anchored receptors (30, 33, 34, 36).

For this report, we analyzed the function of Mim1 and surprisingly found that Mim1 is required for the assembly pathway of the signal-anchored receptors. Mim1 promotes insertion of the precursors of Tom20 and Tom70 into the outer membrane while tail-anchored Tom22 does not require Mim1. Mim1 associates with a fraction of SAM_{core} complexes to a larger SAM complex, explaining its function in the assembly pathway of both Tom40 and signal-anchored receptors.

EXPERIMENTAL PROCEDURES

Yeast Strains and Isolation of Mitochondria—The *mim1*Δ strain was generated by a plasmid loss approach (47, 48). The open reading frame of *MIM1* was inserted between the *MET25* promotor and the *CYC1* terminator of the *URA3* marker-containing plasmid Yep352 and transformed into the *Saccharomyces cerevisiae* strain YPH499 (49). Afterward the chromosomal copy of the *MIM1* open reading frame was disrupted by homologous recombination with *ADE2*. The *mim1*Δ strain was isolated by growth on 5-fluoroorotic acid, ensuring loss of the *MIM1* containing *URA3* plasmid, and maintained on non-fermentable glycerol medium. The strain ProtA-Mim1 (background BY4741, N-terminal protein A tag fused to Mim1) was generated by homologous recombination utilizing a PCR product from an affinity tag replacement cassette (29). Yeast strains expressing $Sam35_{HA}$ or $Mdm10_{His}$ were described (33, 36). Yeast cells lacking Mim1 were grown at 20 °C in YPG medium (1% (w/v) yeast extract, 2% (w/v) bactopeptone and 3% (w/v) glycerol). Yeast cells expressing protein A-tagged Mim1 were grown at 24 °C in YPS medium (1% (w/v) yeast extract, 2% (w/v) bactopeptone, and 2% (w/v) sucrose). The isolation of mitochondria was performed by differential centrifugation (50). Aliquots of mitochondria, adjusted to a protein concentration of 10 mg/ml in SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS/KOH, pH 7.2) were stored in aliquots at −80 °C.

In Vitro Protein Import—PCR-based constructs for *in vitro* translation were transcribed using SP6 polymerase (Ambion). Radiolabeled proteins were synthesized in rabbit reticulocyte lysate in the presence of [^{35}S]methionine (GE Healthcare) (51). Import into isolated mitochondria was started by addition of the translation product (5–10% (v/v) reticulocyte lysate in the total import reaction) and performed in BSA buffer (3% (w/v) fatty acid free BSA, 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 10 mM MOPS/KOH, pH 7.2, 5 mM methionine, 2 mM KH_2PO4) in the presence of 2 mM ATP, 2 mM NADH, 100 μg/ml creatine kinase, and 5 mM creatine phosphate. After the indicated time points, the import of precursor proteins was stopped by transfer on ice. The import of precursor proteins destined for the mitochondrial inner membrane and matrix was stopped by addition of 1 μM valinomycin to dissipate the membrane potential. In control reactions, 1 μM valinomycin was added before the import reaction. Mitochondria were re-isolated and washed with SEM buffer. Samples to be treated for alkaline extraction were incubated with freshly prepared 0.1 M Na_2CO_3 (pH 11.5) for 30 min on ice (51). Total membranes were isolated by ultracentrifugation, subsequently solubilized in Laemmli buffer and subjected to SDS-PAGE.

Blue Native Electrophoresis—Mitochondrial pellets were resuspended in ice-cold digitonin-containing buffer (0.5–1.5% (w/v) digitonin, 20 mM Tris/HCl, pH 7.4, 0.1 mM EDTA, 50 mM NaCl, 10% (w/v) glycerol) to a final protein concentration of 1 mg/ml and incubated for 15 min on ice (13, 51–53). Insoluble material was removed by centrifugation (20,000 × *g*, 15 min, 4 °C) and 1 volume of sample buffer (5% (w/v) Coomassie Brilliant Blue G-250, 100 mM Bis-Tris/HCl, pH 7.0, 500 mM ϵ-amino-n-caproic acid) was added to nine volumes of supernatant. Samples were separated on a 4–16% polyacrylamide gradient gel at 4 °C. The mobility of molecular weight markers was determined on parallel lanes under identical conditions. The radiolabeled proteins were detected by digital autoradiography. Antibody shift blue native electrophoresis was performed as described (51).

