

Identification of the essential yeast protein MIM17, an integral mitochondrial inner membrane protein involved in protein import

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Abstract

We analyzed four *Saccharomyces cerevisiae* mutants defective in mitochondrial protein import and found that they are complemented by a novel gene encoding a 17 kDa protein. The protein is integrally located in the mitochondrial inner membrane and is termed MIM17. It shows significant homology to MIM23/Mas6p, a previously identified mitochondrial inner membrane protein required for the import of preproteins. Like MIM23, the precursor of MIM17 is synthesized without a presequence. A deletion of *MIM17* is lethal. MIM17 thus joins the small group of mitochondrial proteins that are essential for the viability of yeast. We propose that MIM17 is an essential component of the preprotein import machinery of the mitochondrial inner membrane.

Key words: Mitochondrial inner membrane; MIM17; Protein translocation; Preprotein

1. Introduction

Import of preproteins into mitochondria involves translocation of the polypeptide chains across two membranes, the mitochondrial outer membrane and inner membrane. Each membrane contains an import machinery that mediates the selective passage of the preproteins. Originally it was assumed that both import machineries were stably connected by a sealed channel at membrane contact sites. We now know that both machineries can function independently of each other. Preproteins in transit from the outer membrane to the inner membrane pass through the intermembrane space, and a membrane potential $\Delta\psi$ is only needed for preparation translocation across the inner membrane [1–5]. Characterization of the structure and function of these transport machineries is a central aspect of the studies on mitochondrial biogenesis.

The mitochondrial outer membrane contains a high molecular weight complex, the mitochondrial receptor complex, that is responsible for the specific recognition and membrane translocation of preproteins. It consists of at least eight different mitochondrial outer membrane (MOM) proteins: the two import receptors MOM19 (MAS20) and MOM72 (MAS70); the transfer protein MOM22; and the general insertion pore GIP which is formed by MOM38 (ISP42), MOM30, MOM8, MOM7, and ISP6p [6–10].

The detailed information available on the MOM machinery is contrasted by the limited knowledge reported about components of the mitochondrial inner membrane import machinery (MIM). The MIM machinery must translocate hundreds of different preproteins, but is selective enough that the inner membrane maintains a proton gradient even during the translocation of polypeptide chains [11,12]. By a genetic screen for *S. cerevisiae* mutants defective in mitochondrial protein uptake, we recently identified two genes encoding mitochondrial inner membrane proteins of 23 and 44 kDa (MIM23 and MIM44) [13–15]. With an independent genetic selection, Emtage and Jensen [16] identified Mas6p that turned out to be identical to MIM23. Using a polyspecific antiserum, the inner membrane protein ISP45 was characterized and was found to be identical to MIM44 [17,18]. MIM23/Mas6p and MIM44/ISP45 are essential for the viability of yeast, and the available evidence indicates that they are directly involved in the import of preproteins. It was not known if they represented the only components of the inner membrane import machinery.

Here we present new *S. cerevisiae* mutants defective in mitochondrial protein import. The gene complementing the mutants encodes an integral protein of the mitochondrial inner membrane of 17 kDa, termed MIM17. MIM17 is homologous to MIM23 and is essential for the viability of yeast. We propose that MIM17 is a new component of the protein import machinery of the mitochondrial inner membrane.

2. Materials and methods

2.1. Yeast strains

Yeast strains used in this study are listed in Table 1. The *MIM17*

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Abbreviations: MIM17, MIM23, MIM44, mitochondrial inner membrane proteins of 17, 23 and 44 kDa, respectively; mt-hsp70, heat shock protein of 70 kDa in the mitochondrial matrix; *S. cerevisiae*, *Saccharomyces cerevisiae*.

mutant allele in strain MB3-33 is termed *mim17-1*. The mutant strains MB3-31, MB3-56 and MB3-81 have the same genotype as MB3-33 except that the *MIM17* alleles have not been named so far. The heterozygous *MIM17* diploid MB2-3 was obtained by transforming MB2 with a 5.8 kb *XbaI*–*PstI* fragment from pUC18-*mim17::LYS2* and selecting a *Lys*⁺ transformant with one disrupted *MIM17* allele by Southern blot analysis.

