Mba1, a Novel Component of the Mitochondrial Protein Export Machinery of the Yeast *Saccharomyces cerevisiae*

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**Abstract.** The biogenesis of mitochondria requires the integration of many proteins into the inner membrane from the matrix side. The inner membrane protein Oxa1 plays an important role in this process. We identified Mba1 as a second mitochondrial component that is required for efficient protein insertion. Like Oxa1, Mba1 specifically interacts both with mitochondrial translation products and with conservatively sorted, nuclear-encoded proteins during their integration into the inner membrane. Oxa1 and Mba1 overlap in function and substrate specificity, but both can act independently of each other. We conclude that Mba1 is part of the mitochondrial protein export machinery and represents the first component of a novel Oxa1-independent insertion pathway into the mitochondrial inner membrane.

Key words: mitochondria • protein translocation • Mba1 • Oxa1 • membrane insertion

**Introduction**

The inner membrane of mitochondria has a very high protein content and might accommodate roughly half of all mitochondrial polypeptides. A small number of these proteins are synthesized in the mitochondria, whereas the majority are synthesized in the cytosol. Import and sorting of the latter into mitochondria are achieved by translocases in the outer and inner membrane of the organelle (for review see Schatz, 1996; Neupert, 1997; Pfanner et al., 1997; Herrmann and Neupert, 2000). Although all matrix proteins appear to be imported on a single transport route, three different pathways have been identified in the past years that lead to a localization in the inner membrane. First, polytopic proteins with internal signals are transported by the translocase of the outer membrane (TOM) complex to the intermembrane space, from where they are inserted into the inner membrane by the recently identified TIM22 machinery (Sirrenberg et al., 1996; Koehler et al., 1998). Second, proteins with typical presequences can be arrested at the level of the TIM23 complex and laterally inserted into the lipid bilayer. This “stop–transfer mechanism” seems to be typical for monotypic membrane proteins whose NH₂ terminus faces the matrix (Van Loon and Schatz, 1987; Rojo et al., 1998). The third group of inner membrane proteins is completely transported into the matrix from where it reinserts into the inner membrane. Thus, domains of these proteins that are exposed to the intermembrane space have to traverse the inner membrane twice (Rojo et al., 1995). This reinsertion process resembles the membrane insertion of mitochondrially encoded proteins (Herrmann et al., 1995). Since this route seems to have evolved from the insertion process of the bacterial progenitors of mitochondria it was named the “conservative sorting” pathway (Hartl et al., 1986).

The inner membrane protein Oxa1 plays an important role in this export process. Oxa1 is conserved from bacteria to chloroplasts and mitochondria, and appears to mediate protein insertion in all of these systems (Bauer et al., 1994; Bonnefoy et al., 1994; Moore et al., 2000; Samuelson et al., 2000). Oxa1 directly interacts with insertion intermediates (Hell et al., 1997, 1998, 2001). In the absence of Oxa1, the mitochondrially encoded subunit 2 of cytochrome oxidase (Cox) accumulates in the matrix and its intermembrane space domains cannot traverse the inner membrane (He and Fox, 1997; Hell et al., 1997). Similarly, in *oxa1* mutants conservatively sorted proteins like subunit 9 of the F₀F₁-ATP synthase of *Neurospora crassa* or Oxa1 itself show significant export defects and end up in the matrix after import (Hell et al., 1998). This indicates that Oxa1 represents a component of a general protein export machinery in the mitochondrial inner membrane, the OXA translocase.

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However, there are several observations suggesting that the strict dependence of the export of Cox2 on Oxa1 may be exceptional and most inner membrane proteins can insert also in the absence of Oxa1. (a) oxal deletion mutants can be rescued by reintroduction of the OXAl gene (Bonnfey et al., 1994). If a functional OXA translocase would be absolutely required for the insertion of Oxal into the inner membrane, the Oxal protein synthesized in the transformed Δoxal mutant should accumulate exclusively in the matrix and therefore not be able to form a functional translocase. (b) In Δoxal strains, the FpF1-ATPase is still present although at reduced levels (Lemaire et al., 2000). This protein complex contains three mitochondrially encoded transmembrane subunits that obviously can insert independently of Oxal. Deletion of the inner membrane protease Yme1 restores wild-type levels of the FpF1-ATPase in Δoxal mutants (Lemaire et al., 2000). Presumably, when protein degradation is reduced Fp subunits have sufficient time to find their way into the membrane, even in the absence of Oxal. (c) It has been shown that mutations in cytochrome c make Oxal dispensable, and Oxal-independent export pathways obviously have to be used in these strains (Hamel et al., 1998). These observations indicate that at least one alternative pathway for the insertion of inner membrane proteins must exist.

Here we analyzed the function of the inner membrane protein Mba1, which, together with Oxal, was originally described as a multicopy suppressor of a mutant lacking Yta10 (Afg3), a protease in the inner membrane; loss of Mba1 was reported to cause decreased cytochrome aa3 levels and a slow growth phenotype on nonfermentable carbon sources (Rep and Grivell, 1996; Rep et al., 1996). We show that Mba1 is required for efficient membrane insertion of several mitochondrial translation products. Moreover, Mba1 interacts physically with translocation intermediates. Our results suggest that Mba1 represents a novel component of a protein insertion machinery in yeast mitochondria that, dependent on conditions and substrates, can act either cooperatively with or independent of Oxal.

