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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 polyclonal 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 gene was cloned by a two-hybrid selection using the Y2H Gold System (Clontech Laboratories, Inc., Palo Alto, CA).
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 Xba/-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 YCPplcl11-MIM17-c-
mvc 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]. YCplcl11::ADHI-MIM17 (Fig.
1 A, construct 7) was generated in two steps. Firstly, a 0.7 kb EcoRV-
BamHI fragment, liberated from pBPH1 [19], was cloned into
YCplcl11 [20], restricted with Smal and BamHI. Secondly, this plasmid,
YCplcl11::ADHIp, was linearized with Smal and ligated with the
blunt-ended 1045 bp Hinfl-HincII MIM17 fragment. The corre-
sponding YCplcl11 derivative without the ADHI promoter was
obtained by deletion of the 723 bp EcoRI fragment from
YCplcl11::ADHI-MIM17 (Fig. 1 A, construct 6). The BstEII-
HincII subclone with the T-0 A transversion at position 1115 (Fig. 1 A,
construct 8), was obtained in two steps. Firstly, the 1360 bp XbaI-
HindIII MIM17 fragment was cloned into pSF1-FCT-1 and mutagen-
ized using the Altered Sites in vitro mutagenesis system (Promega).
Secondly, the 184 bp Ncol fragment of the wild-type BstEII-HincII
subclone (Fig. 1 A, construct 4) was replaced with the corresponding
Ncol fragment bearing the mutation.

pUC18-mim17::LYS2 was constructed by cloning a blunted 1140 bp
BstEII-HincII MIM17 fragment into the HincII site of pUC18 and
replacing the 194 bp Ncol MIM17 fragment with the blunt-ended 4858
by XbaI-HincII LYS2 fragment liberated from pDP6 [21]. To con-
struct the MIM17-c-mvc fusion gene, the XbaI-HindIII fragment, car-
rying the complete MIM17 coding sequence, was blunt-ended with T4
DNA polymerase and cloned into the blunt-ended BamHI site of
YCpmyc11 [22], creating YCplcl11-MIM17-c-mvc.

To synthesize MIM17 in vitro, a blunt-ended 1045 bp Hinfl-HincII
MIM17 fragment was cloned into the Smal site of the PE30 transcrip-
tion vector [13]. The PE30-MIM17 construct was linearized with
BamHI 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 sepa-
rating into outer membrane and inner membrane fractions [14,25],
treatment with 100 mM Na2CO3 (pH 11.5) and separation of supema-
brate potential, energy substrates were omitted and 0.5 μM valinomy-
cin, 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 nitrocel-
lose, 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 chro-
mosomal URA3 gene was transformed with a plasmid
encoding a chimeric protein which consisted of a mito-
ochondrial matrix targeting sequence and the URA3 gene
product (orotidine 5'-phosphate decarboxylase). The chimeric protein was efficiently targeted into the mito-
ochondrial matrix and thus the transformed strain re-
mained 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 retrans-
formation with non-mutagenized plasmid DNA. The
analysis of two complementation groups of recessive nu-
clear mutations led to the identification of the mitochon-
drial 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 p.96 clone (construct 1) were subcloned
into the centromeric vector YCPplcl11 (constructs 2-6,8). The Hinfl-HindII (isochizomer of SalI) fragment was also cloned downstream of the ADHI
promoter in YCplcl11::ADHIp (construct 7). Thick arrows indicate overlapping reading frames in the BstEII-HindIII region. The ability to
complement the Ura+ phenotype of mutant MB3-33 harboring the SOD-URA test plasmid is indicated by + (full complementation), − (no
complementation) or × (+) (very weak complementation). B, BamHI; Bs, BstEII; H3, HindIII; N, Ncol; P, PstI, Pr, PvuI, Rl, EcoRI, R5, EcoRV;
S, SacI; Sa, SacII; X, XbaI; *1-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 X77786 in the EMBL/GenBank/DDBJ data banks.
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. F.β, F.β-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 complementing gene was located on a 615 bp BstEII-HindIII 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 HindIII-Sall 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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>MB3</td>
<td>MATa ade2-101 his3 d200 leu2-Δ1 lys2-801 ura3::LYS2 rho+</td>
<td>[13]</td>
</tr>
<tr>
<td>MB3 33</td>
<td>MATa ade2-101 his3 d200 leu2-Δ1 lys2-801 ura3::LYS2 mim17-1 rho+</td>
<td>This study</td>
</tr>
<tr>
<td>MB2</td>
<td>MATa/tX ade2-101/ΔADE2 his3/Δhis3 d200 leu2/Δleu2-Δ1 lys2-801 lys2-801 trp1-289/trp1 ura3-52/trp1 ura3-52 rho+</td>
<td>[13]</td>
</tr>
<tr>
<td>MB2-3</td>
<td>MATa/tX ade2-101/ΔADE2 his3/Δhis3 d200 leu2/Δleu2-Δ1 lys2-801 lys2-801 trp1-289/trp1 ura3-52/trp1 ura3-52 mim17::LYS2/MIM17 rho+</td>
<td>This study</td>
</tr>
<tr>
<td>MB16</td>
<td>ade2-101 his3 leu2-801 trp1-289 ura3-52 mim17::LYS2 + YCplac111-MIM17-c-myc (LEL2) rho+</td>
<td>This study</td>
</tr>
</tbody>
</table>
(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 *Hin*II-*Sau*II 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 (F1-ATPase subunit β (F1β) 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 F1β 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 mitochondrial 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 Δψ across the mitochondrial inner membrane.
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 hydrophobic segment 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, 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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