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The Mitochondrial Receptor Complex: Mom22 Is Essential for Cell Viability and Directly Interacts with Preproteins†

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A multisubunit complex in the mitochondrial outer membrane is responsible for targeting and membrane translocation of nuclear-encoded preproteins. This receptor complex contains two import receptors, a general insertion pore and the protein Mom22. It was unknown if Mom22 directly interacts with preproteins, and two views existed about the possible functions of Mom22: a central role in transfer of preproteins from both receptors to the general insertion pore or a more limited function dependent on the presence of the receptor Mom19. For this report, we identified and cloned *Saccharomyces cerevisiae MOM22* and investigated whether it plays a direct role in targeting of preproteins. A preprotein accumulated at the mitochondrial outer membrane was cross-linked to Mom22. The cross-linking depended on the import stage of the preprotein. Overexpression of Mom22 suppressed the respiratory defect of yeast cells lacking Mom19 and increased preprotein import into *mom19*Δ mitochondria, demonstrating that Mom22 can function independently of Mom19. Overexpression of Mom22 even suppressed the lethal phenotype of a double deletion of the two import receptors known so far (*mom19*Δ *mom72*Δ). Deletion of the *MOM22* gene was lethal for yeast cells, identifying Mom22 as one of the few mitochondrial membrane proteins essential for fermentative growth. These results suggest that Mom22 plays an essential role in the mitochondrial receptor complex. It directly interacts with preproteins in transit and can perform receptor-like activities.

Most mitochondrial proteins are synthesized as preproteins in the cytosol (4, 20, 25, 43). Many preproteins carry amino-terminal signal sequences (presequences) that, upon import into mitochondria, are cleaved off by the matrix-processing peptidase. These cleavable preproteins are typically imported via the main import receptor, Mom19 (mitochondrial outer membrane protein of 19 kDa) (19, 38, 39, 45, 55). Mom19 (Mas20) is part of a high-molecular-weight complex in the mitochondrial outer membrane that contains a second import receptor, Mom72 (Mas70), and at least six additional proteins (28, 37, 58). Mom72 has a partially overlapping specificity with Mom19 yet preferentially acts as receptor for noncleavable preproteins with internal signal sequences (23, 56, 59). Five of the additional proteins of the receptor complex, Mom38 (Isp42), Mom30, Mom8a, Mom8b (Isp6), and Mom7, seem to be involved in forming a general insertion pore (GIP) embedded in the outer membrane that mediates membrane insertion and translocation of preproteins (32, 58). The sixth protein, Mom22, carries a cytosolic domain as do the two receptors and in addition a segment exposed to the intermembrane space (31); its possible functions are discussed below.

The genes for several Mom proteins, including Mom22, were cloned from the fungus *Neurospora crassa* (31). In *Saccharomyces cerevisiae*, the genes of four of the Mom proteins were identified, and thus the importance of these Mom proteins for respiratory and fermentative cellular growth could be determined. At that point, Mom38 was the only Mom protein found to be essential for growth of yeast cells also on fermentable carbon sources (3). A deletion of *MOM19* blocked growth of yeast cells only on nonfermentable carbon sources, demonstrating that the receptor Mom19 was required for respiratory competence of yeast cells (38, 45). A deletion of *MOM72* led to a slightly reduced cell growth on nonfermentable carbon sources at higher temperatures (23, 59). A deletion of *ISP6* alone revealed no appreciable growth defect (28). The importance of the receptors was underscored by the finding that cells lacking both Mom19 and Mom72 were inviable also on fermentable carbon sources (40, 45). Together with biochemical data on the functions of Mom proteins (19, 23, 35, 38, 55, 56, 59), the following model was proposed. Two receptor-mediated targeting pathways exist for mitochondrial preproteins: a main route via Mom19 and a more specialized route via Mom72. The receptors show partially overlapping specificities so that they can in part function as backup receptors; however, Mom72 is not able to take over all functions of Mom19. The import pathways then converge at the GIP into a common import pathway. Therefore, Mom38, the major constituent of the GIP, is essential under all growth conditions. One result did not fit into this general model of preprotein targeting. Ramage et al. (45) reported that overexpression of Mom72 suppressed the respiratory defect of Mom19-deficient yeast cells, suggesting a more general role for Mom72. A clarification by Lithgow et al. (35) was now possible. They provided convincing evidence that *mom19*Δ mutants were not rescued by overexpression of Mom72, but that some *mom19*Δ yeast strains could regain respiratory competence (i.e., growth on a nonfermentable medium) by a mechanism independent of Mom72. They speculated that when up-regulated, at least one additional mitochondrial surface protein can act as an import

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† Dedicated to Karl Decker on the occasion of his 70th birthday.

TABLE 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Reference
BMA1	*MAT***a**/α *his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1 trp1-Δ63/trp1-Δ63 ura3-52/ura3-52*	5
OL551	*MAT***a**/α *his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1 trp1-Δ63/trp1-Δ63 ura3-52/ura3-52 MOM22/mom22::HIS3*	This study
PK82	*MAT*α *his4-173 lys2 ura3-52 Δtrp1 leu2-3,112*	17
YPH500	*MAT*α *ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801*	54
MM112	*MAT*α *ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801 mom19::URA3*	38
MM112-A	*MAT*α *ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801 mom19::URA3* + pRS415(*LEU2*)::*MOM19*	38
MM112-B	*MAT*α *ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801 mom19::URA3* + pG-1(*TRP1*)::*MOM19*	This study
MM112-C	*MAT*α *ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801 mom19::URA3* + pG-1(*TRP1*)::*MOM22*	This study
MM208	*MAT***a** *ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801 mom72::HIS3*	38
MM412	*MAT***a**/α *ade2-101/ade2-101 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1 ura3-52/ura3-52 trp1-Δ63/trp1-Δ63 lys2-801/lys2-801 MOM72/mom72::HIS3 MOM19/mom19::URA3*	This study
MM412-C	*MAT***a**/α *ade2-101/ade2-101 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1 ura3-52/ura3-52 trp1-Δ63/trp1-Δ63 lys2-801/lys2-801 MOM72/mom72::HIS3 MOM19/mom19::URA3* + pG-1(*TRP1*)::*MOM22*	This study
MM120-C	*MAT*α *ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801 mom72::HIS3 mom19::URA3* + pG-1(*TRP1*)::*MOM22*	This study

receptor. This putative receptor-like component was not identified.