Purification of Protein Complexes—Mitochondria isolated from yeast cells expressing Mim1 fused to an N-terminal protein A tag were solubilized in digitonin buffer, including 1% digitonin and 250 mM NaCl, to a final protein concentration of 1 mg/ml for 15 min on ice. After a clarifying spin (20,000 × *g*, 10 min, 4 °C), the supernatant was incubated with pre-equilibrated IgG-Sepharose for 2 h at 4 °C. Subsequently, the column material was washed with an excess volume of digitonin buffer, including 0.1% digitonin and 250 mM NaCl. Bound proteins were eluted by incubation with TEV-protease for 12 h at 4 °C. For purification of protein complexes via Mdm10-His tag, mitochondria were solubilized in digitonin buffer, including 1% digitonin and 250 mM NaCl, at a final protein concentration of 1 mg/ml for 15 min on ice. After a clarifying spin (20,000 × *g*, 10 min, 4 °C), the supernatant was incubated with pre-equilibrated Ni-NTA-agarose (Qiagen) for 2 h at 4 °C. Subsequently, the column material was washed in two steps with an excess

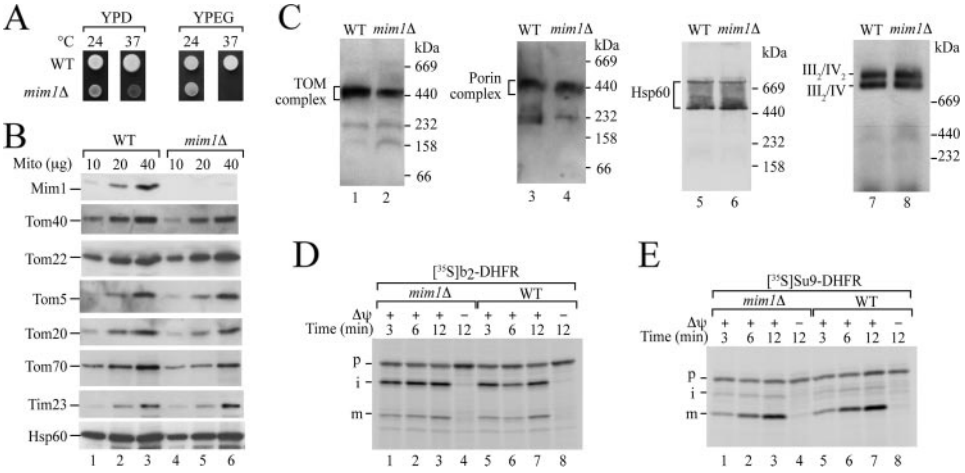

FIGURE 1. **Mim1-deficient yeast cells are respiratory-competent but impaired in levels of the TOM complex.** *A*, growth of *S. cerevisiae* cells on fermentable medium (YPD, yeast extract, bactopeptone, glucose), and non-fermentable medium (YPEG, yeast extract, bactopeptone, ethanol, glycerol). *B*, mitochondria (μg of protein) isolated from WT and yeast cells lacking Mim1 (*mim1*Δ) grown at 20 °C were subjected to SDS-PAGE and Western blot analysis. *C*, mitochondria (50 μg of protein) isolated from WT and *mim1*Δ yeast cells grown at 20 °C were subjected to blue native electrophoresis and Western blot analysis. *Lanes 1* and *2*, antibodies against Tom40; *lanes 7* and *8*, antibodies directed against Cox4 were used to decorate respiratory chain supercomplexes. *D* and *E*, WT and *mim1*Δ mitochondria were incubated with the ^{35}S-labeled precursors of b_2-DHFR and Su9-DHFR for the indicated times at 25 °C, subjected to SDS-PAGE and analyzed by digital autoradiography. Δψ, membrane potential. Precursor (*p*), intermediate (*i*), and mature (*m*) forms are marked.

volume of digitonin buffer, including 0.1% digitonin, 250 mM NaCl, and 20 mM imidazole in the first step and 40 mM imidazole in the second washing step. Bound proteins were eluted by incubation by digitonin buffer containing 250 mM imidazole.

Miscellaneous—Western transfers were performed onto polyvinylidene difluoride membranes and immunodecoration was performed according to standard techniques. Enhanced chemiluminescence was used for detection (GE Healthcare). Antibodies against Mim1 were raised against a peptide covering the C-terminal 18 residues of Mim1.

RESULTS AND DISCUSSION

Yeast Cells Lacking Mim1 Have Reduced Levels of TOM Complex but Retain the Respiratory Chain—We developed a mild procedure to delete the *MIM1* gene in yeast. The reason is that so far the function of Mim1 in assembly of mitochondrial proteins has been studied by the use of galactose-inducible promoters to deplete the levels of Mim1 after shift of the cells to galactose-free medium (32, 40). As the gene for Mim1 is still retained under these conditions, it is possible that residual Mim1 is left in the analysis. Ishikawa *et al.* (32) obtained yeast cells lacking *MIM1* by a sporulation approach, however, the cells became respiratory-deficient. Since a loss of mtDNA can frequently happen as a secondary effect during handling of yeast mutants of mitochondrial proteins (54, 55) and in consequence leads to many pleiotropic defects of mitochondria, we used mild conditions to delete the chromosomal copy of *MIM1* while the wild-type protein was still expressed from a plasmid. Subsequently the plasmid was removed and we obtained *mim1*Δ cells that were respiratory-competent. The mutant cells stopped growth at elevated temperature (37 °C) on non-fermentable medium (Fig. 1*A*).

