MBA1 encodes a mitochondrial membrane-associated protein required for biogenesis of the respiratory chain

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Abstract **The yeast *MBA1* gene (*M*ulti-copy *B*ypass of *A*FG3) is one of three genes whose overexpression suppresses *afg3-null* and *rca1-null* mutations. Bypass of *AFG3* and *RCA1*, whose products are essential for assembly of mitochondrial inner membrane enzyme complexes, suggests a related role for *MBA1*. The predicted translation product is a 30 kDa hydrophilic protein with a putative mitochondrial targeting sequence and no homology to any sequence in protein or EST databases. Gene disruption leads to a partial respiratory growth defect, which is more pronounced at temperatures above 30°C. Concomitantly, amounts of cytochromes *b* and *aa3* are reduced. A C-terminal c-*myc*-tagged *MBA1* gene product is functional and is found associated with the mitochondrial inner membrane, from which it can be extracted by carbonate, but not by high salt. These observations give further support to a role of *MBA1* in assembly of the respiratory chain.**

Key words: Respiratory chain; Assembly; Cytochrome; *AFG3*; *RCA1*; *Saccharomyces cerevisiae*

1. Introduction

About half of the proteins of the mitochondrial inner membrane form part of enzyme complexes involved in oxidative phosphorylation [1]. The biogenesis of these complexes requires regulation of mitochondrial and nuclear gene expression, involving a host of nuclear-encoded factors acting at different levels [2]. Some of these factors act at a post-translational, post-sorting level; these affect assembly of subunits into holoenzymes and/or their degradation. The *AFG3 (YTA10)* gene of *S. cerevisiae* has been implicated in both these processes; it is required for degradation of prematurely terminated and complete mitochondrial translation products [3,4] and for assembly of complexes III, IV and V [5]. Afg3p belongs to the FtsH subgroup of the AAA family of ATPases [6,7]. A direct role in protein breakdown of members of this subgroup, which contains mitochondrial and prokaryotic members, is supported by conserved sequence motifs that suggest ATP-dependent metallo-endoprotease activity [8]. Such an activity has been demonstrated in vitro for *E. coli* FtsH [9]. However, a mutation in the metalloprotease active site motif of *AFG3* that strongly reduces proteolysis does not affect growth on non-fermentable carbon sources, suggesting that the involvement of Afg3p in assembly of enzyme complexes of the mitochondrial inner membrane is independent of its protease activity [4].

In an effort to obtain more information on *AFG3* function, we isolated multi-copy suppressor genes of an *afg3-null* mutation. By examining the function of genes that can bypass *AFG3* when overexpressed, we aimed to better define the primary defect caused by inactivation of *AFG3*. One of these *afg3-null* suppressors is *MBA1* (Multi-copy Bypass of *AFG3*), whose sequence was identified previously in the yeast sequencing project (*YBR1307* in [10]), but whose function was unknown. Here, we present a further characterization of *MBA1* and its gene product. Our results suggest that *MBA1*, like *AFG3* and *RCA1*, affects assembly of the respiratory chain of yeast mitochondria.

2. Materials and methods

2.1. *Strains of* S. cerevisiae *and growth media*

The wild-type *Saccharomyces cerevisiae* strains used in this study are W303/1A (*MATa, ade2-1, his3-11,-15, leu2-3,-112, ura3-1, trp1-1, can1-100*) [11] and D273UK (*MATα, met6, ura3, lys2*) [12]. DDM1 (*MATα, met6, ura3, lys2, mba1::URA3*) was derived from D273UK by disruption of the *MBA1* gene with *URA3* (see Fig. 2).

The following media were used for the propagation of yeast: YPD (2% glucose, 1% peptone, 1% yeast extract); lactate (1.5% lactic acid, 2% sodium lactate, 8 mM $MgSO_4$, 45 mM $(NH_4)_2HPO_4$, 0.5% yeast extract); WO (2% glucose, 0.67% yeast nitrogen base without amino acids (Difco)). Where required, media were supplemented with nutritional requirements to the appropriate concentrations. Solid media contained 2% agar.