**Materials and Methods**

**Yeast Strains and Cell Growth**

Yeast strains used in this study were isogenic to the wild-type strains W303a and YPH499 (Siikorski and Hieter, 1989). In the Δoxal strain, the complete OXAl reading frame was replaced by a kanamycin resistance cassette by homologous recombination of a PCR product. The temperature-sensitive oxal+ strain was generated by replacing the HIS3 gene in the Δoxal strain with the oxal+ allele of the petl402 mutant (Bauer et al., 1994). To generate the Δmba1 strain, the complete MBA1 reading frame was replaced by the HIS3 gene by homologous recombination of a PCR product. The Δmba1 Δoxal mutant was generated by sporulation and tetrad dissection after crossing the Δmba1 strain with the Δoxal strain. The Δmba1 oxal+ mutant was generated by sporulation after crossing the Δmba1 strain with a strain in which oxal carried the petl402 mutation. The imp1 mutant strain was a gift from E. Pratje (University of Hamburg, Hamburg, Germany). Standard genetic manipulations were used throughout (Sherman et al., 1986). Yeast strains were cultivated at 30 or 24°C (for oxal+ strains) on lactate medium or on YM medium (1% yeast extract, 2% peptone) supplemented with 2% galactose and 0.5% potassium hydroxide-buffered lactate (Sherman et al., 1986). Then 140 μg/ml cycloheximide, 60 μCi/ml [35S]methionine, and 40 μg/ml of the other 19 proteinogenic amino acids were added. After incubation for 30 min at 30°C the cells were lysed by vigorous mixing with glass beads in 0.2% SDS, and the resulting extract was subjected to SDS-PAGE.

**Recombinant DNA Techniques**

For construction of the in vitro transcription construct, MBA1 gene was amplified from genomic yeast DNA by PCR using the primers 5'-GGGCTCTAGAGTGGATTTAAGCATC-3' and 5'-GGGAAAGCTTGGCTTAGCTTTGAGTAAACG-3' and subcloned into the XbaI-HindIII sites of the vector pGEM4 (Promega). For overexpression of Mba1, the MBA1 coding region was cut out from the pGEM4 vector and subcloned into the EcoRI and HindIII sites of the expression vector pYX122 (Novagen).

**Mitochondrial Subfractionation**

The procedures used for subfractionation of mitochondria were described previously (Leonhard et al., 2000). Swelling and proteinase K treatment were controlled by Western blotting using cytochrome c peroxidase, Dld1, Oxal, and Mge1 as marker proteins.

**Flotation of Integral Membrane Proteins**

Proteins were synthesized in the presence of [35S]methionine in isolated mitochondria for 20 min as described (Herrmann et al., 1994a). Mitochondria were resolated and incubated for 30 min at 4°C in 200 μl 0.1 M sodium carbonate. The samples were split and proteins from one half were TCA precipitated. The other half were adjusted to 1.6 M sucrose, transferred into a 650-μl SW60 centrifugation tube (Beckman Coulter), and overlaid with 250 μl 1.4 M sucrose, 0.1 M sodium carbonate and 100 μl 0.25 M sucrose, and 0.1 M sodium carbonate. After centrifugation at 485,000 g for 2 h at 2°C, the floated membrane fraction was collected. Proteins were TCA precipitated, separated by SDS-PAGE, and transferred to nitrocellulose. Signals for mitochondrial translation products were detected by autoradiography and quantified using a PhosphoImaging station (BAS-1500; Fuji).

**Cross-linking and Immunoprecipitation**

Cross-linking analysis of mitochondrial translation products was performed essentially as described previously (Herrl et al., 1998). Proteins were synthesized in the presence of [35S]methionine in isolated mitochondria as described (Herrmann et al., 1994a), with the exception that BSA was omitted from the buffer and Hepes was used instead of Tris to prevent quenching of the cross-linkers. For cross-linking, 1.5-difluoro-2,4-dinitrobenzene (DFDNB) or diithio(succinimidylpropionate) (DSP) was added for 15–30 min.

For cross-linking of imported Oxa1p, radiolabeled Oxa1 was incubated with wild-type mitochondria in import buffer lacking BSA for 10 min at 25°C as described (Herrl et al., 1997). Mitochondria were resolated and incubated in 400 μl of 0.6 M sorbitol, 2 mM ATP, 2 mM NADH, and 20 mM Hepes, pH 7.4 in the presence of 400 μM disuccinimidyl glutarate (DSG) for 30 min.

Cross-linking was stopped by the addition of 100 mM glycine. Mitochondria were resolated, washed, and lysed in 0.1% SDS. After a clarifying spin for 10 min at 20,000 g, the extract was diluted 100-fold in 1% Triton X-100, 300 mM KCl, 5 mM EDTA, 1 mM PMSF, 10 mM Tris/HCl, pH 7.4, and used for immunoprecipitation according to published procedures (Herrmann et al., 1994b).

