MOLECULAR AND CELLULAR BIOLOGY, Nov. 1995, p. 6196–6205 Vol. 15, No. 11
0270-7306/95/$04.00+0

The Mitochondrial Receptor Complex: the Small Subunit Mom8b/Isp6 Supports Association of Receptors with the General Insertion Pore and Transfer of Preproteins

AGUSTÍN ALCONADA,† MICHAEL KÜBRICH, MARTIN MOCZKO, ANGELIKA HÖNLINGER, AND NIKOLAUS PFANNER*

Biochemisches Institut, Universität Freiburg, D-79104 Freiburg, Germany

Received 12 May 1995/Returned for modification 22 June 1995/Accepted 10 August 1995

The mitochondrial outer membrane contains import receptors for preproteins and a multisubunit general insertion pore. Several small outer membrane proteins (<10 kDa) have been identified by their association with receptors or the general insertion pore, yet little is known about their function. Here, we present evidence that the biochemically identified Mom8b and the genetically identified Isp6 are identical. A deletion of Mom8b/Isp6 in *Saccharomyces cerevisiae* leads to (i) a delay of import of preproteins, (ii) stabilization of preprotein binding to receptors and the general insertion pore, and (iii) destabilization of the interaction between receptors and the general insertion pore. These results suggest that Mom8b supports the cooperativity between receptors and the general insertion pore and facilitates the release of preproteins from import components and thereby promotes efficient transfer of preproteins.

Targeting of nucleus-encoded preproteins to mitochondria is mediated by the mitochondrial receptor complex, a multisubunit protein complex in the outer membrane of the organelle. The receptor complex contains import receptors and a general insertion pore (GIP). The import receptors characterized best so far are the mitochondrial outer membrane proteins of 19 kDa (Mom19) and 72 kDa (Mom72) (also termed Mas20 and Mas70, respectively) (2, 38). Mom22 (Mas22) and Mas37 seem to be additional subunits of import receptors (11, 17, 23, 26–28, 34). The GIP with Mom38 (Isp42) as the core component mediates translocation of preproteins into or across the outer membrane (24, 36, 37, 52).

In addition to these components, three small proteins have been found associated with the receptor complex, termed Mom8a, Mom8b, and Mom7 (17, 31, 49). These small subunits have not been characterized on a molecular level, and their function is unknown.

It was therefore of particular interest that Kassenbrock et al. (22) identified the *Saccharomyces cerevisiae* mitochondrial outer membrane protein Isp6 by a genetic interaction with Mom38. Isp6 is anchored in the mitochondrial outer membrane by a C-terminal hydrophobic segment (5) and can be coimmunoprecipitated with anti-Mom38 antibodies. On the basis of gel mobility, it was concluded that Isp6 was not related to any of the known components of the translocation machinery of the mitochondrial outer membrane (22).

For this study, we investigated the function of Isp6. Unexpectedly, we found that it is identical to a known subunit of the receptor complex, namely, Mom8b. We demonstrate that Mom8b modulates the functions of other subunits of the receptor complex. It promotes interaction between receptors and the GIP but reduces the stability of binding of preproteins to the receptor complex. Mom8b thus enhances the efficiency of preprotein transfer across the mitochondrial outer membrane.

* Corresponding author. Mailing address: Biochemisches Institut, Universität Freiburg, Hermann-Herder-Straße 7, D-79104 Freiburg, Germany. Phone: 49-761 203 5224. Fax: 49-761 203 5261.

† Present address: European Molecular Biology Laboratory, D-69012 Heidelberg, Germany.

MATERIALS AND METHODS

Construction of plasmids and yeast mutants. Plasmid pGEM4-ISP6 was constructed by insertion of the coding region of the *S. cerevisiae ISP6* gene into the *Eco*RI-*Bam*HI sites in pGEM4 (Promega), downstream of the SP6 RNA polymerase promoter. The presence of the correct sequence was verified by dideoxy sequencing of both DNA strands. Standard procedures were used for DNA manipulations (ligation using T4 DNA ligase, digestion with restriction endonucleases, and *Escherichia coli* transformation) (1, 42).