2.2. *Construction of an* MBA1-c-myc *fusion gene*

For the construction of an *MBA1*-c-*myc* fusion gene we made use of the vector YEpmyc181 which contains a synthetic c-*myc* epitope cloned in the polylinker [13]. Plasmid pJN-C4 contains a 2.9 kb genomic DNA fragment which includes the *MBA1* gene (Rep et al., submitted). A 1.75 kb fragment containing *MBA1* with an *Xma*I linker at its 3′ end was generated by PCR using plasmid pJN-C4 as the template and the following primers: M13 (GTTTTCCCAGTCACGAC) and MBA#1 (TAATCCCGGGGGCTTGGAGGTAAACG; *Xma*I linker underlined). This fragment was cut with *Xma*I and cloned into *Xma*I-linearized YEpmyc181, yielding pE-MBAcmyc. The correct orientation was verified by restriction analysis and the in-frame fusion of *MBA1* to c-*myc* coding sequences was confirmed by sequence analysis. pC-MBAcmyc was created by subcloning a *Bam*HI-*Nar*I fragment containing the *MBA1*-c-*myc* fusion gene into the low-copy vector YCplac111.

2.3. *Isolation and fractionation of mitochondria*

Cells were grown until early stationary phase in lactate medium. Mitochondria were isolated by lysis of spheroplasted cells in breaking buffer (BB; 0.65 M sorbitol, 20 mM KP_i pH 7.5, 1 mM $MgCl_2$, 1 mM EDTA), using a Dounce homogenizer. Unbroken cells and cell debris were removed by a low-speed spin (4000 rpm for 5 min in a Sorvall SS-34 rotor). Mitochondria were pelleted by a high-speed spin (12 000 rpm for 10 min), washed once in BB and suspended in BB to a protein concentration of 10 mg/ml. Before application on gel, mitochondrial proteins were always precipitated with trichloroacetic acid (TCA) and resuspended in Laemmli sample buffer (LSB) [14]. Proteins in the supernatant from the high-speed spin were precipitated with TCA and resuspended in LSB; this is the cytoplasmic fraction. To fractionate mitochondria into membrane and soluble fractions, they were suspended in 10 mM Tris-HCl pH 7.4, 1 mM EDTA and sonicated

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6 times for 5 s at 0°C, followed by centrifugation at 165 000 × *g* for 60 min in a Beckman type SW50.1 rotor. Pellet and supernatant represent the membrane and soluble fractions, respectively. Alternatively, mitochondria were pelleted and resuspended in 0.1 M $NaCO_3$ to a protein concentration of 1 mg/ml and left on ice for 45 min with occasional vortexing. Membrane and soluble fractions were separated as above. A mix of protease inhibitors (1 mM PMSF, 1 mM EDTA, 2 μg/ml pepstatin, 2 μg/ml chymostatin) was added before fractionation of mitochondria to avoid protein breakdown.

Mitoplasts were obtained by diluting the mitochondrial suspension 3.25-fold in 20 mM Tris-HCl pH 7.5, leaving the suspension for 10 min on ice, and sedimenting the mitoplasts at 15 000 rpm for 15 min in a Heraeus Sepatech Biofuge 17RS. For treatment with proteinase K (0.33 mg/ml; 30 min at 0°C), mitochondria or mitoplasts were suspended in BB to a protein concentration of 5 mg/ml. After TCA precipitation, samples corresponding to 75 μg of mitochondrial protein were applied on gel.

2.4. Western blot analysis

Protein electrophoresis was carried out according to Laemmli [14]. HRP-conjugated secondary antibodies were used for immunoblotting as described in the Promega Protocols and Applications Guide, second edition, 1991. A monoclonal antibody for detection of the human c-*myc* epitope was obtained form ICI, Cambridge Biochemicals (clone 9E10). Polyclonal antibodies raised against hexokinase, the mitochondrial ADP/ATP carrier and the β subunit of ATP synthase were used.

2.5. Miscellaneous

Standard methods were used for manipulation of DNA, transformation of *E. coli* (strain DH5α) and Southern blotting [15]. Transformation of yeast cells was done according to [16], DNA sequence analysis by the method of Sanger [17].