**Generation of Antiserum**

Antiserum against the COOH terminus of Mba1 were raised in rabbits by injecting the chemically synthesized peptide CEDDVKAVHRMK, representing amino acid residues 259–271 coupled to keyhole limpet hemocyanin (Pierce Chemical Co.). The antiserum against Cox2, Cox20, Oxal, and
Yta10 were described previously (Pajic et al., 1994; Herrmann et al., 1995, 1997; Hell et al., 2000).

**Miscellaneous**

Import of in vitro–synthesized proteins into isolated mitochondria (Herrmann et al., 1997) and enzymatic measurement of the activities of the bc1, Cox, and ATPase complexes were performed essentially as described previously (Hell et al., 2000).

**Results**

*Mba1 Is a Matrix Protein Associated with the Inner Membrane*

To assess the localization of Mba1, we raised an antiserum against a peptide representing amino acid residues 259–271 of Mba1. In Western blots of mitochondrial extracts, this serum specifically recognized a 24-kD band which was absent in *Δmba1* mutant mitochondria (Fig. 1 A). The *Δmba1* mitochondria contained wild-type levels of the proteins Oxa1 and Cox20. In contrast, the amount of Cox2 was strongly reduced (Fig. 1 A), which explains the low cytochrome aa3 levels that are reported for this mutant (Rep and Grivell, 1996).

A mitochondrial subfractionation experiment is shown in Fig. 1 B. Mba1 was found to be resistant to added protease both in mitochondria and in mitoplasts in which the outer membrane was ruptured by hypotonic swelling (lanes 2 and 3). In contrast, the outer membrane protein Tom70 and the intermembrane space protein cytochrome b2 (Cyt b2) were degraded under these conditions. After detergent lysis of mitochondrial membranes, Mba1 was protease sensitive (lane 4). This indicates a localization of Mba1 in the mitochondrial matrix or in the inner membrane. Upon sonication of mitochondria, Mba1 was exclusively found in the membrane fraction (lanes 5 and 6). Even under the rigid extraction conditions of treatment with 0.1 M unbuffered carbonate, a significant fraction of Mba1 fractionated with membranes (lanes 7 and 8). A very similar behavior was reported for the overexpressed myc-tagged Mba1 (Rep and Grivell, 1996). Thus, although Mba1 does not expose protease-sensitive domains to the intermembrane space, it is tightly associated with the inner membrane. The sequence of Mba1 contains two adjacent hydrophobic stretches (amino acid residues 70–86 and 89–102) which might be embedded deeply in the membrane and lead to an extraction pattern that resembles that of the transmembrane protein Tom70.

The mitochondrial localization of Mba1 was further supported in vitro by import experiments of a 35S-labeled Mba1 precursor (Fig. 1 C, pMba1). Upon incubation with isolated yeast mitochondria (lanes 2–5), a proteolytically processed mature form of Mba1 was generated (mMba1) that remained protease resistant in mitochondria and mitoplasts (lanes 3 and 4). In the absence of a membrane potential, no Mba1 was imported into mitochondria and the Mba1 precursor remained protease accessible (lanes 7–10, −Δψ).

The reduced amounts of Cox2 in *Δmba1* mitochondria might be due either to an impaired synthesis rate or to an instability of Cox2. To differentiate between these two possibilities, mitochondrial translation products were labeled with [35S]methionine in wild-type or *Δmba1* mutant cells for 30 min. Then mitochondrial translation was

![Figure 1. Localization of Mba1 on the matrix side of the mitochondrial inner membrane.](image-url)
Mba1 Is Required for Efficient Membrane Insertion of Cox2

The isolation of both Mba1 and Oxa1 in the same genetic screen (Rep and Grivell, 1996) might indicate a similar function of both proteins. Therefore, we tested whether Mba1 plays a role in the membrane insertion process of mitochondrial translation products. The mitochondrial genome of yeast encodes eight major proteins, out of which seven are integral membrane subunits of respiratory chain complexes (Borst and Grivell, 1978). One of these proteins, Cox2, is synthesized with an NH2-terminal presequence that is removed by the Imp1 protease in the intermembrane space across translocation across the inner membrane. Therefore, the accumulation of the precursor form of Cox2 is characteristic of defects in the protein export process from the matrix and is associated with oxa1 mutations, for example (Bauer et al., 1994; He and Fox, 1997; Hell et al., 1997). To look for an accumulation of Cox2 precursor in the matrix, we synthesized proteins in isolated wild-type or Δmba1 mitochondria in the presence of [35S]methionine. In wild-type mitochondria no precursor of Cox2 was observed (Fig. 2 A, top). In contrast, in Δmba1 mitochondria a significant fraction of Cox2 remained unprocessed (Fig. 2 A, bottom). To test whether this fraction accumulated in the mitochondrial matrix, we performed the translation reaction in mitoplasts and treated them with protease to degrade translation products that expose domains into the intermembrane space (lane 4). The mature form of Cox2 was completely degraded both in the wild-type and mutant sample. In contrast, the Cox2 precursor (pCox2) formed in the Δmba1 mitoplasts was not degraded, indicating an export defect in the Δmba1 mitochondria. In addition to the Cox2 precursor, the translation products cytochrome b and Cox1 were partially protease inaccessible in Δmba1 mitoplasts. This suggests that Mba1 is involved in the insertion of several mitochondrially encoded proteins.