MB16 was constructed by transformation of YCplac111-*MIM17-c-myc* into MB2-3 and selecting a meiotic segregant harbouring the *mim17* nuclear deletion (marked by *LYS2*) and the rescuing plasmid (marked by *LEU2*).

2.2. DNA manipulations

The SOD-URA test plasmid used for selection of yeast import mutants was described previously [13]. UCplac111::*ADH1p-MIM17* (Fig. 1A, construct 7) was generated in two steps. Firstly, a 0.7 kb *EcoRV*–*Bam*HI fragment, liberated from pBPH1 [19], was cloned into YCplac111 [20], restricted with *Sma*I and *Bam*HI. Secondly, this plasmid, YCplac111::*ADH1p*, was linearized with *Sma*I and ligated with the blunt-ended 1045 bp *Hinf*I–*Hinc*II *MIM17* fragment. The corresponding YCplac111 derivative without the *ADH1* promoter was obtained by deletion of the 723 bp *Eco*RI fragment from YCplac111::*ADH1p-MIM17* (Fig. 1A, construct 6). The *Bst*EII–*Hinc*II subclone with the T-to-A transversion at position 1115 (Fig. 1A, construct 8), was obtained in two steps. Firstly, the 1360 bp *Xba*I–*Hind*III *MIM17* fragment was cloned into pSELECT-1 and mutagenized using the Altered Sites in vitro mutagenesis system (Promega). Secondly, the 194 bp *Nco*I fragment of the wild-type *Bst*EII–*Hinc*II subclone (Fig. 1A, construct 4) was replaced with the corresponding *Nco*I fragment bearing the mutation.

pUC18-*mim17::LYS2* was constructed by cloning a blunt-ended 1140 bp *Bst*EII–*Hinc*II *MIM17* fragment into the *Hinc*II site of pUC18 and replacing the 194 bp *Nco*I *MIM17* fragment with the blunt-ended 4858 bp *Xba*I–*Hind*III *LYS2* fragment liberated from pDP6 [21]. To construct the *MIM17-c-myc* fusion gene, the *Xba*I–*Hind*III fragment, carrying the complete *MIM17* coding sequence, was blunt-ended with T4 DNA polymerase and cloned into the blunt-ended *Bam*HI site of YCpmyc111 [22], creating YCplac111-*MIM17-c-myc*.

To synthesize *MIM17* in vitro, a blunt-ended 1045 bp *Hinf*I–*Hinc*II *MIM17* fragment was cloned into the *Sma*I site of the pEP30 transcription vector [13]. The pEP30-*MIM17* construct was linearized with *Bam*HI before transcription with T7 RNA polymerase.

2.3. Isolation and subfractionation of mitochondria

The following procedures were performed as published: growth of *S. cerevisiae*, isolation of mitochondria, and formation of mitoplasts by swelling [14,23,24]; preparation of submitochondrial vesicles and separation into outer membrane and inner membrane fractions [14,25]; treatment with 100 mM Na₂CO₃ (pH 11.5) and separation of supernatant and membrane pellet [14,26]; treatment with protease [27,28].

2.4. Import of the precursor of *MIM17* into isolated mitochondria

After in vitro transcription with T7 RNA polymerase, the precursor of *MIM17* was synthesized in rabbit reticulocyte lysates in the presence of [³⁵S]methionine [28]. Import into isolated mitochondria was performed in the presence of 1 mM ATP, 8 mM potassium ascorbate, and 0.2 mM *N,N,N',N'*-tetramethylphenylenediamine (TMPD), and bovine serum albumin containing buffer as described [9,14,28,29]. Where indicated, the mitochondria were treated with protease (trypsin or proteinase K) [28]. The mitochondria were re-isolated, washed, and the proteins were separated by SDS-PAGE. For dissipation of the mem-

brane potential, energy substrates were omitted and 0.5 μM valinomycin, 8 μM antimycin A, and 20 μM oligomycin were included [27].

2.5. Miscellaneous

The following procedures were performed essentially according to published methods: manipulation of nucleic acids [30]; DNA sequence analysis with chain elongation inhibitors [31]; EMS mutagenesis and sporulation of diploid yeast cells [32]; transformation of yeast cells [33]. Standard procedures were used for SDS-PAGE, transfer to nitrocellulose, immunodecoration, autoradiography, fluorography, and scanning densitometry [28,34].