3. Results

3.1. Characterization of an MBA1-disruption strain

The *MBA1* gene was isolated as a multi-copy suppressor of the respiratory growth defect caused by an *afg3-null* mutation. The gene also suppresses *rca1-null* and *afg3/rca1* double mutations (data not shown). *MBA1* encodes a putative 278 amino acid protein whose N-terminal sequence has all the characteristics of cleavable mitochondrial targeting sequences [10] (Fig. 1). The PSORT program [18] yields the mitochondrial matrix as the location of highest probability (certainty = 0.807) and does not list any non-mitochondrial localization. To date, no sequences resembling *MBA1* have been found in any database examined (checked using XDBRef [19] and Fastalert (Biocomputing Basel)). To learn more about its function, *MBA1* was disrupted by insertion of a 1.1 kb DNA fragment containing the *URA3* gene into the *Bgl*II site close to the 5′ end of the open reading frame (Fig. 2). The possibility that the remaining coding sequence is expressed into a functional protein is highly unlikely since the first ATG that could serve to initiate translation is codon 183. Moreover, we have seen that even short N- or C-terminal truncations lead to loss of *afg3-null* suppressor activity (Rep, unpublished observations). The disruption construct was used to inactivate *MBA1* in the wild-type strain D273UK. The resulting strain shows a reduced growth rate on non-fermentable carbon sources, which is more pronounced at high temperature (Table 1). This correlates with reduced levels of cytochromes *b* and *aa3* in mitochondria isolated from the *mba1::URA3* strain (Fig. 3), indicating that Mba1p is involved in biogenesis of respiratory chain complexes. Cytochrome *aa3* is more sensitive to growth at 35°C than cytochrome *b*. Cytochrome *c* oxidase levels may therefore be limiting for respiratory growth at this temperature.

Table 1
Generation times of D273UK (wild-type) and DDM1 (*mba1::URA3*) with lactate as carbon source

	Generation time (h)	
	at 30°C	at 35°C
D273UK	2.5	3.2
DDM1	3.5	5.7

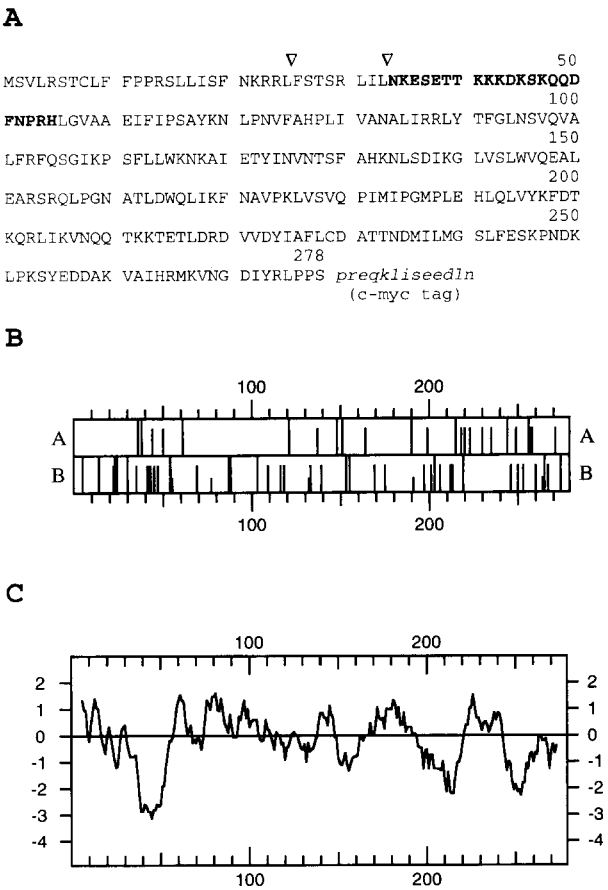

Fig. 1. Sequence characteristics of the Mba1 protein. Mba1p is a 278 amino acid protein (panel A) with a putative mitochondrial targeting sequence (cleavage sites predicted by PSORT [18] are indicated by arrowheads). The acidic and basic amino acid side chain map (panel B; A: acidic side chains, B: basic side chains) and hydropathy profile according to Kyte and Doolittle (panel C; high values indicate hydrophobic regions) were generated with DNA Strider (C. Marck, CEA-FRANCE). The most obvious feature beside the putative mitochondrial targeting sequence is a very hydrophilic region of 22 amino acids following the putative targeting sequence (indicated with bold in panel A). The c-*myc* epitope present in Mba1-cmyc is indicated by italics in panel A.