To minimize potential indirect effects in the *mim1*Δ strain, the cells were grown at low temperature (20 °C) and mitochondria were isolated. Previous studies with deletion mutants of components of the β-barrel assembly pathway (Sam37, Mdm10, Mdm12, and Mmm1) showed that growth of the mutant cells at low temperature only moderately affected the steady-state levels of critical import components and thus prevented indirect inhibitory effects on the different mitochondrial sorting pathways, but allowed a specific functional analysis by monitoring the kinetics of assembly with radiolabeled precursor proteins (25, 35, 36). The steady-state protein levels were determined by immunodecoration. The levels of Tom40, Tom20, and Tom70 were moderately reduced in *mim1*Δ mitochondria while the levels of Tom22, Tom5, and the controls proteins Tim23 of the translocase of the inner membrane and the matrix heat shock protein Hsp60 were similar to that of wild-type mitochondria (Fig. 1*B*).

We used blue native electrophoresis to analyze protein complexes upon lysis of mitochondria with digitonin. The mature TOM complex forms a complex of ~450 kDa that was present in reduced amount in *mim1*Δ mitochondria whereas porin (56) and Hsp60 complexes were present like in wild-type mitochondria (Fig. 1*C, lanes 1–6*). The respiratory chain of *S. cerevisiae* mitochondria contains two proton-pumping complexes, the bc_1-complex (complex III) and cytochrome *c* oxidase (complex IV), that assemble into supercomplexes (57–59). As several subunits of these complexes are encoded by mtDNA, a lack of mtDNA would lead to a loss of the supercomplexes. *mim1*Δ mitochondria contained the supercomplexes in the same amount as wild-type mitochondria (Fig. 1*C, lanes 7* and *8*) in line with the respiratory competence of the cells.

To determine if the lack of Mim1 and the reduced level of TOM complex affected the general protein import pathway to internal mitochondrial compartments, we studied the import of two model preproteins (51, 60). The radiolabeled precursors of the intermembrane space-targeted b_2-DHFR and the matrix-targeted Su9-DHFR were incubated with isolated mitochondria. In the presence of a membrane potential Δψ across the inner membrane the preproteins were processed to the mature forms with the same efficiency in *mim1*Δ and wild-type mitochondria (Fig. 1, *D* and *E*). Upon dissipation of the membrane potential by ionophores, the import of the preproteins was blocked. We conclude that *mim1*Δ mitochondria are competent in generation of a membrane potential and import of preproteins to internal mitochondrial compartments. Thus, the remaining levels of TOM complex upon growth of *mim1*Δ cells at low temperature support an efficient import of precursor proteins.

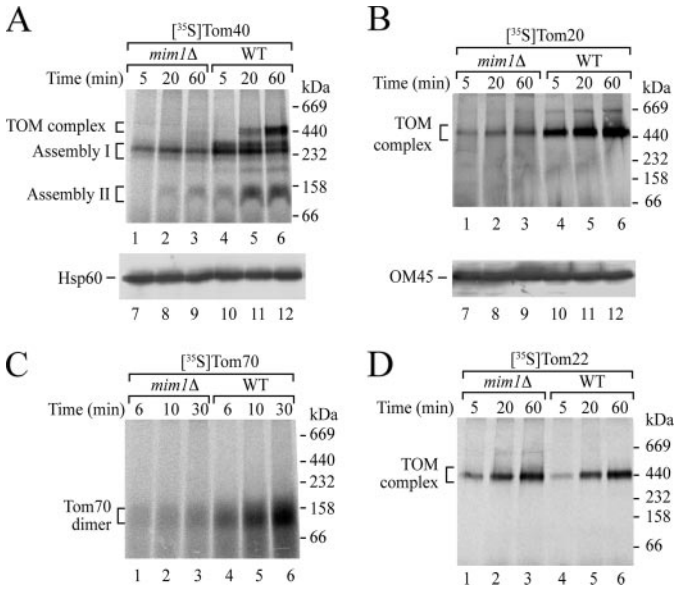

FIGURE 2. **Mitochondria lacking Mim1 are impaired in assembly of Tom40, Tom20, and Tom70, but not Tom22, into the TOM complex.** WT and *mim1*Δ mitochondria were incubated with the ^{35}S-labeled precursors of Tom40 (*A*), Tom20 (*B*), Tom70 (*C*), or Tom22 (*D*) for the indicated time periods at 25 °C. Mitochondria were re-isolated, lysed in a digitonin-containing buffer and subjected to blue native electrophoresis and digital autoradiography. Loading control, the levels of Hsp60 (*A*) and OM45 (*B*) were determined by Western blot analysis.