For disruption of the yeast *MOM8b*(*ISP6*) gene by homologous recombination, the plasmid pGEM4-ISP6 was digested with *Sph*I and *Bam*HI within the polylinker of the vector. The resulting overhanging 5′ ends and 3′ ends were filled in or removed, respectively, by using T4 DNA polymerase, and the vector was religated in order to remove the *Pst*I site inside the polylinker. The modified plasmid was then digested with *Pst*I. The protruding 3′ termini were made blunt with T4 DNA polymerase, and a blunt-ended cassette containing the *URA3* gene was ligated with T4 DNA ligase. The resulting construct was linearized with *Sma*I and used to transform the haploid strain YPH500 (Table 1) as described by Elble (8). Yeast cells that had integrated the DNA were selected by their uracil prototrophy. The presence of the correct genomic insertion was tested by PCR of genomic DNA using primers specific for the *MOM8b*(*ISP6*) gene, both by checking the size of the amplified product and by confirming the existence of an internal *Eco*RV site in the amplified fragment belonging to the *URA3* gene.

The *MOM8b*(*ISP6*) gene in the YEp13 vector was obtained by screening an *S. cerevisiae* genomic DNA library, using as a probe a digoxigenin-labeled DNA fragment corresponding to most of the *MOM8b*(*ISP6*) gene. The presence of the whole open reading frame was checked by restriction analysis.

Mutant strains MM112 (*mom19*Δ) and MM208 (*mom72*Δ) have been described previously (32) (Table 1). To construct doubly deficient yeast strains, heterozygous diploids were constructed. Strain MM413 contained one disrupted copy of *MOM8b* and one disrupted copy of *MOM72*. After sporulation of these cells, four viable spores per diploid were obtained, and their genotypes were analyzed by growth on selective media (10 tetrads were analyzed, yielding 5 tetratype tetrads, 3 parental-ditype tetrads, and 2 nonparental-ditype tetrads). Growth on yeast extract-peptone-dextrose (YPD) medium (2% glucose) and yeast extract-peptone-glycerol (YPG) medium (3% glycerol) was tested at different temperatures. Strain MM415 contained one disrupted copy of *MOM8b* and one disrupted copy of *MOM19*. The tetrads were classified and the genotypes of the viable spores were determined (described in the legend to Fig. 6B).

Isolation of mitochondria. Mitochondria were isolated from the yeast strains grown at 30°C in YPD medium until mid-logarithmic phase by previously described procedures (6, 13). For isolation of ^{35}S-labeled mitochondria, an analogous procedure was used, except that the yeast cells were grown in complete synthetic medium containing 10 μCi of $[^{35}S]SO_4^{2-}$ per ml (41). Unless otherwise indicated, the yeast strain YPH500 (wild type) was used.

Immunoprecipitation of the receptor complex. Isolated mitochondria were pelleted by a 10-min centrifugation at 16,000 × *g* and 2°C and lysed by repeated pipetting in ice-cold digitonin buffer (0.25 M sucrose, 10 mM MOPS [morpholinepropanesulfonic acid] [pH 7.2], 0.13 M NaCl, 0.5% digitonin, 10% glycerol, 3% bovine serum albumin [BSA]) containing 1 mM phenylmethylsulfonyl fluo-

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

Strain	Genotype or relevant characteristic	Reference
YPH500	*MAT*α *ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801*	45
MM307	*MAT*α *ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801 mom8b*(*isp6*)::*URA3*	This study
MM307-A	*MAT*α *ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801 mom8b*(*isp6*)::*URA3* + YEp13-MOM8b	This study
MM112	*MAT***a** *ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801 mom19*::*URA3*	32
MM208	*MAT***a** *ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801 mom72*::*HIS3*	32
MM413	*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 MOM8b*(*ISP6*)/*mom8b*(*isp6*)::*URA3*	This study
MM415	*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 MOM19*/*mom19*::*URA3 MOM8b*(*ISP6*)/*mom8b*(*isp6*)::*URA3*	This study
MM415-A	*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 MOM19*/*mom19*::*URA3 MOM8b*(*ISP6*)/*mom8b*(*isp6*)::*URA3* + YEp13-MOM8b	This study
MM310	*MAT***a** *ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801 mom72*::*HIS3 mom8b*(*isp6*)::*URA3*	This study