3. Results and discussion

We previously devised a genetic screen for *S. cerevisiae* mutants with defects in mitochondrial protein import [13]. The approach was based on the mislocalization of a cytosolic enzyme into an organelle and the selection of import mutants that prevented the mislocalization [35]. Briefly, the yeast strain MB3 with a deletion of the chromosomal *URA3* gene was transformed with a plasmid encoding a chimeric protein which consisted of a mitochondrial matrix targeting sequence and the *URA3* gene product (orotidine 5'-phosphate decarboxylase). The chimeric protein was efficiently targeted into the mitochondrial matrix and thus the transformed strain remained a uracil auxotroph like the parent strain MB3. The cells were mutagenized with ethylmethanesulfonate. Mutations that impair mitochondrial protein import are expected to allow growth of the transformed strain also in the absence of added uracil. The mutants obtained were then selected for temperature-sensitivity of growth. To eliminate mutants arising from the defective targeting information of the chimeric protein mutant strains were cured of the plasmid and tested again after retransformation with non-mutagenized plasmid DNA. The analysis of two complementation groups of recessive nuclear mutations led to the identification of the mitochondrial inner membrane proteins *MIM44* and *MIM23* [13–15]. A third complementation group was represented by mutants of the gene for the heat shock protein hsp70 in the mitochondrial matrix [15,36].

Here we report on new mutants found in this screen (MB3-31, MB3-33, MB3-56, MB3-81) that represent a fourth complementation group. To identify the mutated gene affecting mitochondrial protein import, the mutant strain MB3-33 was transformed with a library of yeast

Fig. 1. Primary sequence of *MIM17*. (A) Identification of the *MIM17* gene. The indicated fragments from the p366 clone (construct 1) were subcloned into the centromeric vector YCplac111 (constructs 2–6,8). The *Hinf*I–*Hinc*II (isoschizomer of *Sal*I) fragment was also cloned downstream of the *ADH1* promoter in YCplac111::*ADH1p* (construct 7). Thick arrows indicate overlapping reading frames in the *Bst*EII–*Hind*III region. The ability to complement the *Ura*⁺ phenotype of mutant MB3-33 harbouring the SOD-URA test plasmid is indicated by + (full complementation), – (no complementation) or –/(+) (very weak complementation). B, *Bam*HI; Bs, *Bst*EII; H3, *Hind*III; N, *Nco*I; P, *Pst*I; Pv, *Pvu*I; R1, *Eco*RI; R5, *Eco*RV; S, *Sal*I; Sa, *Sau*3A; X, *Xba*I; *T-to-A transversion at position 1115. (B) Nucleotide sequence of the *MIM17* gene and derived amino acid sequence (single letter code). The accession number for the sequence reported here is X77796 in the EMBL/GenBank/DBJ data banks.

[illegible]