3.2. Epitope-tagged Mba1p localizes to the mitochondrial inner membrane

For determination of the subcellular location of Mba1p, a c-*myc* epitope was added to its C-terminus, allowing visualization of the fusion protein with anti-c-*myc* antibody (see Section 2). Mba1-cmyc is likely to have the same location as Mba1p, as it suppresses equally well the *afg3-null* mutation (not shown). Yeast strains transformed with either a high or low copy number plasmid encoding Mba1-cmyc contain a protein of 30 kDa in the mitochondrial fraction that is recog-

nized by the anti-c-*myc* antibody (Fig. 4A). This corresponds to the predicted size of Mba1-cmyc (33.3 kDa) minus a putative cleavable presequence of about 3 kDa.

As detection in strains carrying a low copy *MBA1* plasmid was difficult, we proceeded with the strain carrying the high copy *MBA1* plasmid to identify the submitochondrial location of Mba1-cmyc. Fig. 4B shows that Mba1-cmyc is present in the membrane fraction upon sonication of mitochondria, and can only be extracted from the membrane with carbonate, but not with NaCl in concentrations up to 1 M. Even with carbonate, part of Mba1-cmyc remains in the membrane fraction, while the F1β subunit of ATP synthase is completely solubilized (Fig. 4B, last 2 lanes). Furthermore, Mba1-cmyc was protected in mitoplasts from externally added proteinase K, under conditions where the ADP/ATP carrier (AAC2) is sensitive to proteolysis (Fig. 4C). The fact that most Mba1-cmyc was extracted with carbonate precludes a transmembrane topology, in line with the absence from the sequence of a hydrophobic region of sufficient length to span the membrane (Fig. 1). We conclude that Mba1p is firmly attached to the inner side of the mitochondrial inner membrane when expressed from a multi-copy plasmid; the condition under which it is active as a suppressor of *afg3-null*. In view of the effect of Mba1p depletion on the levels of inner membrane complexes, this location is in agreement with its presumed site of action under normal conditions. However, we cannot exclude the possibility that overproduction of Mba1p influences its location, for example by inducing membrane association.

4. Discussion

We show here that *MBA1*, a multi-copy suppressor of *afg3-null*, encodes a mitochondrial protein of about 30 kDa that is required for biogenesis of the respiratory chain. As mitochondrial protein synthesis is not affected in an *MBA1* disruption strain (not shown), Mba1p is likely to act post-translationally. Thus, it can be added to the list of proteins that act at a post-translational level in the assembly of mitochondrial inner membrane complexes, where it falls within the category of factors that affect the assembly of more than one complex [2]. Other proteins in this category are *MRS2* [20], *OXA1* [21,22], *AFG3*, *RCA1* [5] and *YME1* [23,24]. The latter three belong to the same subfamily of the AAA family of ATPases [6,7] with (putative) ATP-dependent metalloprotease activity (see Section 1). Interestingly, as a rule, complex IV is more affected than complex III in strains mutated for either of the above genes. Like *YME1*, *MBA1* is more important for respiratory growth at high temperature.

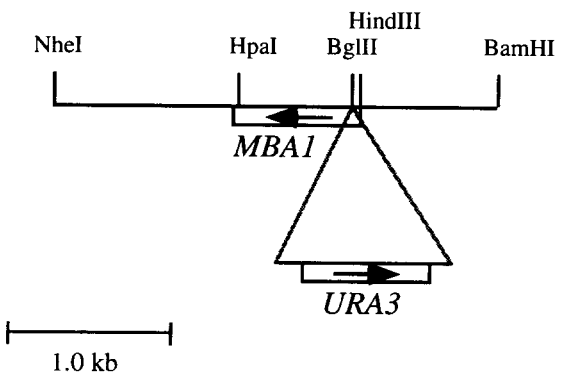

Fig. 2. Disruption of *MBA1*. The *MBA1* gene, present on a 2.9 kb genomic DNA fragment in pUC18, was disrupted by insertion of a 1.2 kb *URA3* fragment from pfl38 into the *Bgl*II site near the 5′ end of the open reading frame. Transformation of the linearized construct to wild-type strain D273UK yielded DDM1 (α, *met6, ura3, lys2, mba1::URA3*).