ride and incubation for 10 min at 0°C. The insoluble material was removed by centrifugation at 18,000 × *g* and 2°C for 10 min. The supernatant was incubated with a protein A-Sepharose suspension, to which antibodies had been allowed to adsorb for 1 h at 4°C in the same buffer (where indicated, antibodies were covalently coupled to the protein A-Sepharose by using the homobifunctional cross-linker dimethyl pimelimidate at a concentration of 20 mM). Preimmune sera (antibodies) were obtained from rabbits before immunization with a specific antigen. The binding reaction was allowed to proceed for 1 h at 4°C, and then the beads were collected and successively washed three times with digitonin buffer without BSA and once with 10 mM Tris, pH 7.5. The complexes were dissociated by being heated at 95°C in electrophoresis sample buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In order to visualize the low-molecular-weight components of the complex, either urea-SDS-PAGE (19) or Tricine-SDS-PAGE (43) was used. For the urea-SDS-PAGE, the separating gel contained 18.2% T, 1.3% C, and 5 M urea (Tris at pH 8.85); with the Tricine, the separating gel contained 16.5% T and 6% C and a spacer gel of 10% C and 3% C (T and C as defined by Schägger and von Jagow [43]).

Import of preproteins into isolated mitochondria. Radiolabeled preproteins were obtained by in vitro transcription and translation using rabbit reticulocyte lysate (Amersham) in the presence of [^{35}S]methionine as previously described (48). Import reaction mixtures included BSA-containing buffer, reticulocyte lysate (20 to 50% [vol/vol] for Mom8b and 1% [vol/vol] for other preproteins), 2 mM NADH plus 2 mM ATP or 8 mM potassium ascorbate (pH 7) plus 0.2 mM *N*,*N*,*N*′,*N*′-tetramethylphenylenediamine (TMPD), and isolated *S. cerevisiae* mitochondria (0.3 mg of mitochondrial protein per ml) (48). Import was performed at 25°C for the times indicated below, and then the mitochondria were reisolated by centrifugation for 10 to 12 min at 16,000 × *g* and 2°C. Where indicated, a treatment with proteinase K (50 to 250 μg/ml) was performed. After being washed with SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS [pH 7.2]), the pelleted mitochondria were either analyzed by SDS-PAGE or lysed in ice-cold digitonin buffer and then subjected to coprecipitation of the receptor complex. Where indicated, surface receptors were removed by treatment of the mitochondria prior to the import reaction with 50 μg of trypsin per ml for 20 min at 0°C, and the protease was inhibited with a 30-fold weight excess of soybean trypsin inhibitor (5 min at 0°C). Dissipation of the membrane potential (Δψ) was accomplished by omitting NADH or ascorbate-TMPD from the import reaction mixture and adding a mixture of valinomycin, oligomycin, and antimycin A (final concentrations of 0.5, 20, and 8 μM, respectively). To accumulate the precursor of the ADP-ATP carrier at the receptor stage, reticulocyte lysate and mitochondria were pretreated with apyrase (5 U/ml) for 20 min at 25 or 0°C, respectively; incubation with mitochondria was performed in the absence of a Δψ (39, 48). For generation of the GIP intermediate, radiolabeled ADP-ATP carrier was incubated with isolated mitochondria in the absence of a Δψ and the presence of 2 mM ATP. Surface-bound preproteins were removed by a treatment with trypsin (20 μg/ml) for 20 min at 0°C (37, 48). All samples were made chemically identical by addition of solute-free solvents.

Miscellaneous. Antibodies against the C-terminal 14 amino acid residues of Isp6 were generated in rabbits by using a chemically synthesized peptide that was coupled to keyhole limpet hemocyanin via an N-terminal cysteine (12). Standard procedures were used for immunoblotting by using the enhanced chemiluminescence system (Amersham), assessment of the Δψ by using the fluorescent dye 3,3′-dipropylthiadicarbocyanine iodide [$DiSC_3(5)$; Molecular Probes, Inc.] (7, 10, 46), and storage phosphor imaging technology (Molecular Dynamics).

RESULTS

Evidence for identity of Isp6 with Mom8b. The coding region of *ISP6* was cloned into the vector pGEM4. By in vitro transcription and translation in rabbit reticulocyte lysate in the presence of [^{35}S]methionine, the precursor of Isp6 was synthesized. We incubated the precursor with isolated *S. cerevisiae* mitochondria and found association of Isp6 with the mitochondria. In order to address the question of whether in vitro-imported Isp6 was associated with the outer membrane receptor complex, we tested for coprecipitation with antibodies directed against Mom19 or Mom38. Mitochondria with in vitro-imported Isp6 were lysed with digitonin, and the mitochondrial receptor complex was coprecipitated (24, 31, 49). Isp6 was indeed coprecipitated with antibodies directed against Mom19 or against Mom38 but not with preimmune serum (Fig. 1A, lanes 1 to 3). The import of Isp6 was not inhibited by dissipation of the Δψ across the inner membrane (Fig. 1A, lane 4), demonstrating that its import, like that of other outer membrane proteins, is independent of a Δψ (14). We conclude that in vitro-synthesized Isp6 is targeted to the mitochondrial receptor complex.