Mim1 Is Required for Assembly of Tom40, Tom20, and Tom70, but Not Tom22—To study a role of Mim1 in the assembly of the TOM complex, we followed the assembly pathway of radiolabeled Tom precursors in isolated mitochondria by blue native electrophoresis. In wild-type mitochondria, the precursor of Tom40 is assembled into the mature 450-kDa TOM complex via two intermediate stages (Fig. 2*A, lanes 4–6*). The assembly intermediate I of ~250 kDa represents the interaction of the precursor with the SAM_{core} complex while in assembly intermediate II, Tom40 associates with Tom5 (24, 25, 30–33, 35, 36). By using Mim1-depleted mitochondria isolated from yeast strains with different galactose-inducible promoters, different views on the stage dependence of Tom40 assembly on Mim1 were reported. Waizenegger *et al.* (40) showed that the mutant mitochondria were impaired in formation of assembly intermediate II whereas Ishikawa *et al.* (32) reported that the subsequent step, formation of the mature TOM complex, was affected. The differences were likely caused by different residual amounts of Mim1 left in the mitochondria that influenced the kinetics of Tom40 assembly. We thus used *mim1*Δ mitochondria, which are completely devoid of Mim1, and imported the precursor of Tom40. Assembly intermediate I was still formed in the mutant mitochondria whereas the formation of assembly intermediate II was strongly inhibited (Fig. 2*A, lanes 1–3*). Thus Mim1 is required on the Tom40 biogenesis pathway in the step following the SAM_{core} stage.

As shown in Fig. 1*B*, the steady-state levels of Tom40 as well as Tom20 and Tom70 were moderately reduced in *mim1*Δ mitochondria, raising the possibility that Mim1 was also involved in the biogenesis of these receptors. We used the radiolabeled precursor of Tom20 and imported it into isolated mitochondria. Tom20 was efficiently integrated into the TOM complex of wild-type mitochondria but strongly impaired in *mim1*Δ mitochondria (Fig. 2*B*). It has to be emphasized that neither the SAM subunits, Sam50, Sam37, and Sam35, nor Mdm10 are required for assembly of Tom20 (30, 33, 34, 36), indicating a specific role of Mim1 in biogenesis of Tom20. We asked if the second signal-anchored Tom receptor, Tom70, also depended on Mim1. Tom70 is only loosely associated with the TOM complex and migrates as homodimer on blue native electrophoresis (Fig. 2*C, lanes 4–6*) (61–64). The assembly of Tom70 was strongly inhibited in *mim1*Δ mitochondria (Fig. 2*C, lanes 1–3*). We thus wondered if the biogenesis of all Tom receptors required Mim1 and used the radiolabeled precursor of Tom22. Remarkably, Tom22 assembly into the TOM complex of *mim1*Δ mitochondria was not inhibited (Fig. 2*D*). This demonstrates that the mitochondrial outer membrane and the TOM complex of the mutant mitochondria are fully competent in the assembly pathway of this tail-anchored precursor, excluding an unspecific damage of the outer membrane by the lack of Mim1. We conclude that mitochondria lacking Mim1 are defective in the assembly pathway of Tom40 and signal-anchored Tom receptors but not of tail-anchored Tom22.

Mim1-deficient Mitochondria Are Impaired in Membrane Insertion of Tom20, Tom70, and Tom40—The blue native assay revealed that Mim1 was required for the assembly pathway of Tom20 and Tom70. As these precursors do not interact with the SAM_{core} complex, an early intermediate like in case of Tom40 cannot be separated by blue native gels. We thus used a further assay to determine if Mim1 was already involved in the early import stage of precursor insertion into the lipid phase of the outer membrane. Thus after the import reaction, the mitochondria were treated at alkaline pH (sodium carbonate) to extract soluble and peripheral membrane proteins while integral membrane proteins remain in the membrane sheets (51, 65–66). To validate the assay we first analyzed the precursors of Tom40 and Tom22. The membrane insertion of Tom40 in *mim1*Δ mitochondria occurred with reduced efficiency (Fig. 3) in agreement with the requirement of Mim1 at a post-SAM stage. The integration of Tom22 into the membrane was not affected by the lack of Mim1 (Fig. 3). The precursors of Tom20 and Tom70, however, were impaired in membrane insertion. After an import time of 60 min into *mim1*Δ mitochondria, an integration efficiency was obtained that corresponded to a short import time in wild-type mitochondria (Fig. 3). The levels of control proteins of outer and inner membranes did not differ between wild-type and mutant mitochondria (Fig. 3, *lanes 7–12*), indicating that the resistance of membrane proteins to extraction at alkaline pH was not altered in *mim1*Δ mitochondria. We conclude that the lack of Mim1 does not completely block membrane insertion of the signal-anchored receptors but significantly delays it.