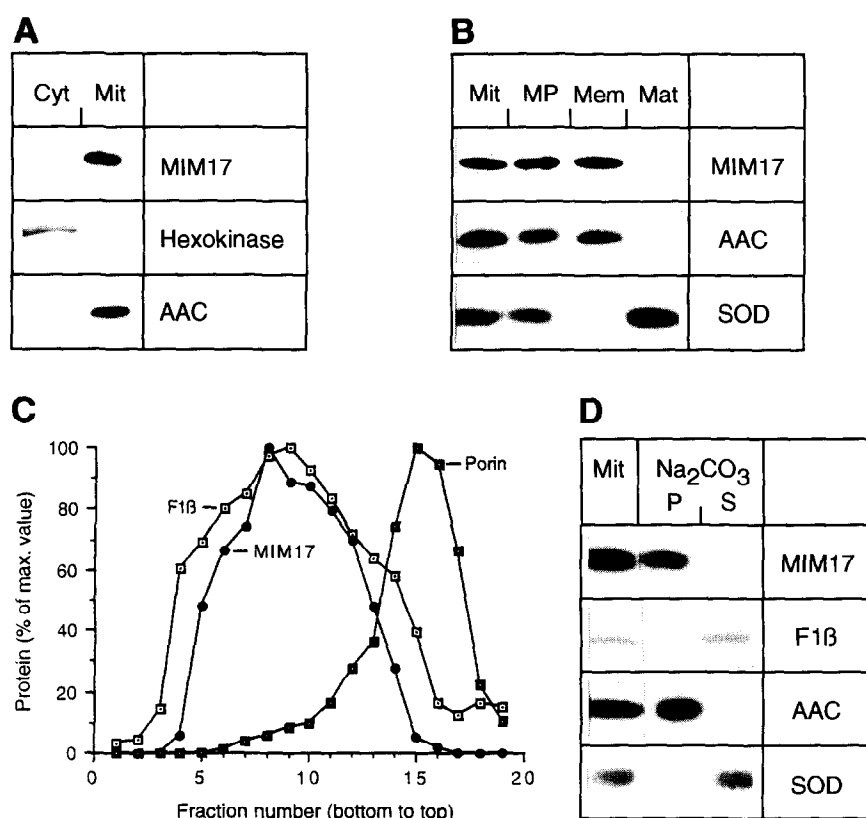


Fig. 2. MIM17 is an integral protein of the mitochondrial inner membrane. (A) MIM17 is located in mitochondria. Yeast cells from the strain MB16 (containing MIM17 with a human c-Myc epitope) were fractionated. A cytosolic fraction (Cyt) and a mitochondrial fraction (Mit) were analyzed by SDS-PAGE and immunodecoration with antibodies directed against c-Myc (i.e. recognizing the tagged MIM17), hexokinase, and ADP/ATP carrier (AAC). (B) Association of MIM17 with the mitochondrial membranes. Mitochondria were swollen to form mitoplasts (MP) and were then separated by sonication and centrifugation into membranes (Mem) and matrix fraction (Mat). SOD, superoxide dismutase. (C) Fractionation of MIM17 with the mitochondrial inner membrane. Submitochondrial membrane vesicles were separated on a linear sucrose gradient (0.85–1.6 M sucrose). The fractions were analyzed by SDS-PAGE and immunodecoration. F1 β , F₁-ATPase subunit β . (D) MIM17 is not extracted from the membranes at pH 11.5. Mitochondria were treated with 100 mM Na₂CO₃. Pellets (P) and supernatants (S) were separated by centrifugation.

genomic DNA in the centromeric shuttle vector p366 [37]. Uracil auxotrophic cells, i.e. cells that efficiently imported the chimeric protein into mitochondria, were selected by growth on medium containing glucose, uracil and 5-fluoro-orotic acid (cells containing orotidine 5'-phosphate decarboxylase activity in the cytosol convert 5-fluoro-orotic acid to the toxic 5-fluoro-uracil and thus die) [38]. A clone with 8.2 kb genomic DNA and several subclones derived thereof were able to complement the mutant MB3-33 and also the mutant strains MB3-31, MB3-56, and MB3-81, indicating that the complement-

ing gene was located on a 615 bp *Bst*EII–*Hind*III fragment (Fig. 1A). The nucleotide sequence of this fragment revealed two overlapping open reading frames on opposite strands, encoding putative proteins of 158 amino acids (16.6 kDa; ORF16.6) and 88 amino acids (9.3 kDa; ORF 9.3), respectively. The following evidence demonstrates the ORF16.6 contains the complementing activity. (i) A *Hinf*I–*Sal*I subclone lacking most of the 5'-flanking sequences of ORF16.6 (except of 50 bp), but retaining 600 bp of 5'-flanking sequence of ORF 9.3, showed only very weak complementing activity

Table 1
S. cerevisiae strains used in this study

Strain	Genotype	Source
MB3	<i>MATα ade2-101 his3-Δ200 leu2-Δ1 lys2-801 ura3::LYS2 rho⁺</i>	[13]
MB3-33	<i>MATα ade2-101 his3-Δ200 leu2-Δ1 lys2-801 ura3::LYS2 mim17-1 rho⁺</i>	This study
MB2	<i>MATα ade2-101/ADE2 his3/his3-Δ200 leu2/leu2-Δ1 lys2-801/lys2-801 trp1-289/TRP1 ura3-52/ura3-52 rho⁺</i>	[13]
MB2-3	<i>MATα ade2-101/ADE2 his3/his3-Δ200 leu2/leu2-Δ1 lys2-801/lys2-801 trp1-289/TRP1 ura3-52/ura3-52 mim17::LYS2/MIM17 rho⁺</i>	This study
MB16	<i>ade2-101 his3 leu2 lys2-801 trp1-289 ura3-52 mim17::LYS2 + YCplac111-MIM17-c-myc (LEL2) rho⁺</i>	This study