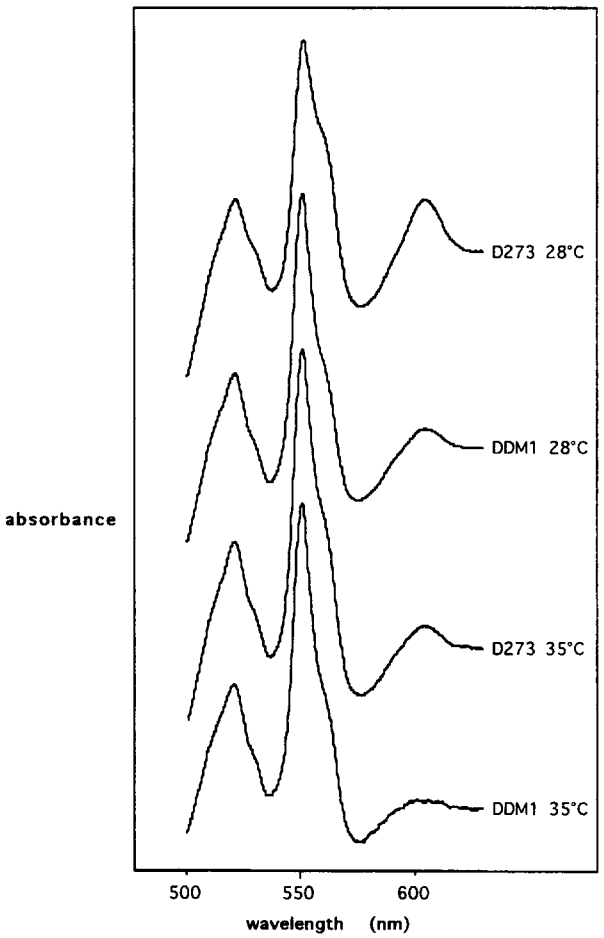

Fig. 3. Spectral analysis of mitochondria from wild-type and *mba1::URA3* cells. Mitochondria isolated from cells grown in Lac medium at either 28°C or 35°C were suspended in 100 mM KP_i pH 7.4, 250 mM sucrose, 0.5% cholate to a protein concentration of 2–4 mg/ml. Difference spectra were recorded at room temperature with an Aminco DW2000 spectrophotometer after oxidizing one half of the suspension with ferricyanide and reducing the other half with sodium dithionite. Cytochrome *c* and *c1* absorb maximally at 550 nm, cytochrome *b* at 562 nm and cytochrome *a* and *a3* at 605 nm. D273: wild-type (D273UK), DDM1: mutant (*mba1::URA3*).

Although the exact molecular function of Mba1p has yet to be resolved, our finding that Mba1p can associate with the matrix side of the inner membrane is consistent with a direct involvement in assembly processes. A similar function has been proposed for *OXA1*, which was also found as a multi-copy suppressor of *afg3-* and *rca1-null* mutations (Rep et al., manuscript in preparation). Disruption of *OXA1* leads to several mitochondrial defects: loss of cytochrome *aa3* and reduction of cytochrome *b*, an RNA splicing and/or stability defect [21], a block in processing of CoxII and an overall reduction of mitochondrial biogenesis [22]. In contrast to *MBA1*, *OXA1* has the characteristics of a membrane-spanning protein, and homologues have been found in the plasma membrane of