The mitochondrial outer membrane contains a third signal-anchored protein, OM45, that is not associated with the TOM complex (15, 23, 67). We analyzed the import of OM45 by both blue native electrophoresis and extraction at alkaline pH. Both assays revealed that the import of this signal-anchored protein did not depend on the presence of Mim1 (Fig. 4*A*). We thus analyzed several more mitochondrial outer membrane proteins that contain transmembrane α-helical segments in different

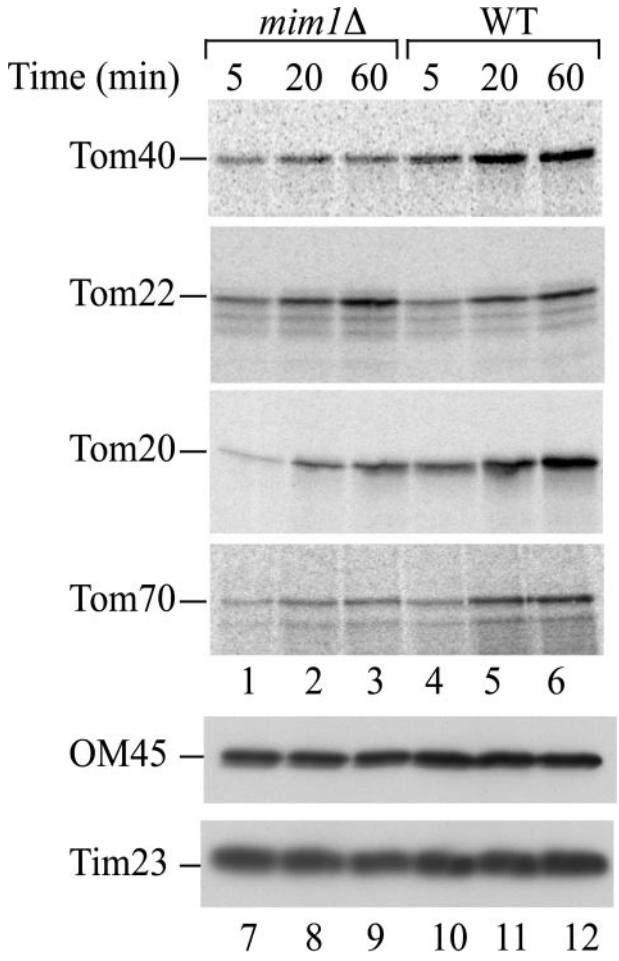

FIGURE 3. **Mim1-deficient mitochondria are impaired in membrane insertion of Tom40, Tom20, and Tom70.** Membrane insertion of Tom40, Tom22, Tom20, and Tom70 was analyzed by Na_2CO_3 extraction. WT and *mim1*Δ mitochondria were incubated with the ^{35}S-labeled precursors of Tom40, Tom22, Tom20, and Tom70 at 25 °C for the indicated times. The re-isolated mitochondria were treated with Na_2CO_3 (0.1 M, pH 11.5) for 30 min on ice. Membrane sheets were isolated by ultracentrifugation, solubilized by Laemmli buffer and analyzed by SDS-PAGE and autoradiography (*lanes 1–6*). For control, the levels of OM45 and Tim23 were determined by Western blot analysis (*lanes 7–12*).

portions of their polypeptide chain, including the precursors of Mim1, Mdm12, Mmm1, and the fusion component Ugo1 (68–73). The radiolabeled precursors were inserted into isolated *mim1*Δ mitochondria and resistant to extraction at alkaline pH like in wild-type mitochondria (Fig. 4*B*). These results suggest that Mim1 plays a specific role in the assembly pathway of TOM subunits.

Mim1 Associates with the SAM Complex—To study a possible relation of Mim1 to the SAM complex, we used blue native electrophoresis. Two forms of the SAM complex have been described. A core complex of ~200 kDa, containing Sam50, Sam37, and Sam35, and a larger SAM complex of ~350 kDa, which contains Mdm10 in addition to the three SAM_{core} subunits (25, 36, 37). Fig. 5*A* shows that in wild-type mitochondria both SAM forms, SAM_{core} and the larger SAM complex (indicated as SAM*) are decorated with antibodies against Sam50, Sam37, and Sam35. In *mim1*Δ mitochondria, each of these antibodies decorated an additional complex migrating at ~300 kDa, indicating the presence of a third SAM form, termed SAM**, in the absence of Mim1 (Fig. 5*A*, *lanes 2*, *4*, and *6*).