During the biogenesis of Cox2, both its NH2 and COOH termini have to be translocated across the membrane. To assess whether the export of the COOH terminus is also affected in Δmba1, mitochondrial translation products were radiolabeled in mitoplasts before an incubation without or with protease. The mitochondria were reisolated, lysed, and subjected to immunoprecipitation using antibodies against Cox2. In wild-type mitochondria only mature, protease-accessible Cox2 was detected, indicating that Cox2 was exclusively in an NH2out topology (Fig. 2 B, lanes 1 and 2). Depletion of the membrane potential strongly reduced the export efficiency of both the NH2 and COOH termini resulting both in a protease-inaccessible...
preprotein form of Cox2 (N<sub>out</sub>–C<sub>in</sub>) and a characteristic COOH-terminal fragment which is generated from a species in N<sub>out</sub>–C<sub>in</sub> topology by digestion of the exposed NH<sub>2</sub>COOH-terminal fragment which is generated from a spe-

To exclude the possibility that a decreased activity of the Imp1 protease in Δmba1 mitochondria may cause the observed Cox2 precursor accumulation, we analyzed the kinetics of the Imp1-dependent processing of the cytochrome b<sub>2</sub> presequence in wild-type and mutant mitochondria after in vitro import experiments. Cytochrome b<sub>2</sub> is synthesized in the cytosol with a bipartite presequence which is removed after import in two sequential steps, first by the mitochondrial-processing peptidase and then by Imp1. Both the import kinetics into the Δmba1 mitochondria (not shown) and the kinetics of the maturation by Imp1 (Fig. 2 C) resembled that of wild-type mitochondria. This excludes a diminished Imp1 processing in the Δmba1 strain.

**Mba1 Interacts with Mitochondrial Translation Products**

Is Mba1 in direct contact with inserting polypeptides? We used chemical cross-linking reagents to answer this question, as they had been very useful to study the interaction of Oxa1 with its substrates (Hell et al., 1997, 1998). Translation products were radiolabeled with [35S]methionine in the presence of Oxa1 with its substrates (Hell et al., 1997, 1998). Trans-

Figure 3. Mba1 is in direct proximity to mitochondrial translation intermediates. (A) Mitochondrial translation products were radiolabeled for 15 min in the absence (−Δψ, lanes 1–8) or the presence (+Δψ, lanes 9–16) of 1 μM valinomycin. Then the samples were mock treated (lanes 1–4 and 9–12) or treated with 200 μM DSP (other lanes) for 30 min. Cross-linking and translation were stopped by addition of glycine and unlabeled methionine. Mitochondria were reisolated, washed, and further treated as outlined in A. Lanes labeled T show 10% of the extract used for immunoprecipitation. (C) Transient interaction of Mba1 and Oxa1 with newly synthesized Cox2. Mitochondrial translation products were radiolabeled in three separate reactions for 25 min (Pulse). The labeling was stopped by the addition of 25 μg/ml puromycin and 5 mM methionine, and the samples were further incubated for 30 min (Chase). During this procedure 400 μM of the cross-linker DSP was present for 15 min and then quenched with 100 μM glycine. As depicted in the insert, sample A was cross-linked during the labeling period, B directly afterwards, and C 15 min after the beginning of the chase reaction. The mitochondria were reisolated, lysed, and either directly applied to SDS-PAGE (Total) or used for immunoprecipitation with preimmune serum (p.i.) or antiserum against Mba1, Oxa1, and Cox20 as indicated. Lanes labeled “Total” show 10% of the extract used for immunoprecipitation. The amounts of immunoprecipitated Cox2 were quantified and are shown as a percentage of total Cox2.
tides of a wide size range, indicating the interaction of all three proteins with uncompleted translation products.

These cross-linked polypeptides might represent nascent chains that are bound to ribosomes or uncompleted translation products that are prone to protein degradation. To test this we blocked the translation reaction with chloramphenicol before DFDNB was added (lanes 11–20). Chloramphenicol leads to an arrest of protein synthesis and nascent chains remain firmly associated with the ribosomes. Under these conditions, no cross-linking of translation products to Yta10 was found, indicating that this protease may preferentially bind to uncompleted polypeptides which are no longer bound to ribosomes. In contrast, an increase of the cross-linking efficiency to Mba1 was observed, suggesting that Mba1 interacts with translation products predominantly during their synthesis. This is further indicated by the wide size range of the cross-link products, which is typical for cross-linked nascent chains and which would not be expected if cross-linking occurred only to completely synthesized and inserted proteins.