Different views on the possible roles of Mom22 exist. (i) Kiebler et al. (31) reported that both preproteins using Mom19 and preproteins using Mom72 needed the function of Mom22 to enter the GIP. This central role of Mom22 might imply that Mom22, like Mom38, should be essential for cellular growth. (ii) Harkness et al. (19) suggested that preproteins imported via Mom19 require functional Mom22, whereas in the absence of Mom19, preproteins should enter the outer membrane mainly without the help of Mom22, implying that a function of Mom22 in protein import depended on the presence of Mom19. Moreover, it was unknown if Mom22 directly interacts with preproteins.

In this study, we cloned *S. cerevisiae* mitochondrial *MOM22* and found that it is essential for cell viability. A central role of Mom22 is supported by its ability to function independently of Mom19; upon overexpression, Mom22 suppresses the respiratory defect of *mom19*Δ yeast cells and the lethality of a double deletion, *mom19*Δ *mom72*Δ. By chemical cross-linking, we provide evidence that Mom22 directly interacts with preproteins. We propose that Mom22 mediates transfer of preproteins from both receptors to the GIP by direct interaction with the polypeptides and that its preprotein-binding activity leads to receptor-like activities (at least when Mom19 is missing).

MATERIALS AND METHODS

***S. cerevisiae* strains and growth media.** The *S. cerevisiae* strains used in this study are listed in Table 1. For isolation of outer membranes and receptor complex and for cross-linking experiments, strain PK82 was used and grown in YP medium containing 3% glycerol. For import experiments with *mom19*Δ mitochondria, strains YPH500, MM112, and MM112-C were used and grown in synthetic complete medium containing 2% glucose (and lacking L-tryptophan in the case of MM112-C).

DNA sequencing. DNA sequencing was performed with an automatic sequencing apparatus (Applied Biosystems model 373A) (36). Cosmid α14-12, containing an insert localized on chromosome XIV, was obtained from Philippsen. A shotgun strategy using DNase I partial digestion was used to produce the fragment to be sequenced (14). The two strands were completely sequenced; gaps were filled in by oligonucleotide-directed sequencing. The complete sequence was determined with an average 4.8-fold redundancy.

Gene disruption. Deletion of the *S. cerevisiae MOM22* gene was performed with a PCR-based strategy (5). Two oligonucleotides containing part of the *MOM22* sequence (positions 37 to 71 and 391 to 425) and part of the *HIS3* sequence (17 bases) were used to generate the *HIS3* gene from a *HIS3* plasmid. The amplified fragment was used to transform the diploid strain BMA1 deleted of *HIS3* (5). Disruption of one *MOM22* gene in the resulting strain OL551 was confirmed by PCR analysis and Southern blotting.

Expression of Mom22 and Mom19 from plasmids. For overexpression of Mom22, a 0.46-kb DNA fragment containing the open reading frame (ORF) of *MOM22* was subcloned into the high-copy-number plasmid pG-1 (49). This DNA fragment was amplified from yeast genomic DNA isolated from strain YPH500 by using primers 2735 (5′-GAATTCGGATCCATGGTCGAATTAACTGAAA TT-3′) and 2734 (5′-AAGCTTGTCGACTTAATTGGCTGTTGCTGC-3′), cut with *Bam*HI and *Sal*I, and ligated into the *Bam*HI-*Sal*I site of pG-1, placing the insert immediately downstream of the glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter.

A 0.56-kb DNA fragment containing the ORF of *MOM19* was subcloned into plasmid pG-1. This DNA fragment was amplified from yeast genomic DNA isolated from strain YPH500 by using primer 478 (5′-GAGAGGATCCATTAT GTCCCAGTCGAACCC-3′) and primer 479 (5′-AAGCTTGTCGACTCAGT CATCGGATATCGTTAGC-3′), cut, and ligated into pG-1 as described above.

Isolation of mitochondria, outer membranes, and receptor complex and generation of antisera. Yeast mitochondria and outer membranes were isolated as described previously (12, 21, 55, 59). The receptor complex was affinity purified from digitonin-lysed mitochondria by using protein A-Sepharose carrying covalently coupled antibodies against Mom38; after washing in digitonin-containing buffer, bound proteins were eluted at pH 2.5 (37). Rabbit antisera against Mom22 were generated from the Mom22 band excised from nitrocellulose carrying purified outer membranes (57).

Amino acid sequence analysis. For amino-terminal sequence analysis, purified receptor complex was separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride membranes (Immobilon P; Millipore). The Mom22 band was sequenced by using a pulsed liquid-phase 477A sequencer equipped with an on-line model 120A PTH amino acid analyzer (Applied Biosystems) (15).

In vitro import of preproteins into isolated mitochondria and cross-linking. Preproteins were synthesized in rabbit reticulocyte lysates in the presence of [^{35}S]methionine and incubated with isolated yeast mitochondria in the presence or absence of a membrane potential $\Delta\psi$ for 5 to 20 min as described previously (6, 57). To perform import at low ATP, the mitochondria were depleted of ATP by a pretreatment with apyrase (1 U/ml), and import was performed in the presence of a $\Delta\psi$ but absence of ATP (33).

For cross-linking, mitochondria containing accumulated ^{35}S-labeled preproteins were reisolated through a sucrose cushion, washed, and incubated with disuccinimidyl suberate (DSS) as described previously (6, 33). After precipitation with trichloroacetic acid, the samples were analyzed by SDS-PAGE or lysed in SDS-containing buffer and subjected to immunoprecipitation in Triton X-100 buffer (6, 33).