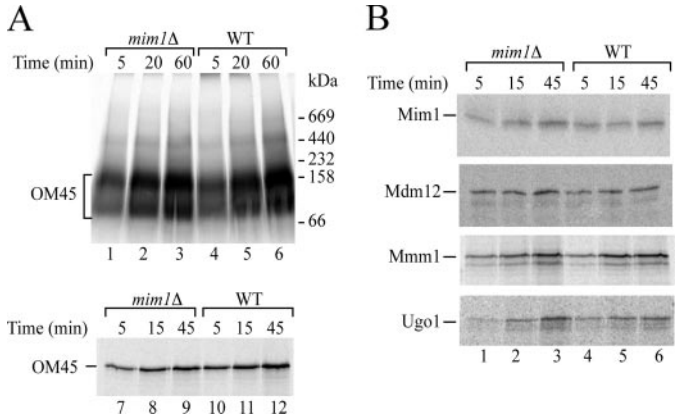

FIGURE 4. **Import of outer membrane proteins in Mim1-deficient mitochondria.** *A*, WT and *mim1*Δ mitochondria were incubated with ^{35}S-labeled OM45 at 25 °C. For assembly studies, the re-isolated mitochondria were lysed in a digitonin-containing buffer and analyzed by blue native electrophoresis and digital autoradiography (*lanes 1–6*). Membrane insertion was detected by Na_2CO_3 extraction. After import, mitochondria were treated with Na_2CO_3 for 30 min on ice. Membrane sheets were isolated by ultracentrifugation, solubilized by Laemmli buffer and analyzed by SDS-PAGE and autoradiography (*lanes 7–12*). *B*, WT and *mim1*Δ mitochondria were incubated with the ^{35}S-labeled precursors of Mim1, Mdm12, Mmm1, and Ugo1 at 25 °C. Subsequent Na_2CO_3 extraction was performed as described under *A*.

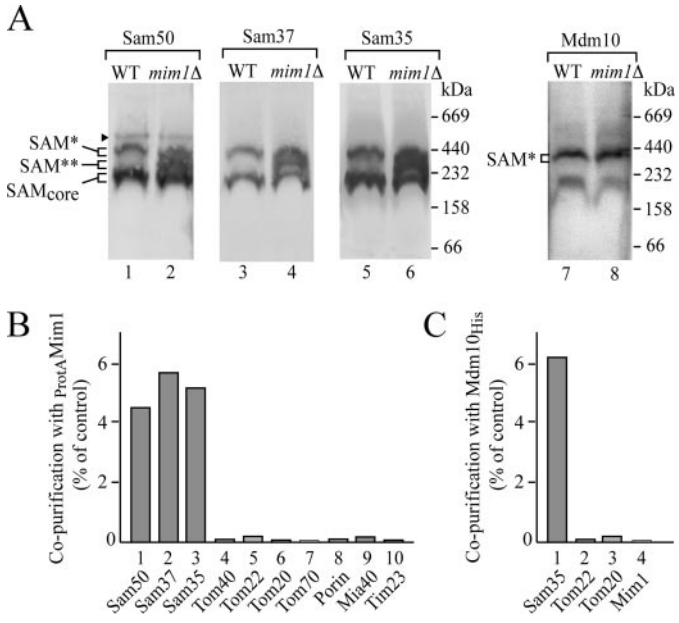

FIGURE 5. **Association of Mim1 with the SAM complex.** *A*, mitochondria (50 μg of protein) isolated from WT and *mim1*Δ yeast cells were subjected to blue native electrophoresis and Western blot analysis. *Arrowhead,* unspecific band decorated by anti-Sam50. *B*, mitochondria (1 mg of protein) were isolated from WT cells or yeast cells expressing Mim1 fused to an N-terminal, TEV-cleavable protein A tag. The mitochondria were lysed in 1% digitonin buffer and subjected to IgG affinity chromatography. After washing, bound proteins were eluted by cleavage with TEV protease, detected by immunodecoration and quantified by ImageQuant 5.2. The efficiency of purification of Mim1 from the protein A-tagged mitochondria was set to 100% (control) compared with the load of Mim1 in WT mitochondria. For Tom proteins, porin, Mia40, and Tim23, no specific co-purification above the background signal (eluate from WT mitochondria) was observed. *C*, mitochondria (1 mg of protein) were isolated from WT cells or yeast cells expressing Mdm10 fused to a C-terminal His tag. The mitochondria were lysed in 1% digitonin buffer and subjected to Ni-NTA affinity chromatography. After washing, bound proteins were eluted by imidazole, detected by immunodecoration and quantified by ImageQuant 5.2. The quantification of the co-purified proteins was determined as described under *B*. *Control*, efficiency of purification of Mdm10 (100%).