Which proteins are substrates of Mba1? To answer this question we used the cleavable cross-linker DSP. During protein synthesis in isolated mitochondria, rather low amounts of DSP were added so that translation was allowed to continue during the cross-linking reaction, leading to the completion of the synthesis of cross-linked adducts (Hell et al., 1998). The mitochondria were lysed, and the extracts were used for immunoprecipitation with anti-sera against Mba1 and Oxa1 or with preimmune serum. Then the cross-links were cleaved and the samples were applied to SDS-PAGE. Four out of the eight translation products could be specifically pulled down together with Mba1 and Oxa1: subunits 1, 2, and 3 of the cytochrome c oxidase and, with lower efficiency, cytochrome b (Fig. 3 B, lanes 6 and 7). Thus, these proteins are in close proximity to Mba1 and Oxa1 at some stage of their synthesis. The other translation products do not interact with Mba1 and Oxa1, or do not expose lysine residues which would allow cross-linking. A similar result was obtained when the membrane potential was depleted during synthesis (lanes 9–16). Thus, the membrane potential is not required for the interaction of mitochondrial translation products with Mba1 and Oxa1.

At which time point of the Cox2 biogenesis does the interaction to Mba1 and Oxa1 occur? To address this question translation products were radiolabeled in wild-type mitochondria for 25 min in three parallel reactions. Then the labeling reaction was stopped by addition of puromycin and an excess of cold methionine and the mitochondria were further incubated (“chase”). The cleavable cross-linker DSP was present for 15 min either during the labeling period (A) or at an early (B) or late (C) stage of the chase period as depicted in Fig. 3 C. Then the mitochondria were reisolated, lysed, and subjected to immunoprecipitation with sera against Mba1, Oxa1, Cox20, or preimmune serum. Cross-links to Mba1 and Oxa1 were mainly formed during or directly after the labeling period. This indicates a transient interaction of Cox2 with both Mba1 and Oxa1 very early in the Cox2 biogenesis. In contrast, the cross-link efficiency to Cox20 was much higher and was maximal at later time points. Cox20 was reported to represent a chaperoning factor that forms a stable interaction with unassembled Cox2 (Hell et al., 2000).

**Mba1 Facilitates Export of Nuclear-encoded Proteins**

Efficient membrane insertion of Cox2 appears to depend on the recruitment of the ribosomes to the inner membrane (Sanchirico et al., 1998) which may allow a cotranslational translocation process. If Mba1 is required to recruit ribosomes to the inner membrane, it should be dispensable for a posttranslational export of the Cox2 NH2 terminus. To test this we performed import experiments with the in vitro–synthesized fusion protein pSu9(1-66)Cox2(1-74)-DHFR, comprising a mitochondrial targeting signal followed by the first 74 amino acid residues of Cox2 and mouse dihydrofolate reductase (Fig. 4 A). This protein is imported into the matrix where the mitochondrial targeting signal is removed so that the NH2 terminus of Cox2 can be inserted into the inner membrane (Herrmann et al., 1995). Upon insertion, the NH2-terminal 39 amino acid residues are exposed to the intermembrane space, which leads to the generation of a characteristic fragment after protease treatment of mitoplasts. This allows the quantification of the posttranslational insertion of the NH2 terminus of Cox2. Both Mba1 and functional Oxa1 were required for efficient insertion of Cox2(1-74)-DHFR (Fig. 4 A). This suggests that, similar to Oxa1, Mba1 plays a role in the protein export process from the mitochondrial matrix.

Is Mba1 also involved in the insertion of nuclear-encoded proteins? Oxa1 has been used as a model protein to analyze the export of nuclear-encoded proteins from the matrix into the inner membrane (Herrmann et al., 1997). After import into the mitochondrial matrix, Oxa1 is initially protected against protease, even after opening of the outer membrane. In contrast, after export of the NH2 terminus of Oxa1 protease, treatment of mitoplasts leads to a characteristic 27-kD fragment. To analyze the export efficiency in various strains, radiolabeled Oxa1 was imported into isolated mitochondria for 10 min. Then mitochondria were reisolated, converted into mitoplasts, treated with protease, and the ratio of protease accessible (i.e., inserted) to total protein was determined (Fig. 4 B). Complete opening of the intermembrane space was controlled by Western blotting. In wild-type mitochondria, 61% of Oxa1 was inserted. The insertion was slightly reduced in Δmba1 and oxa1ts mitochondria (the mitochondria were not pretreated at 37°C, and thus the temperature-sensitive phenotype was not induced). However, Oxa1 was inserted with significantly reduced efficiency in mitochondria of a Δmba1 oxa1ts double mutant (Fig. 4 B). This indicates that even at permissive conditions Oxa1ts function depends on the presence of Mba1. Thus, in the background of this partially impaired Oxa1 protein, Mba1 is required for the insertion of a nuclear-encoded protein, which indicates that Oxa1 and Mba1 cooperate in the process of Oxa1 insertion.