(Fig. 1A). When the yeast *ADH1* promoter was placed in front of ORF16.6, full complementation was restored, suggesting that the reduced complementation activity of the *HinfI*–*Sall* fragment was caused by lack of promoter activity of ORF16.6. (ii) A premature stop codon was introduced at codon 19 of ORF9.3, leading to a codon for tyrosine instead of phenylalanine at position 75 of ORF16.6 (Fig. 1A). Full complementation was observed, strongly suggesting that ORF16.6 was responsible for the complementing activity. (iii) As shown below, ORF16.6 encodes a protein of 17 kDa that is located in the mitochondrial inner membrane and is named MIM17.

Fig. 1B shows the nucleotide sequence of the gene and derived amino acid sequence of MIM17. The amino-terminal region of MIM17 has a net negative charge and thus does not show the typical feature of a positively charged mitochondrial targeting sequence (presequence) [39,40]. A computer search did not reveal a significant homology of MIM17 to any known protein except to the hydrophobic domain of MIM23/Mas6p, including four hydrophobic segments, that will be discussed in the accompanying paper [41]. *MIM17* is located on the opposite strand and 389 bp downstream of the gene *YAK1*, encoding a protein kinase involved in cell-cycle regulation [42]. *YAK1* and thus also *MIM17* are located on chromosome X [43].

To determine whether *MIM17* encodes an essential mitochondrial protein, one of the two copies of the *MIM17* gene in the homozygous *lys2* diploid strain MB2 was disrupted with the *LYS2* gene. Cells of the resulting strain MB2-3 were sporulated and the spores dissected. Each tetrad yielded maximally two viable spores on both non-fermentable and fermentable carbon sources. All viable spores were *Lys*[−], indicating that spores carrying the null allele of *MIM17* were inviable. When the diploid MB2-3 cells were transformed with a centromeric plasmid containing either wild-type *MIM17* or *MIM17* with the mutation described above (introducing a premature stop in ORF9.3), sporulation yielded both *Lys*⁺ and *Lys*[−] spores. All *Lys*⁺ spores carried the plasmid marker. We conclude that *MIM17* is essential for the viability of yeast cells.

A centromeric plasmid encoding MIM17 with an epitope tag (human c-Myc) at the extreme carboxy-terminus complemented the mitochondrial import defect of the mutant MB3-33 and rescued the viability of a strain with a disruption of chromosomal *MIM17*. The MIM17-c-Myc chimeric protein thus functionally substituted for authentic MIM17. Cells of the haploid strain MB16, which expressed the chimeric protein while the chromosomal *MIM17* gene was disrupted, were used for immunodetection of MIM17 with anti-c-Myc monoclonal antibodies. yeast cells were fractionated into a cytosolic fraction, marked by hexokinase, and a mitochondrial fraction, marked by the inner membrane protein

ADP/ATP carrier. MIM17 was exclusively found in the mitochondrial fraction (Fig. 2A). MIM17 was not released from mitochondria by opening of the intermembrane space (formation of mitoplasts by swelling) and remained membrane-associated also after sonication of the mitoplasts, like the ADP/ATP carrier. The soluble matrix protein superoxide dismutase was released to the supernatant by sonication (Fig. 2B). Mitochondrial vesicles were separated by sucrose density gradient fractionation into outer membrane vesicles (porin as marker protein) and inner membrane vesicles (*F*₁-ATPase subunit β (*F*₁ β) as marker protein). MIM17 fractionated with the inner membrane vesicles (Fig. 2C). To test if MIM17 is an integral protein of the mitochondrial inner membrane, mitochondria were treated with carbonate at pH 11.5. This treatment is known to release soluble and peripheral membrane proteins to the supernatant, whereas integral membrane proteins are retained in the membrane sheets [14,26]. MIM17 remained in the membrane fraction like the ADP/ATP carrier, while superoxide dismutase and the peripheral membrane protein *F*₁ β were extracted (Fig. 2D). The prediction of hydrophobic sequences from the primary sequence and the resistance to alkaline extraction thus indicate that MIM17 is an integral membrane protein.