Three possibilities were conceivable. (i) Mim1 and Mdm10 are present in the same large SAM complex. This possibility appeared less likely as in *mim1*Δ mitochondria the 350-kDa SAM form was still observed despite the complete absence of Mim1. (ii) Two different large SAM forms exist, one associating with Mdm10, the other one with Mim1. (iii) Mim1 is not a subunit of a large SAM complex but only required for its assembly or stability and thus in the absence of Mim1 the complex would partially dissociate leading to the SAM** form. We used antibodies against Mdm10 to decorate the known large SAM complex (36, 37). The blue native mobility of Mdm10 was not affected by the presence or absence of Mim1, in particular Mdm10 was not found in the 300-kDa SAM** form (Fig. 5*A, lanes 7* and *8*) (Mdm10 additionally forms a smaller complex not containing SAM subunits (37)). Thus Mdm10 is present in the 350-kDa SAM* complex independently of Mim1, excluding the first possibility. Moreover, this result shows that the stability of the Mdm10-containing 350-kDa SAM complex was not affected by the lack of Mim1, arguing also against the third possibility. Since the available antibodies against Mim1 do not decorate a defined band on blue native gels (an observation known for several other subunits of mitochondrial protein translocases, like Tim50, Tim44, and mtHsp70 (53, 60, 74, 75)), we generated a yeast strain expressing Mim1 with an N-terminal protein A tag to test the association of Mim1 with the SAM complex. Mitochondria were lysed with digitonin and subjected to IgG affinity chromatography. A TEV cleavage site between protein A and Mim1 allowed a specific release of the bound proteins. Fig. 5*B* demonstrates indeed that a fraction of Sam50, Sam37, and Sam35 were co-purified with tagged Mim1 while neither the TOM subunits nor control proteins like porin, Mia40 of the intermembrane space and Tim23 of the inner membrane were found in the eluate. Moreover, isolation of His-tagged Mdm10 from digitonin-lysed mitochondria led to a co-purification of a fraction of Sam subunits (shown here for Sam35) but not Tom subunits as expected (Fig. 5*C, columns 1–3*) (35, 36). Mim1 was not co-purified with $Mdm10_{His}$ (Fig. 5*C, column 4*), indicating that Mim1 and Mdm10 are present in different complexes.

Taken together, these findings show that two large SAM complexes exist, one associating with Mdm10 and the other one with Mim1. The SAM_{core} subunits Sam50, Sam37, and Sam35 are present in both complexes.

A Role of Mim1 in Biogenesis of Small Tom Proteins—The lack of Mdm10 has been shown to impair late steps in the assembly of small Tom proteins, leading to an accumulation of 100 kDa intermediate forms (36). We asked if Mim1 was also involved in the assembly pathway of small Tom proteins. In Mim1-deficient mitochondria, the assembly of Tom5 and Tom7 was partially impaired, leading to accumulation of 100 kDa intermediate forms (Fig. 6*A* and *B, upper panels*). The membrane integration of Tom5 and Tom7, determined by the resistance to extraction at alkaline pH, was not inhibited in the absence of Mim1 (Fig. 6*A* and *B, lower panels*). The late steps of TOM assembly involve the association of Tom40 with small Tom proteins (36), and thus Mim1 seems to affect these steps in case of Tom5 and Tom7.