Next we used chemical cross-linking to test whether Mba1 physically interacts with the imported Oxa1 during its insertion (Fig. 4 C). After import of Oxa1, mitochondria were incubated with the cross-linker DSG, lysed, and the resulting extract was used for immunoprecipitation with Mba1-specific or preimmune serum. In the presence
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Figure 4. Mba1 interacts with nuclear-encoded proteins. (A) Posttranslational insertion of the Cox2 NH2 terminus. In the inset, the export of the Cox2 NH2 terminus after import of pSu9(1-66)pCox2(1-74)-DHFR is depicted. N, NH2 terminus; C, COOH terminus; OM, outer membrane; IMS, intermembrane space; IM, inner membrane; MPP, cleavage site of the mitochondrial processing peptidase; Su9, subunit 9 presequence; TM1, first transmembrane domain of Cox2. (Middle and bottom panels) Wild-type, Δmba1, and oxalits mitochondria were pretreated for 10 min at 37°C and incubated with pSu9(1-66)pCox2(1-74)-DHFR for 4, 10, and 30 min at 25°C. The mitochondria were converted into mitoplasts and treated with protease K (50 μg/ml) to digest the NH2 terminus of inserted Cox2. Lanes 1–3 show the generation of the fragment in wild-type mitochondria; lanes 4 and 5 show the 30 min reactions of Δmba1 and oxalits mitochondria, respectively. The graph shows a quantification of the fraction of inserted Cox2 relative to total imported protein at various times of import. The numbers were corrected for the methionine residues contained in the different Cox2 species. (B) Oxal precursor (pOxa1) was synthesized in reticulocyte lysate and incubated for 20 min at 25°C with

wild-type, Δmba1, oxalits, oxalits Δmba1, or Δoxa1 mitochondria. Mitochondria were then converted to mitoplasts (MP) and treated with 50 μg/ml protease K (PK) as indicated to convert inserted Oxa1 into a characteristic fragment. The ratio of fragment to total imported Oxa1 was determined by densitometry. (C) Radiolabeled Oxal precursor was imported into wild-type mitochondria for 10 min. Then the reaction was split and one half was mock treated, and the other treated with 100 μM DSG for 20 min. The mitochondria were resolated, lysed, and either directly analyzed by SDS-PAGE (10%) or used for immunoprecipitation with Mba1 antiserum or with preimmune serum. The cross-link product specifically precipitated with Mba1 serum is indicated by an arrow.

of the DSG a cross-link product of ~60 kD was specifically pulled down with the Mba1 serum. This corresponds to a size shift of ~24 kD, as would be expected for a cross-link to Mba1.

Mba1 Can Function Independently of Oxa1

What is the mode of cooperation of Mba1 and Oxa1? Both complexes may either work in parallel or have overlapping but independent functions. Alternatively, they might function sequentially. For example, Mba1 might pass on a substrate protein to the OXA complex. We used the advantage of yeast genetics to differentiate between both possibilities. If Mba1 would function upstream or downstream of Oxa1, the phenotype of a Δoxa1 Δmba1 double and a Δoxa1 single mutant should be similar. In contrast, we observed severe synthetic growth defects of the double mutant even on glucose (Fig. 5 A); this strain hardly grew on glucose at 24°C and grew slowly at 30°C (Fig. 5 A, top). This synthetic effect indicates that Mba1 can function independently of Oxa1 and can facilitate protein insertion on a pathway that works in parallel to the Oxa1 route. A similar strong growth defect was observed when Mba1 was deleted in an oxalits background at restrictive conditions (37°C). Even at permissive conditions (24°C) this mutant was unable to grow on glycerol (Fig. 5 A, bottom), although the steady state levels of Oxa1 were unchanged (not shown). In addition, a complete block of the Cox2 processing was observed in the oxalits Δmba1 strain even at 24°C (not shown). Thus, the oxalits strain requires Mba1 for Cox2 export and respiration competence even at the permissive temperature. This suggests that Oxa1 is partially defective at all temperatures, but that Mba1 can compensate for the defects at lower temperatures.

Mba1 Is Not Part of the OXA Complex

Oxa1 has been reported to be part of an oligomeric complex (Hell et al., 1998). Is Mba1 a subunit of this complex? No cross-links of endogenous Mba1 and Oxa1 could be detected and Mba1 was not coimmunoprecipitated with Oxa1 or vice versa, even when mitochondria were lysed with the very mild detergent digitonin (not shown). Both proteins did not cofractionate upon gel filtration chromatography, as shown in Fig. 5 B. Mba1 is part of a 200-kD complex that is slightly but distinctly smaller than the OXA complex (250–300 kD). In addition, deletion of Mba1 did not affect the levels of Oxa1, which might be expected if both proteins are subunits of one complex (Fig. 1 A). This also excludes that the effects observed in the
mba1 and oxa1 Mutations Cause Synthetic Defects in Protein Export

Next we determined the efficiency of membrane integration in the different mutants. Mitochondrial translation products were radiolabeled for 20 min. Then a fraction containing integral membrane proteins were isolated by carbonate extraction and flotation and compared with the total amount of synthesized proteins (Fig. 6). The membrane insertion of cytochrome b was significantly reduced in the absence of Mba1, and almost completely abolished in Δmba1 Δoxa1 mitochondria. The insertion of Cox1 and Cox3 was also impaired in Δmba1 mitochondria. This fractionation protocol probably underestimates the defects in the mutants since it does not allow verification of a correct topology and functionality of the membrane-associated proteins.