Miscellaneous. Standard techniques were used for manipulation of DNA, *Escherichia coli*, and *S. cerevisiae* (2, 18, 47, 53), SDS-PAGE, immunodecoration (31), and storage phosphor imaging technology (Molecular Dynamics).

Nucleotide sequence accession number. The accession number for the sequence reported here is Z46843 in the EMBL/GenBank/DDBJ data banks.

RESULTS

Cloning and primary sequence of yeast *MOM22*. A screening for the *S. cerevisiae MOM22* gene with the *N. crassa MOM22* DNA or with antibodies prepared against *N. crassa* Mom22 (31) was not possible because of lack of cross-hybridization and antibody cross-reaction. The most likely explanation was the relatively low homology that is usually observed between Mom proteins from *N. crassa* and *S. cerevisiae* (32, 38, 59). We thus prepared the yeast mitochondrial receptor com-

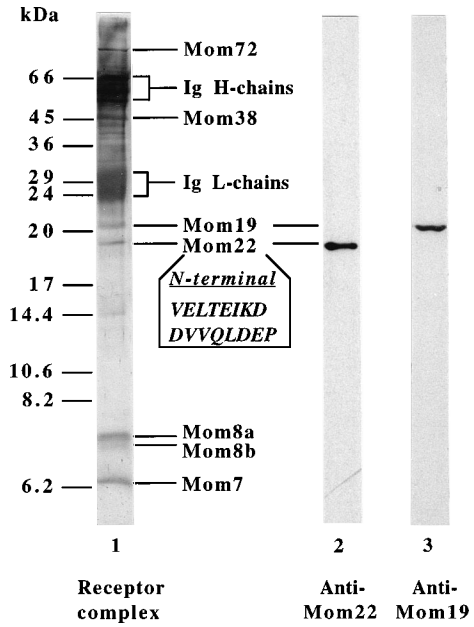

FIG. 1. Characterization of Mom22 of the yeast mitochondrial receptor complex. The mitochondrial receptor complex was affinity purified from isolated *S. cerevisiae* mitochondria with anti-Mom38 antibodies covalently coupled to protein A-Sepharose. Bound proteins were eluted at pH 2.5, separated by urea-SDS-PAGE, and stained with Coomassie brilliant blue R250 (lane 1). Some of the immunoglobulin (Ig) heavy (H) and light (L) chains were also released at low pH. The Mom22 band was subjected to amino-terminal amino acid sequencing as described in Materials and Methods; the sequence is shown in the one-letter code. For lanes 2 and 3, affinity-purified receptor complex was transferred to nitrocellulose and immunodecorated with antibodies directed against yeast Mom22 (see Materials and Methods) or yeast Mom19 (39).

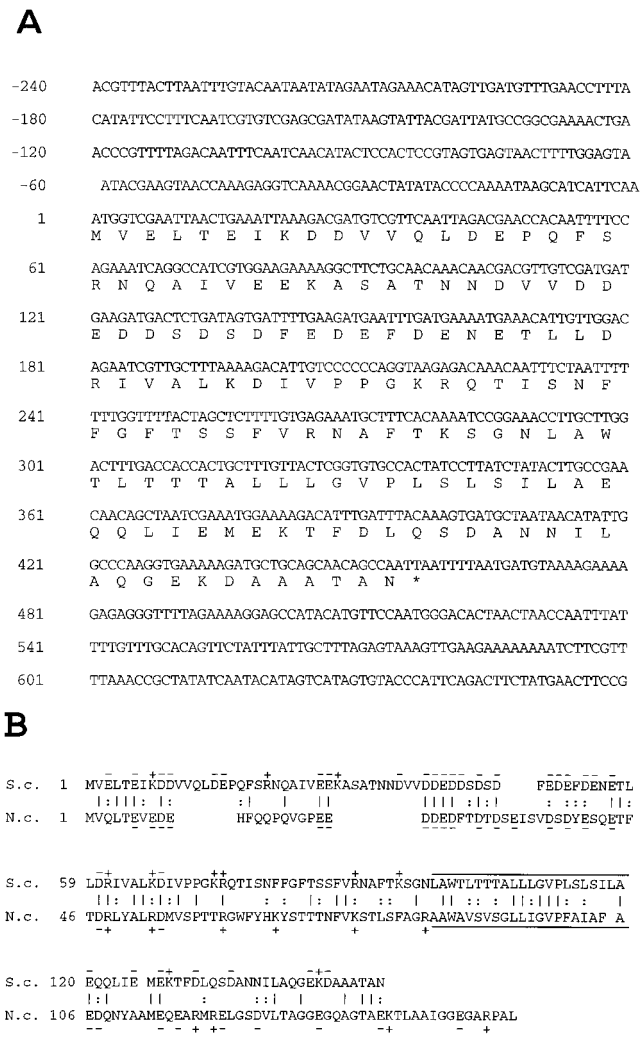

FIG. 2. Gene and deduced amino acid sequences of *S. cerevisiae MOM22*. (A) Nucleotide sequence of the *MOM22* gene. The amino acid sequence is given in the one-letter code. (B) Comparison of the deduced amino acid sequences of *S. cerevisiae* (S.c.) and *N. crassa* (N.c.) MOM22. Identical residues are indicated by vertical lines; isofunctional residues are indicated by double dots. The hydrophobic segment is indicated by a horizontal line.

plex in chemical amounts. Antibodies directed against yeast Mom38 were covalently coupled to protein A-Sepharose. Isolated mitochondria were lysed in buffer containing digitonin, and the receptor complex was affinity purified. Lane 1 of Fig. 1 shows the typical protein pattern of the yeast receptor complex, including Mom72, Mom38, Mom19, Mom22, Mom8a, Mom8b, and Mom7 (37). During the acid-induced release of the complex subunits from the affinity column, some of the antibody chains were also released; Mom30 was thereby masked by immunoglobulin light chains. On the basis of the gel mobility in comparison with that of the *N. crassa* receptor complex, Moczko et al. (37) designated the complex subunit running below Mom19 in a urea-SDS-polyacrylamide gel as Mom22 (the numbers of the Mom proteins are derived from their gel mobilities in a standard SDS-polyacrylamide gel [32]). Below we show that this assignment of yeast Mom22 was correct. Mom22 was subjected to amino-terminal sequencing. Sixteen amino acids could be determined (Fig. 1). In addition, we prepared an antiserum in rabbits against the protein band excised from purified outer membranes. The antiserum selectively reacted with Mom22 (Fig. 1, lane 2).