We questioned whether Isp6 corresponded to one of the known small components of the mitochondrial receptor complex. ^{35}S-labeled yeast mitochondria were lysed in digitonin-containing buffer, and the receptor complex was coprecipitated with anti-Mom38 antibodies and resolved by urea-SDS-PAGE to visualize the small subunits Mom8a and Mom8b (Fig. 1B, lane 4) (31) (Mom7 from ^{35}S-labeled yeast cells cannot be detected by autoradiography because of a lack of sulfur-containing amino acids besides the posttranslationally removed initial methionine [16, 31]). In parallel, the ^{35}S-labeled precursor of Isp6 was imported into unlabeled mitochondria, and then the receptor complex was coprecipitated with anti-Mom38 antibodies. Surprisingly, Isp6 showed the same gel mobility as Mom8b (Fig. 1B, lanes 2 and 4), although Kassenbrock et al. (22) had reported that Isp6 ran below the 6-kDa marker. We noticed that this experiment and our previous experiments were analyzed by urea-SDS-PAGE, whereas Kassenbrock et al. (22) had used Tricine-SDS-PAGE. Separation of a ^{35}S-labeled receptor complex on a Tricine gel indeed resulted in a larger mobility difference between Mom8a and Mom8b. In vitro-imported Isp6 again ran at the same position as the lower of the two bands (Fig. 1B, lanes 6 and 8), raising the possibility that Isp6 is identical to Mom8b.

We thus disrupted the *ISP6* gene with the *URA3* gene. In agreement with the results of Kassenbrock et al. (22), the mutant *S. cerevisiae* cells were viable on fermentable and nonfermentable carbon sources at 30°C. When ^{35}S-labeled *isp6*Δ mitochondria were subjected to coprecipitation with anti-Mom38 antibodies, the band corresponding to Mom8b was indeed lacking, whereas Mom8a was unaffected (Fig. 1C, lane 2).

To obtain further evidence for the identity of Isp6 and Mom8b, we raised an antiserum against the C-terminal 14