Mba1 Can Partially Compensate for the Loss of Oxa1

To further analyze this Oxa1-independent function of Mba1, we asked whether an interaction of Mba1 with mitochondrial translation products can be observed in the absence of Oxa1. We performed chemical cross-linking using either wild-type, Δoxa1, or Δimp1 mitochondria (Fig. 7 A). Translation products were labeled in isolated mitochondria in the presence of [35S]methionine. After incubation with the cleavable cross-linker DSP (lanes 5–11) or mock treatment (lanes 1–4), the mitochondria were lysed and cross-linked adducts to Mba1 or Oxa1 were isolated by immunoprecipitation. Then the cross-links were broken and the samples were analyzed by SDS-PAGE and autoradiography. In wild-type mitochondria, Cox1, Cox2, and Cox3 were specifically cross-linked to both Mba1 and Oxa1, but in Δoxa1 mitochondria, Oxa1 is not required for this interaction. The levels of Cox1 synthesized in Δoxa1 mitochondria are strongly reduced, so that its interaction to Mba1 cannot be assessed. However, Cox2 is efficiently synthesized, but no cross-linking to Mba1 was observed. Thus, Oxa1 is dispensable for the interaction of Mba1 with Cox3, but is required for the contact of Mba1 to Cox2. It is unlikely that this is due to the Cox2 processing defect in Δoxa1 mitochondria since the cross-linking efficiency of Cox2 to Mba1 was unaffected in Δimp1 mitochondria that also accumulate Cox2 precursor (Fig. 7 A, bottom).

Defects in Protein Export

To analyze the nature of the synthetic growth phenotypes observed for mba1 and oxa1 mutants, we measured the levels of activity of the bc1 and the Cox complexes of the respiratory chain in single and double deletion strains (Fig. 5, C and D). The absence of Mba1 alone led to a reduction in Cox activity by >90%, but did not affect bc1 activity. Δoxa1 mitochondria still contained ~30% of the wild-type bc1 activity, yet almost no activity was detectable in the double deletion mutant. Defects in the biogenesis of the F0 part of the F0F1-ATPase cause a strong reduction of the percentage of oligomycin-sensitive mitochondrial ATPase activity (Schatz, 1968). As shown in Fig. 5 E, deletion of both MBA1 and OXA1 reduced the level of oligomycin-sensitive ATPase to ~20%, which is similar to amounts found in rho0 strains, e.g., Δfzo1 (Rapaport et al., 1998), which do not contain any functional F0F1-ATPase (Ackerman and Tzagoloff, 1990). Thus, in the presence of Oxa1, Mba1 is dispensable for the biogenesis of the bc1 and ATPase complex, but Mba1 is absolutely required if Oxa1 is absent.

mba1 and oxa1 Mutations Cause Synthetic Defects of Respiratory Chain Enzymes

To analyze the nature of the synthetic growth phenotypes observed for mba1 and oxa1 mutants, we measured the levels of activity of the bc1 and the Cox complexes of the respiratory chain in single and double deletion strains (Fig. 5, C and D). The absence of Mba1 alone led to a reduction in Cox activity by >90%, but did not affect bc1 activity. Δoxa1 mitochondria still contained ~30% of the wild-type bc1 activity, yet almost no activity was detectable in the double deletion mutant. Defects in the biogenesis of the F0 part of the F0F1-ATPase cause a strong reduction of the percentage of oligomycin-sensitive mitochondrial ATPase activity (Schatz, 1968). As shown in Fig. 5 E, deletion of both MBA1 and OXA1 reduced the level of oligomycin-sensitive ATPase to ~20%, which is similar to amounts found in rho0 strains, e.g., Δfzo1 (Rapaport et al., 1998), which do not contain any functional F0F1-ATPase (Ackerman and Tzagoloff, 1990). Thus, in the presence of Oxa1, Mba1 is dispensable for the biogenesis of the bc1 and ATPase complex, but Mba1 is absolutely required if Oxa1 is absent.
To directly prove an Oxa1-independent function of Mba1, we tested whether overexpression of Mba1 allows the loss of Oxa1. Expression of Mba1 under control of the strong TPI1 promoter did not suppress the growth defect of a \( \Delta oxa1 \) mutant on glycerol, indicating that Mba1 overexpression does not make Oxa1 dispensable. However, overexpression of Mba1 clearly restored the competence of the mitochondria to integrate newly imported Oxa1 into the inner membrane (Fig. 7, B and C). In vitro–synthesized Oxa1 precursor was imported into wild-type, \( \Delta oxa1 \), or \( \Delta mba1 \) mitochondria. The samples were divided into three parts. Mitochondria (M) were then either mock treated, treated with proteinase K (PK), or converted to mitoplasts (MP) and proteinase K treated. Lane 1 shows 10% of the preprotein used per reaction. (C) The ratio of proteinase-accessible Oxa1 to total imported Oxa1 was quantified from four independent experiments and depicted in the diagram. Error bars show the standard deviation (\( n = 4 \)).