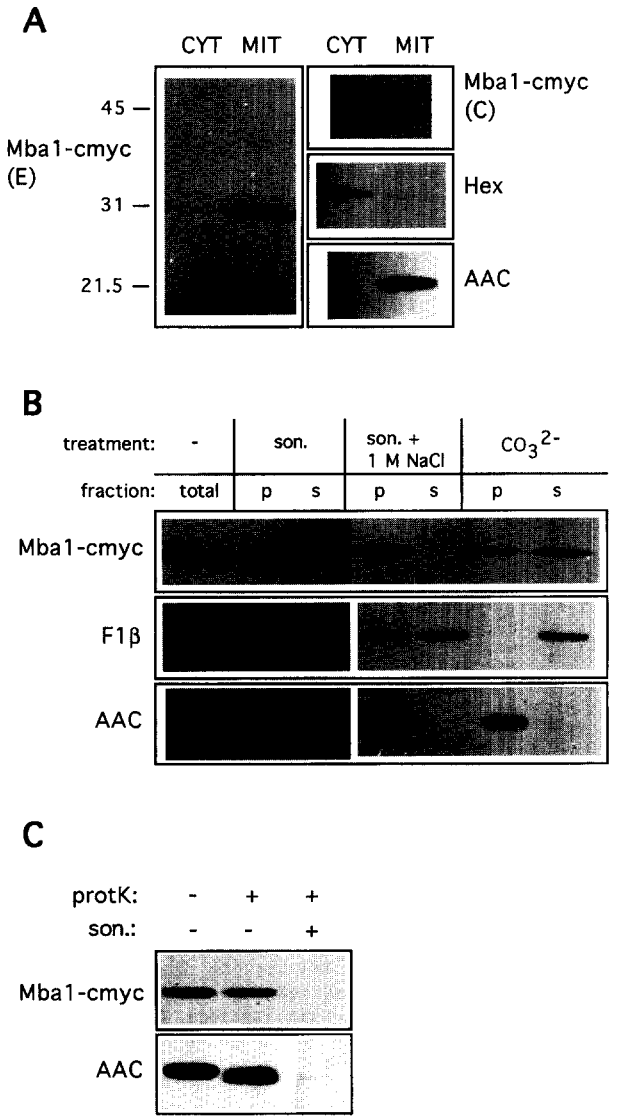

Fig. 4. Mba1-cmyc localizes to the matrix side of the mitochondrial inner membrane. Mitochondria from wild-type strain W303/1A transformed with pC-MBAcmyc (C) or pE-MBAcmyc (E) were isolated and fractionated as described in Section 2. Samples were separated on 15% SDS-polyacrylamide gels, blotted and probed with antibodies. A: Detection of Mba1-cmyc in the mitochondrial fraction. Mba1-cmyc expressed from low copy plasmid pC-MBAcmyc was only detected when applying five times the standard amount of protein to the gel (375 μg instead of 75 μg; top right panel). Antibodies against hexokinase (Hex) and the major ADP/ATP carrier of the inner membrane (AAC) mark the cytoplasmic and mitochondrial fraction of pE-MBA1cmyc transformed cells, respectively. B: Mitochondria were fractionated into membrane (p) and soluble (s) fractions either by sonication (son.) in the absence or presence of 1 M NaCl, or by carbonate extraction (CO_3^{2-}). F1β: blot probed with antiserum raised against the β-subunit of F1-ATPase. C: Detection of Mba1-cmyc and AAC in mitoplasts after incubation with (+) or without (−) proteinase K. As a control for the sensitivity of Mba1-cmyc towards proteinase K, mitoplasts were sonicated after addition of proteinase K and prior to incubation (son.), allowing proteolysis of proteins present in the matrix.

bacteria [21]. As the protease function of Afg3p is not required for mitochondrial biogenesis [4], suppression of *afg3-null* by *MBA1* and *OXA1* is not likely to be mediated by stimulation of protein degradation. Rather, they appear to bypass the role of Afg3p in complex assembly.

The suppression of *afg3-* and *rca1-null* by *MBA1* and *OXA1*, together with the fact that mutations in *AFG3*, *RCA1*, *MBA1* and *OXA1* all result in reduced assembly of several inner membrane enzyme complexes, and the likelihood that all encode proteins that are themselves associated with the inner membrane, suggests that these genes co-operate in the biogenesis of inner membrane complexes.

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