The amino-terminal peptide sequence of yeast Mom22 was compared with sequences deposited in the MIPS protein data bank. The peptide sequence was found to be identical to an amino acid sequence deduced from a 43-kb insert cloned from *S. cerevisiae* chromosome XIV in the framework of the European sequencing project. The nucleotide sequence was determined on both strands by using a shotgun strategy. It revealed an ORF of 152 codons with a predicted M_r of 16,780 (Fig. 2A). The amino-terminal peptide sequence corresponds to residues 2 to 17 of the predicted sequence, indicating that the initial methionine is removed from Mom22 after synthesis on cytosolic ribosomes.

A smaller ORF of 116 codons (ORF116) is present on the complementary DNA strand and is included within the *MOM22* ORF. ORF116 starts at nucleotide position 416 and ends at position 69 (Fig. 2A). The presence of an ORF larger than 100 codons opposite an ORF from an expressed gene has already been found in several instances. These ORFs have never been found to be functional and usually have a low codon adaptation index (52). The codon adaptation index for ORF116 is 0.072, whereas it is 0.284 for the *MOM22* ORF. In addition, the composition of dipeptides of ORF116 differs from the general use of dipeptides in yeast proteins, with a negative score of −137, compared with the positive score 227 obtained with the *MOM22* ORF (60). Thus, ORF116 is most likely not functional.

Yeast Mom22 shows significant sequence similarity to *N. crassa* Mom22 (49% similarity, including 30% identical amino acid residues). A comparison of the two sequences reveals

three typical features (Fig. 2B): segments with an excessively high abundance of negatively charged residues in the amino-terminal half, an uncharged hydrophobic sequence of more than 20 residues in the carboxy-terminal half, and an overall negative character of the carboxy-terminal tail (beyond the hydrophobic sequence) with a net charge of −5 in both *S. cerevisiae* and *N. crassa.*

***MOM22* is an essential gene.** To determine whether *MOM22* encodes an essential mitochondrial protein, one of the two copies of the *MOM22* gene in the homozygous *his3* diploid BMA1 was disrupted by using the *HIS3* gene as marker. The disruption was confirmed by Southern blotting and PCR analysis. Cells of the resulting strain, OL551, were sporulated, and the asci were dissected. Each tetrad yielded maximally two viable spores, even on medium containing a fermentable carbon source. All viable spores were His^- and thus did not carry the null allele of *MOM22*. The other spores underwent maximally 11 to 12 divisions and could not be further expanded, suggesting that after dilution or degradation of preexisting Mom22, the cells were inviable. The diploid OL551 was transformed with a plasmid carrying the *MOM22* coding region under control of the constitutive GPD promoter. Sporulation of the transformed cells yielded both His^+ and His^- spores; all His^+ spores carried the plasmid marker. When a plasmid with the insert in the opposite orientation was used, all viable spores (maximally two spores per tetrad) were His^-, excluding that the lethal phenotype of the *mom22*Δ null mutation was due to deletion of ORF116. We conclude that *MOM22* is essential for the viability of yeast cells. Overexpression of Mom19 from the high-copy-number plasmid pG-1 did not suppress the lethality of a *mom22*Δ null mutation.

A preprotein accumulated at the mitochondrial outer membrane is cross-linked to Mom22. So far, an involvement of Mom22 in protein import has been inferred from its presence in the receptor complex (32, 37) and from the reduction of protein import into isolated mitochondria by antibodies directed against Mom22 (31). However, it has not been shown that Mom22 comes in contact with preproteins.

We investigated if a cleavable preprotein in transit could be cross-linked to Mom22 in intact mitochondria. Cross-linking of a preprotein to mitochondrial surface proteins (the receptors Mom19 and Mom72) was reported only for the precursor of the ADP/ATP carrier, which is special as it does not carry a presequence but rather carries several internal signal sequences (58). The cleavable preprotein Su9-DHFR, a fusion protein between the presequence of F_0-ATPase subunit 9 and dihydrofolate reductase, was successfully used for cross-linking to import components of the mitochondrial inner membrane and matrix, Mim17 (Sms1p), Mim23 (Mas6p), Mim44 (Isp45), and mitochondrial Hsp70 (Ssc1p) (6, 24, 33, 46). We tested whether Su9-DHFR was also a suitable substrate for cross-linking to Mom proteins. The precursor was synthesized in rabbit reticulocyte lysates in the presence of [^{35}S]methionine and accumulated at the outer membrane of isolated yeast mitochondria in the absence of a membrane potential Δψ. The mitochondria were reisolated, and the amino-reactive homobifunctional noncleavable cross-linking reagent DSS was added. After quenching of the DSS, mitochondrial proteins were analyzed by SDS-PAGE. Lane 2 of Fig. 3 shows that several cross-link products were generated that were not present in the absence of DSS (Fig. 3, lane 1). In an immunoprecipitation under stringent conditions, a cross-link product of 48 kDa was selectively recognized by antibodies directed against Mom22 (Fig. 3, lane 4). The size of the cross-link product indicates a reaction between one molecule of Su9-DHFR (29 kDa) and one molecule of Mom22. A cross-link