For in vitro transcription and translation, the coding region for MIM17 was cloned into the vector pEP30. The precursor of MIM17 was synthesized in rabbit reticulocyte lysate in the presence of [³⁵S]methionine and incubated with isolated energized yeast mitochondria. MIM17 was transported into a location protected against externally added protease (Fig. 3, lane 3). Import of the precursor of MIM17 was inhibited by a dissipation of the membrane potential $\Delta\psi$ across the mitochondrial

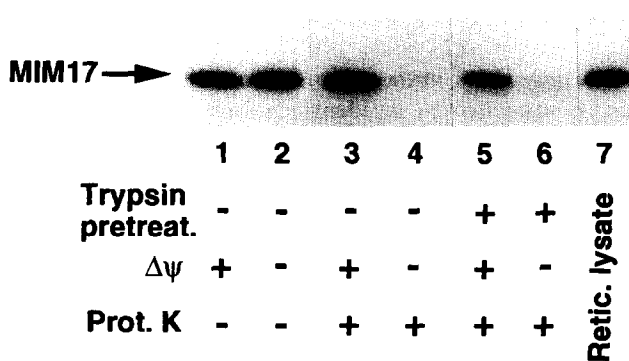


Fig. 3. Import of the precursor of MIM17 into mitochondria requires a membrane potential. The precursor of MIM17 was synthesized in rabbit reticulocyte lysates in the presence of [³⁵S]methionine (lane 7; 50% of amount added to import reaction). The reticulocyte lysate was incubated with isolated yeast mitochondria in the presence or absence of a membrane potential ($\Delta\psi$) for 20 min at 25°C. The mitochondria of samples 5 and 6 were pretreated with trypsin (20 μ g/ml) to remove the surface receptors [28]. After the import reaction, the mitochondria of samples 3–6 were treated with proteinase K (50 μ g/ml). The mitochondria were re-isolated. Analysis was performed by SDS-PAGE and fluorography.

inner membrane (Fig. 3, lane 4). This agrees with a location of MIM17 in the mitochondrial inner membrane, since the import of all inner membrane proteins analyzed so far required the presence of a $\Delta\Psi$ in contrast to the import of outer membrane proteins [40,44]. Removal of the outer membrane import receptors by pretreatment of the mitochondria with trypsin led to a reduction in MIM17 import (Fig. 3, lane 5). As observed for other mitochondrial preproteins [44], removal of the surface receptors did not completely block the import of MIM17, indicating that a fraction of its import occurs independently of receptors. MIM17 imported into mitochondria had the same apparent size as the precursor form (Fig. 3, lane 7), supporting the prediction made from the primary sequence that MIM17 is synthesized without a cleavable amino-terminal presequence.

In summary, we identified a new yeast protein that is located in the mitochondrial inner membrane. MIM17 is not extracted from the membranes at alkaline pH. Together with the presence of hydrophobic segments in the primary sequence [41], this indicates that MIM17 is as integral membrane protein. MIM17 is homologous to MIM23/Mas6p, a previously identified component of the preprotein import machinery of the mitochondrial inner membrane. Both MIM17 and MIM23 are synthesized without a cleavable presequence; they obviously contain the targeting signals within the mature protein part. MIM17 is essential for the viability of yeast cells on fermentable and non-fermentable carbon sources. The MIM17 joins the small group of essential mitochondrial proteins all of which are required for import or folding of cytosolically synthesized preproteins. Included in this group are the outer membrane protein ISP42/MOM38, the inner membrane proteins MIM23/Mas6p and MIM44/ISP45, and the matrix proteins hsp70, mitochondrial GrpE, hsp60, and the α - and β -subunit of the mitochondrial processing peptidase [13,16,36,40,45–47]. A role of MIM17 in the import of mitochondrial preproteins is suggested by its identification via mitochondrial import mutants and the homology to MIM23. In the accompanying paper [41] we provide direct evidence that MIM17, MIM23, MIM44, and mt-hsp70 cooperate in the import of preproteins.

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