**Discussion**

The mitochondrial inner membrane belongs to the protein-richest membranes of the eukaryotic cell. It accommodates a large number of different integral membrane proteins which, typically assembled in multiprotein complexes, perform a variety of functions, like substrate transport or generation of ATP. Upon growth of mitochondria, these proteins have to be efficiently inserted into the inner membrane and the correct topology of each single transmembrane segment must be achieved. Many of these proteins are inserted from the matrix side and intermembrane space domains of these proteins have to be exported across the inner membrane. The OXA complex plays an important role in this insertion (or export) process. However, there may exist other additional components which act together with Oxa1 or independent of Oxa1 to facilitate the membrane insertion of proteins.

We have identified Mba1 as one such other component that is in contact with insertion intermediates as they integrate into the lipid bilayer. Like Oxa1, Mba1 is required for efficient protein insertion of both mitochondrially and nuclear-encoded proteins. Mba1 and Oxa1 overlap not only in substrate specificity, but most likely also in their function. In the presence of Oxa1, Mba1 is largely dispensable, indicating that Oxa1 can function independently of Mba1. In contrast, Mba1 is unable to replace Oxa1 completely even upon overexpression, but restores the defective insertion of the Oxa1 precursor in a \( \Delta oxa1 \) mutant. This partial suppression may be explained by the observation that Mba1 needs Oxa1 to interact with Cox2, whereas its interaction with Cox3 is independent of Oxa1.

Is Cox2 the only protein that exclusively depends on Oxa1 function? It has been shown that mutations in Cox2 suppress the growth defects observed in \( oxa1^{\mu} \) mutants (Meyer et al., 1997), and therefore the integration of other
proteins may not be strictly dependent on Oxa1 function. We showed that the absence of both Mba1 and Oxa1 cause a strong growth defect even on glucose. This synthetic defect points not only to an Oxa1-independent function of Mba1, but suggests that both components can act in parallel pathways which, if both are blocked, lead to severe problems in the biogenesis of mitochondria. The Δoxa1 Δmba1 double mutant is associated with an almost complete inactivation of both the respiratory chain and the F_{2}F_{0}-ATPase, which is known to lead to a reduced growth even on glucose probably due to a dissipation of cellular ATP levels by the uncoupled F_{2}-ATPase (Lai-Zhang et al., 1999).

What is the molecular function of Mba1? Translocation pathways use typically three different types of components: (a) receptors that make contact to the substrates, (b) pore forming proteins that mediate the translocation, and (c) chaperones that assist translocation intermediates or completely translocated proteins until they attain their folded and assembled state. It is not trivial to analyze the precise function of these components, and even in the case of Oxa1, which has now been studied for several years, the exact molecular role is not clear. The localization of Mba1 at the matrix side of the inner membrane and the following observations seem best compatible with a receptor function of Mba1: (a) Mba1 interacts with nascent chains during their synthesis on mitochondrial ribosomes; (b) this interaction is transient and restricted to an early stage in the biogenesis of substrate proteins; (c) a conditional Oxa1ps mutant protein loses function completely in a Δmba1 background, indicating that Mba1 cooperates with the Oxa1 translocase; and (d) overexpression of Mba1 can partially suppress the defects of Δoxa1 mitochondria, and thus Mba1 can improve protein insertion on an Oxa1-independent pathway. Hence, Mba1 might serve as a substrate receptor that delivers proteins both to Oxa1 and to a translocase in parallel. In the absence of Mba1, low local substrate concentrations at the inner membrane might abolish the function of the partially defective Oxa1ps protein. On the other hand, increased Mba1 concentrations might improve the recruitment of substrates to a so far uncharacterized translocase, resulting in the observed suppression of defects of Δoxa1 mitochondria.

Interestingly, the transcription of MBA1 is closely correlated with that of genes encoding subunits of the mitochondrial ribosome. In a comparison with 300 expression profiles, 7 out of the 9 best correlating transcripts to MBA1 were for mitochondrial ribosomal proteins: MRPS9, MRPL24, MRPL6, YDR115w (L34), MRP21, MRPS28, and MRPL9 showed correlation coefficients higher than 0.7 (Hughes et al., 2000). This correlation of ribosomes and Mba1 may ensure an adequate insertion capacity for the level of proteins produced in mitochondria.

Together with Oxa1, Mba1 was first isolated as a protein that upon overexpression suppressed mutations of the inner membrane protease Yta10 (Afg3). What might be the reason for this interaction? It was shown recently that the degradation of membrane proteins by inner membrane proteases requires the active extraction of transmembrane segments (Leonhard et al., 2000). How this extraction or “deinsertion” is achieved is unclear, but it is conceivable that insertion machineries play a crucial role in this process. Increased levels of these machineries in the suppressor strains might improve degradation in the yta10 mutants, perhaps by allowing degradation by the Yta10 homologue Yme1 which faces the intermembrane space.

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