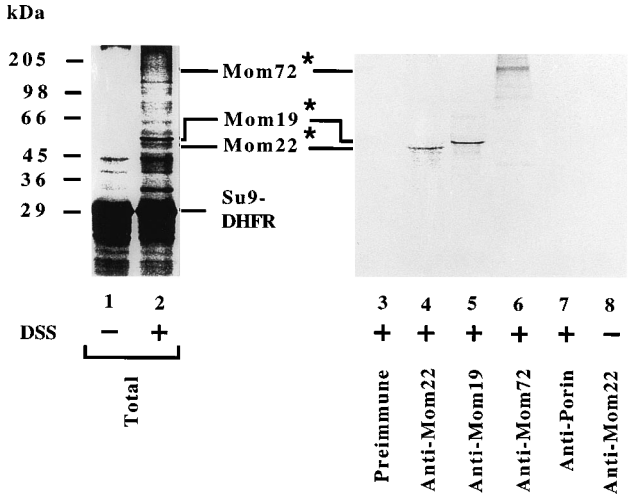

FIG. 3. Cross-linking of a preprotein accumulated at the mitochondrial outer membrane to Mom22. Rabbit reticulocyte lysate containing the ^{35}S-labeled precursor of Su9-DHFR was incubated with isolated yeast wild-type mitochondria for 20 min at 25°C in the absence of a membrane potential Δψ (6). The mitochondria were reisolated and incubated with the cross-linker DSS as indicated. After quenching of the cross-linker with Tris buffer, the proteins were precipitated with trichloroacetic acid (6). Samples 1 and 2 were directly analyzed by SDS-PAGE. Samples 3 to 8 (each representing threefold the amount of sample 1 or 2) were dissolved in SDS-containing buffer, diluted 40-fold in 1% Triton X-100 buffer, immunoprecipitated with protein A-Sepharose and the indicated antisera, and subjected to SDS-PAGE. The cross-link products between Su9-DHFR and Mom proteins are indicated by asterisks.

product of 50 kDa was precipitated by antibodies directed against Mom19 (Fig. 3, lane 5). A cross-link product of about 170 kDa was recognized by antibodies directed against Mom72 (Fig. 3, lane 6). Mom72 has previously been suggested to behave as a homodimer also under stringent conditions (58), so the product of 170 kDa may consist of one molecule of Su9-DHFR and two molecules of Mom72. With regard to the intensities of the three cross-link products formed, the best cross-linking was observed to Mom19, followed by Mom22. The cross-linking to Mom72 was only weak, in agreement with the observation that cleavable preproteins mainly use the receptor Mom19. Antibodies against the by far most abundant outer membrane protein porin did not precipitate any of the cross-link products (Fig. 3, lane 7), excluding unspecific cross-linking to proteins of the outer membrane. Moreover, none of the cross-link bands was precipitated by preimmune serum (Fig. 3, lane 3).

To further control the specificity of the cross-linking to Mom22, the preprotein Su9-DHFR was arrested at different import stages, followed by cross-linking with DSS and immunoprecipitation with anti-Mom22 antibodies. In Fig. 4A, we depict the distinct import stages. (i) In the absence of a membrane potential Δψ, the precursor form of Su9-DHFR was accumulated at the outer membrane (Fig. 4A, lane 1). Treatment of the mitochondria with proteinase K degraded all precursor molecules (Fig. 4A, lane 2), demonstrating that the preproteins were exposed on the mitochondrial surface. (ii) In the presence of a Δψ but at low levels of ATP, Su9-DHFR was accumulated as a membrane-spanning intermediate. From most molecules, the first half of the presequence was removed by the matrix-processing peptidase (Fig. 4A, lane 3), while the rest of the protein spanned the mitochondrial inner membrane (6, 33, 50). About half of the molecules spanned not only the inner membrane but also the outer membrane, as evidenced by the accessibility of part of the intermediates to added protease

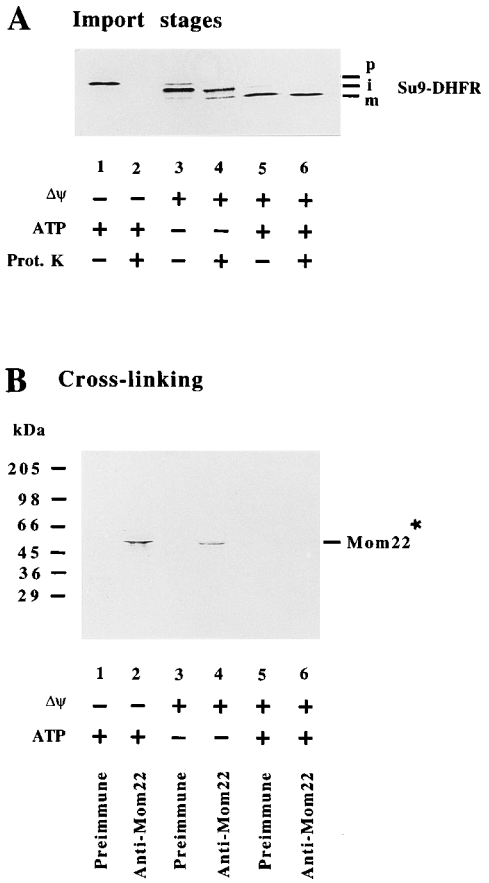

FIG. 4. Cross-linking of a preprotein to Mom22 is dependent on the import stage. ^{35}S-labeled Su9-DHFR was incubated with isolated mitochondria in the absence of a membrane potential $\Delta\psi$ (outer membrane intermediate; as described in the legend to Fig. 3), in the presence of $\Delta\psi$ but low levels of ATP (membrane-spanning intermediate), or in the presence of $\Delta\psi$ and ATP (full import into the matrix) as indicated and described in Materials and Methods. (A) Half of each sample was then treated with proteinase K (50 μg/ml). The mitochondria were reisolated and analyzed by SDS-PAGE. p, i, and m, precursor-, intermediate-, and mature-size forms, respectively, of Su9-DHFR. (B) The mitochondria were reisolated and subjected to cross-linking with DSS and immunoprecipitation with preimmune serum or anti-Mom22 serum as described in the legend to Fig. 3.