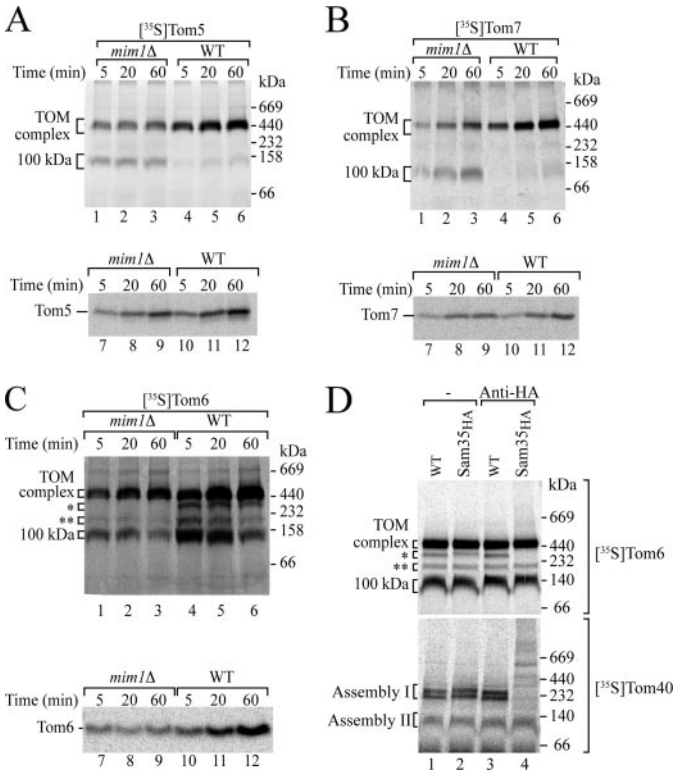

FIGURE 6. **Role of Mim1 in the biogenesis of small Tom proteins.** WT and *mim1*Δ mitochondria were incubated with the ^{35}S-labeled precursors of Tom5 (*A*), Tom7 (*B*), or Tom6 (*C*) for the indicated time periods at 25 °C. The assembly of the small Tom proteins was analyzed by blue native electrophoresis (*lanes 1–6*). The membrane insertion was studied by treatment with Na_2CO_3 (*lanes 7–12*). *D*, WT and $Sam35_{HA}$ mitochondria were incubated with the ^{35}S-labeled precursors of Tom6 (*upper panel*) or Tom40 (*lower panel*) for 30 min at 10 °C or 5 min at 25 °C, respectively. The mitochondria were reisolated and incubated in the absence (*lanes 1* and *2*) or presence (*lanes 3* and *4*) of antibodies directed against the HA epitope for 40 min on ice. Subsequently, mitochondria were reisolated, lysed in a digitonin-containing buffer and subjected to blue native electrophoresis and digital autoradiography.

For Tom6, however, a different dependence on Mim1 was observed. The membrane insertion of the precursor of Tom6 was impaired in *mim1*Δ mitochondria (Fig. 6*C, lanes 7–9*) and consequently the formation of the 100-kDa intermediate and mature TOM complex was delayed compared with wild-type mitochondria (Fig. 6*C, lanes 1–6*). We noted that two further intermediate forms of lower abundance were observed during assembly of Tom6. These intermediates (indicated by asterisks) were formed after a short term incubation of the precursor of Tom6 with wild-type mitochondria, while after longer incubation times the amounts of the intermediates, including the 100-kDa intermediate, decreased and more mature TOM complex was generated (Fig. 6*C, lanes 4–6*). In *mim1*Δ mitochondria, the amount of the lower intermediate** was decreased while, remarkably, the upper intermediate* was absent (Fig. 6*C*, *lanes 1–3*). The size of the upper intermediate would fit to that of a large SAM complex. We thus wondered if this intermediate represented the interaction of Tom6 with a Mim1-dependent large SAM complex. We used a yeast strain that expressed Sam35 with HA tag (33). Upon formation of the Tom6 intermediates, mitochondria were incubated with anti-HA antibodies. Indeed, the upper intermediate* was selectively sensitive to this treatment (Fig. 6*D, upper panel*, *lane 4*), demonstrating

that it represented an association of Tom6 with SAM. As control we show that the assembly intermediate I (SAM intermediate) of Tom40 is similarly shifted by the HA-antibodies (Fig. 6*D*, *lower panel*). We conclude that Mim1 is required for efficient membrane insertion of Tom6. The precursor of Tom6 assembles into the TOM complex via several intermediate steps. One of the intermediates represents a Mim1-dependent large SAM complex, providing further support for a role of Mim1 in the formation and function of a large SAM complex.

CONCLUSIONS

We report a new function for the mitochondrial outer membrane protein Mim1. In addition to its involvement in the assembly pathway of the β-barrel protein Tom40, Mim1 is required for the biogenesis of the two signal-anchored subunits of the TOM complex, the receptors Tom20 and Tom70. Mim1 promotes insertion of the precursor proteins into the lipid phase of the outer membrane. Mim1 is also involved in the assembly pathways of the small Tom proteins. While the precursors of Tom5 and Tom7 require the presence of Mim1 only for the late steps of association with Tom40, Mim1 functions in an early step of the biogenesis of Tom6 by promoting its membrane insertion. The function of Mim1, however, is not required for all α-helical outer membrane proteins because the import of the tail-anchored receptor Tom22 as well as the import of several further outer membrane proteins were not affected by the lack of Mim1.

The function of Mim1 is in part complementary to that of Mdm10. Both outer membrane proteins promote the biogenesis pathway of Tom40 toward the mature TOM complex at a stage after the SAM_{core} complex. However, Mdm10 is critical for the assembly of the tail-anchored receptor Tom22 with Tom40 (36), while Mim1 promotes the assembly of the signal-anchored Tom20 and Tom70. The organization of the SAM complex into several dynamic complexes provides the framework for coordination of these different assembly steps. Besides the SAM_{core} complex with Sam50, Sam37, and Sam35 (25, 32–34), two larger forms of the SAM complex exist that both migrate at ~350 kDa in blue native gels. In addition to the three SAM_{core} subunits, one complex contains Mdm10, while the other interacts with Mim1. The current results suggest that Mim1 is not a stoichiometric subunit of SAM but interacts in a dynamic manner. In summary, the SAM_{core} complex can associate with different partner proteins to complete the final steps in assembly of the TOM complex, *i.e.* the association of the central component Tom40 with the different types of Tom proteins. Mim1 seems to play a dual role. In an early biogenesis step, Mim1 promotes the membrane insertion of signal-anchored Tom receptors and of Tom6. In a late biogenesis step, Mim1 associates with a large SAM complex to promote the assembly of Tom40 with several α-helical Tom proteins.

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