(Fig. 4A, lane 4) (6). (iii) In the presence of $\Delta\psi$ and ATP, Su9-DHFR was completely imported into the mitochondrial matrix, processed to the mature form (Fig. 4A, lane 5), and not accessible to externally added protease (Fig. 4A, lane 6). Figure 4B shows the cross-linking of Su9-DHFR at the distinct import stages to Mom22. As described above, at stage i, cross-linking took place (Fig. 4B, lane 2). At stage ii, the efficiency of cross-linking to Mom22 was considerably decreased (Fig. 4B, lane 4). At stage iii (complete import into the matrix), no cross-link product with Mom22 was observed (Fig. 4B, lane 6). This stage dependence of cross-linking provides further evidence for the specificity of the cross-linking approach used. We conclude that Mom22 is in close proximity to a preprotein accumulated at the mitochondrial outer membrane.

Overexpression of Mom22 suppresses the respiratory defect and import defect of *mom19*Δ yeast cells. Yeast cells lacking Mom19 are strongly impaired in respiration, that is, growth on nonfermentable carbon sources such as glycerol (38, 45) (Fig. 5A, sector 4). Expression of Mom19 from a centromeric plasmid restored respiration of *mom19*Δ cells (Fig. 5A, sector 3), excluding the possibility that the respiratory defect was caused by a secondary mutation or loss of mitochondrial DNA.

We asked if Mom22 may be able to substitute for functions of Mom19. The coding sequence of *MOM22* was cloned into the high-copy-number plasmid pG-1 under the control of the GPD promoter, and *mom19*Δ cells were transformed. This led to a significant overexpression of Mom22. Isolated mitochondria contained a strongly increased amount of Mom22 (Fig. 5B, lane 2). *mom19*Δ cells with overexpressed Mom22 were able to grow on a nonfermentable medium (Fig. 5A, sector 2). The growth on nonfermentable medium (doubling time of 8 to 9 h) was about two- to threefold slower than that of wild-type yeast cells (doubling time of 3.5 h), and the colonies formed were smaller than wild-type colonies. Overexpression of Mom22 thus partially rescues the growth defect of *mom19*Δ yeast cells; in particular, it allows respiratory growth. Transformation of *mom19*Δ cells with plasmid pG-1 not containing *MOM22* did not restore respiratory growth (not shown), excluding the possibility that a rescue of respiration was indirectly caused by the transformation procedure.

*mom19*Δ mitochondria are strongly inhibited in import of presequence-carrying (cleavable) preproteins (19, 38, 45). Does the overexpression of Mom22 restore protein import into the mutant mitochondria? Rabbit reticulocyte lysates containing ^{35}S-labeled preproteins, i.e., the α subunit of the mitochondrial processing peptidase, the β subunit of the F_1-ATPase, cytochrome c_1, and the fusion protein Su9-DHFR, were incubated with isolated yeast mitochondria. In the presence of a membrane potential $\Delta\psi$, the preproteins were imported into wild-type mitochondria, as determined by proteolytic processing (Fig. 6, lanes 2) and protection of the imported proteins against externally added proteinase K (Fig. 6, lanes 8). The import was strongly reduced with *mom19*Δ mitochondria (Fig. 6, lanes 4 and 10). Protein import was significantly increased with *mom19*Δ mitochondria carrying overexpressed Mom22 (Fig. 6, lanes 6 and 12), although it did not reach the import value of wild-type mitochondria. We conclude that overexpression of Mom22 partially rescues the growth defect and protein import defect of cells lacking the receptor Mom19.

A deletion of both *MOM19* and *MOM72* is lethal to yeast cells also on fermentable medium (40, 45). We transformed the heterozygous diploid yeast strain MM412, which contained one disrupted *MOM19* gene and one disrupted *MOM72* gene, with plasmid pG-1 overexpressing Mom22. The resulting diploid, MM412-C (Table 1), was sporulated. After dissection of asci and analysis of the resulting tetrads, we found viable haploid clones (strain MM120-C) with the double deletion *mom19*Δ *mom72*Δ; all these clones contained the plasmid marker (Table 1). The growth on fermentable medium was about twofold slower than that of wild-type cells. The growth on nonfermentable medium (doubling time of about 11 h) was about threefold slower than that of wild-type cells (doubling time of 3.5 h). Thus, overexpressed Mom22 can partially substitute for both import receptors and allow viability of *mom19*Δ *mom72*Δ cells.

DISCUSSION

We have identified the gene for *S. cerevisiae* Mom22 and analyzed the function of this subunit of the mitochondrial receptor complex with mutant cells and by chemical cross-linking to preproteins.

The following findings demonstrate that the correct *MOM22* gene was cloned. (i) The deduced amino-terminal sequence is identical to an amino-terminal peptide sequence determined from the Mom22 band in the purified yeast mitochondrial receptor complex (except that the initial methionine is lacking). (ii) *S. cerevisiae* Mom22 shows significant homology to *N. crassa* Mom22 (31). A comparison of the *S. cerevisiae* and *N.*

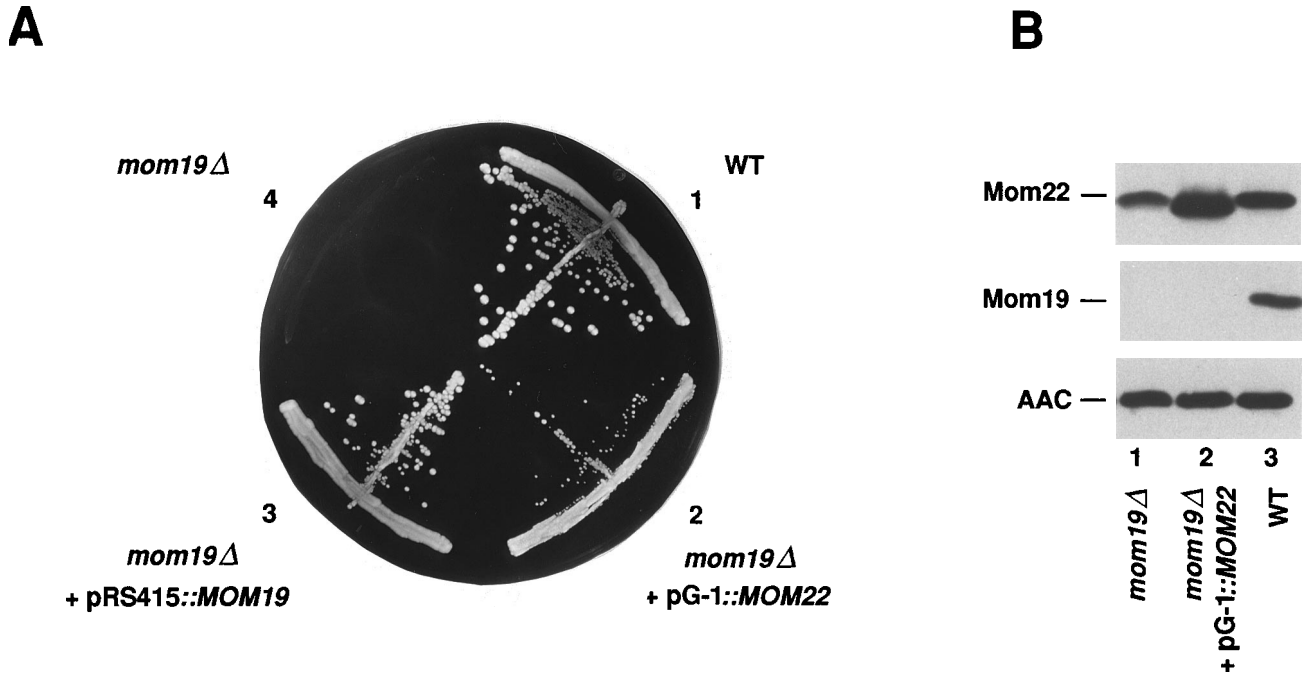

FIG. 5. Overexpression of Mom22 suppresses the respiratory defect of *mom19*Δ cells. (A) *S. cerevisiae* strains were grown on a YPG plate (3% glycerol) for 3 days at 30°C. The genotypes of the strains are listed in Table 1. WT (wild type), strain YPH500; *mom19*Δ, strain MM112; pG-1, high-copy-number plasmid with the GPD promoter; pRS415, centromeric plasmid (*MOM19* under control of its own promoter). Expression of Mom19 from plasmid pG-1 (strain MM112-B; Table 1) also led to a full restoration of growth of *mom19*Δ cells. *mom19*Δ cells (without plasmid) showed very slow growth on the YPG plate after 5 days (not shown). (B) *S. cerevisiae* was grown on glucose-containing medium. Mitochondria were isolated and subjected to immunodecoration with antiserum directed against Mom22, Mom19, or ADP/ATP carrier (AAC; an inner membrane protein mainly imported via Mom72).

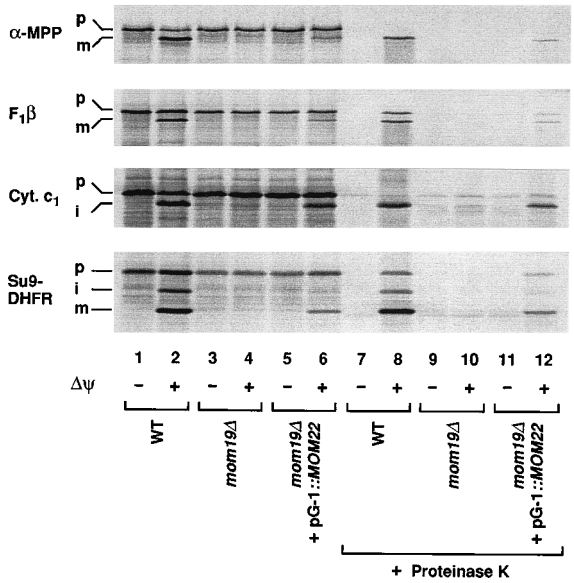

FIG. 6. Overexpression of Mom22 partially restores preprotein import into *mom19*Δ mitochondria. Rabbit reticulocyte lysates containing ^{35}S-labeled preproteins were incubated with isolated mitochondria (described in the legend to Fig. 5) in the presence or absence of a membrane potential Δψ at 25°C. Import was stopped by addition of valinomycin to dissipate the membrane potential (57). Where indicated, the samples were treated with proteinase K (75 μg/ml). The mitochondria were reisolated and analyzed by SDS-PAGE. Under the conditions used here, including short import times, mainly the intermediate-sized form of cytochrome c_1 was generated. α-MPP, α subunit of mitochondrial processing peptidase; Cyt. c_1, cytochrome c_1; $F_1\beta$, β subunit of F_1-ATPase; p, i, and m, precursor-, intermediate-, and mature-size forms, respectively, of a preprotein.

crassa sequences in context with the topology determined for *N. crassa* Mom22 (31) suggests three typical features of Mom22: a highly negatively charged segment in the amino-terminal domain that is located on the cytosolic side, a hydrophobic membrane anchor segment in the carboxy-terminal half, and a carboxy-terminal tail in the intermembrane space with a net negative charge. As the signal sequences of mitochondrial preproteins are characterized by the presence of positively charged residues and the predicted formation of an amphipathic α helix (61), one might speculate that the negatively charged segments of Mom22 are involved in the transfer of the positively charged signal sequences.

Yeast cells lacking the *MOM22* gene are inviable also on fermentable carbon sources, indicating that Mom22 is an essential protein. Mom22 thus joins the group of mitochondrial proteins essential for viability of yeast cells, all of which identified to date are involved in the import of nucleus-encoded mitochondrial preproteins. This group includes Mom38 of the outer membrane (3), Mim17, Mim23, and Mim44 of the inner membrane (43), the two subunits of the mitochondrial processing peptidase (27, 44, 62), and the heat shock proteins Hsp70 (Ssc1p), Hsp60 (Mif4p), and Mge1 (Yge1) (7, 9–11, 26, 34). It is remarkable that Mom22 is only the second essential protein of the mitochondrial outer membrane, whereas cells lacking one of the import receptors Mom19 and Mom72 are viable on fermentable carbon sources.

By chemical cross-linking to a preprotein arrested at the mitochondrial outer membrane, we found that Mom22 is in close proximity to a preprotein in transit. The specificity of the cross-linking approach was underscored by its dependence on the import stage of the preprotein and the lack of cross-linking of the preprotein to the most abundant outer membrane protein porin. This represents the first evidence for a direct inter-

action of Mom22 with preproteins. In addition, the presequence-carrying (cleavable) preprotein was cross-linked to the main import receptor Mom19 and, with low efficiency, to the special import receptor Mom72. This finding is of particular importance for Mom19, as its major function in import of cleavable preproteins had been shown by inhibitory antibodies and the use of mutant mitochondria, yet a demonstration of a direct interaction of cleavable preproteins with Mom19 was lacking. Import studies using Mom72-deficient mutant mitochondria had suggested that some cleavable preproteins require Mom72 for a small fraction of their import. Coimmunoprecipitation experiments with the purified receptor (51) and the cross-linking reported here suggest a direct interaction of Mom72 with cleavable preproteins.

Since Mom22 contains a domain exposed to the cytosol and interacts with preproteins in transit, one might speculate about possible receptor-like functions of Mom22. In view of the observation by Lithgow et al. (35) that a Mom19-deficient yeast strain regained respiration and rescued mitochondrial protein import by an unknown mechanism, for example, up-regulation of an unknown mitochondrial import receptor, MasXp, we asked whether Mom22 was able to rescue *mom19*Δ cells. Overexpression of Mom22 in respiratory-defective *mom19*Δ cells indeed led to respiratory competence. The strongly reduced protein import capability of *mom19*Δ mitochondria was increased by the overexpression of Mom22. Thus, Mom22 seems to be able to fulfill receptor-like functions and might be considered a third mitochondrial import receptor. Mom22 seems to directly interact with preproteins during their transfer from the receptors Mom19 or Mom72 to the GIP. This ability to interact with preproteins on the outer membrane surface may give Mom22 receptor-like properties. In the absence of Mom19, these receptor-like activities of Mom22 can partially substitute and can restore respiratory competence, in contrast to Mom72. *mom19*Δ mitochondria contained a reduced amount of Mom22 (reduction by about 60% compared with wild-type mitochondria; Fig. 5B, lane 1). It is thus possible that the inhibition of protein import into *mom19*Δ mitochondria is partially caused by a reduction of Mom22. Mom19 seems to play at least two roles in the mitochondrial receptor complex: (i) it is a major site for direct interaction with preproteins, as indicated by the cross-linking experiments; and (ii) it is involved in the biogenesis and possibly also in the stabilization of Mom22 (19, 29, 48). The observation that Mom22 can be overexpressed also in the absence of Mom19 demonstrates that Mom19 is not strictly required for the biogenesis or stability of Mom22 and supports the view of a flexible stoichiometry in the mitochondrial receptor complex (37).

Mom22 was found to be functional even in the absence of both receptors Mom19 and Mom72. Whereas a *mom19*Δ *mom72*Δ double mutant is inviable also on fermentable carbon sources, the overexpression of Mom22 permitted slow growth in the absence of Mom19 and Mom72 on both fermentable and nonfermentable media. Mom22 can thus partially substitute for the functions of the two previously characterized import receptors.

Two different views existed about the role of Mom22 in the receptor complex. (i) The first posits a central role of Mom22 in mediating transfer of preproteins to the general insertion pore. At Mom22, the targeting pathways converge, and Mom22 could thus be seen as the main entry gate for the GIP (31). (ii) Preproteins imported via Mom19 require the function of Mom22, whereas in the absence of Mom19, protein import does not require Mom22 (19). Our results argue against the second view, as Mom22 can function in protein import in the absence of Mom19. The observation that Mom22 is an essential protein like the GIP-component Mom38, whereas Mom19 is dispensable on fermentable carbon sources, supports the first view of a central role of Mom22. This includes receptor-like activities of Mom22. The central importance of Mom22 is underscored by the lack of rescue of a *mom22*Δ null mutation by overexpression of Mom19.

The most prominent structural feature of Mom22 is a high abundance of negative charges in the amino-terminal domain which has been shown to be located on the cytosolic side (31). Interestingly, we found that several components of the mitochondrial protein import machinery contained negatively charged segments mostly with a predicted α-helical character, though none of them was as highly negatively charged as the cytosolic domain of Mom22. These segments are located as follows: in the cytosol, in the carboxy-terminal portion of the mitochondrial import stimulation factor MSF (1); on the cytosolic side of the outer membrane, in the carboxy-terminal regions of Mom19 (38, 45) and Mom72 (59) and in the amino-terminal domain of Mom22; on the intermembrane space side of the outer membrane, in the carboxy-terminal tails of Mom22 and Mom38 (4, 30, 32); on the intermembrane space side of the inner membrane, in the amino-terminal domain of Mim23 (13, 16, 33); and in the matrix, in the mitochondrial processing peptidase (22, 27, 44). Recent evidence indeed reinforces the importance of positively charged residues in presequences not only for targeting and membrane translocation of preproteins (8) but also for cleavage by processing peptidase (41, 42). We did not identify a significantly negatively charged segment in an import component at the matrix side of the inner membrane, yet transfer of the presequences across the inner membrane strictly depends on the presence of a membrane potential that is negative on the matrix side. We speculate that the negatively charged segments of import components and the membrane potential are important to guide a stepwise import of the positively charged mitochondrial signal sequences (acid chain hypothesis).

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