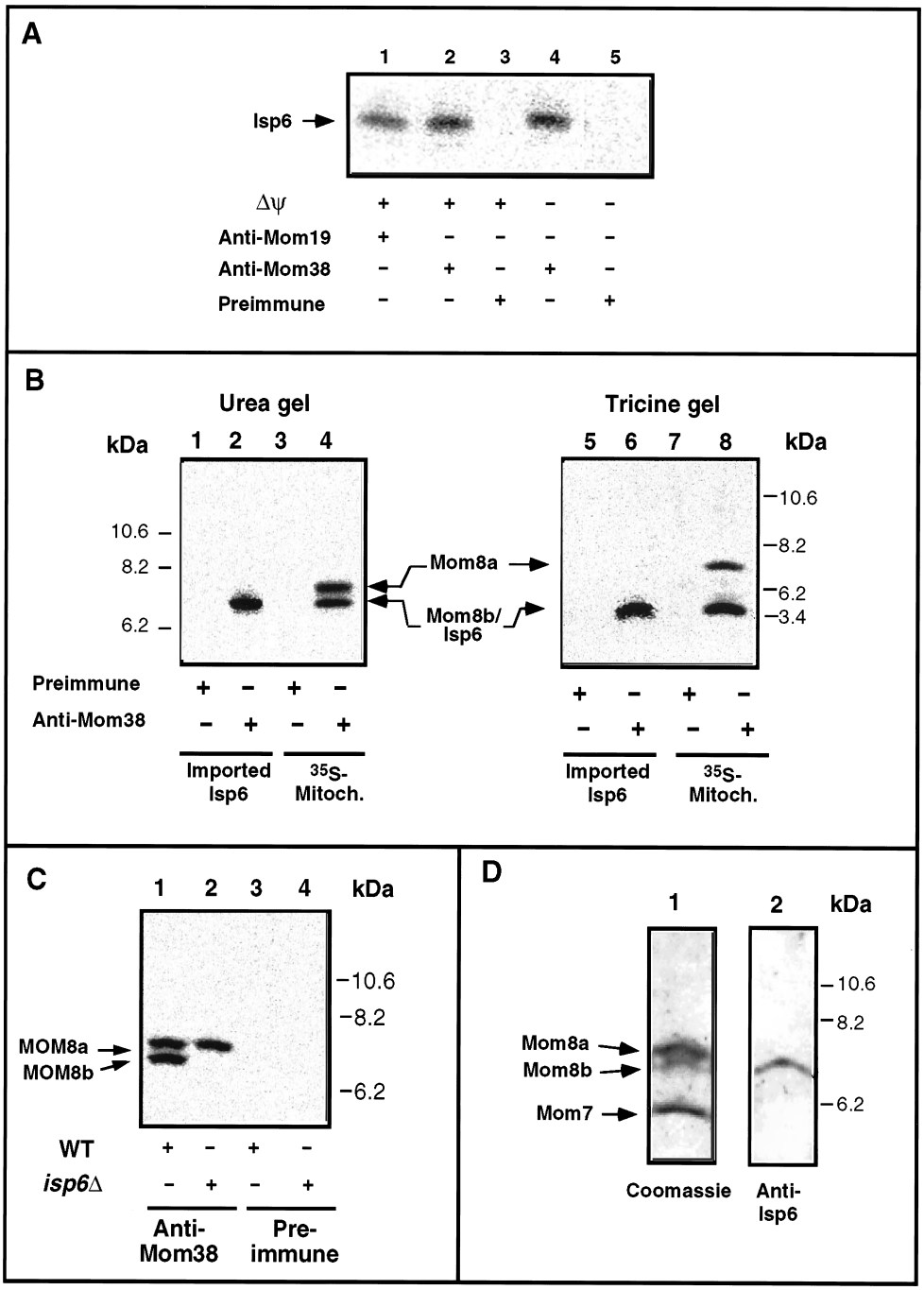

FIG. 1. Evidence for identity of Isp6 and Mom8b. (A) Import of in vitro-synthesized Isp6 into isolated mitochondria and assembly into the receptor complex. Rabbit reticulocyte lysate containing ^{35}S-labeled precursor of Isp6 was incubated with isolated *S. cerevisiae* mitochondria in the presence of either NADH (+ Δψ) or valinomycin, antimycin A, and oligomycin (− Δψ) for 15 min at 25°C. The mitochondria were reisolated and lysed with digitonin, and immunoprecipitations with antibodies directed against Mom19 or Mom38 or with preimmune serum were performed as described in Materials and Methods. The precipitates were analyzed by urea-SDS-PAGE and visualized by storage phosphor imaging technology. (B) Similar mobilities of Mom8b and Isp6 in different gel electrophoresis systems. In vitro-labeled precursor of Isp6 was imported into isolated mitochondria as described above. The mitochondria were isolated and lysed with digitonin buffer, and the solution was split into halves, which were subjected to coprecipitation with anti-Mom38 antibodies (samples 2 and 6) or preimmune antibodies (samples 1 and 5). In parallel, ^{35}S-labeled mitochondria (^{35}S-Mitoch.) were lysed with digitonin buffer and subjected to coprecipitation with anti-Mom38 antibodies (lanes 4 and 8) or preimmune antibodies (lanes 3 and 7). The precipitates were dissociated by being heated in sample buffer for 3 min at 95°C and analyzed by urea-SDS-PAGE (lanes 1 to 4) or Tricine-SDS-PAGE (lanes 5 to 8) following the conditions described in Materials and Methods. (C) Coprecipitation of the receptor complex from mitochondria lacking Isp6. Mitochondria were isolated from wild-type (WT) strain YPH500 and from strain MM307 (disrupted *ISP6* gene, *isp6*Δ) after growth in synthetic medium containing $[^{35}S]SO_4^{2-}$. The mitochondria were solubilized with digitonin, and coprecipitations with antibodies directed against Mom38 or preimmune antibodies were performed. The precipitates were analyzed by urea-SDS-PAGE and storage phosphor imaging technology. (D) Immunodecoration of Mom8b with antibodies directed against Isp6. Unlabeled *S. cerevisiae* wild-type mitochondria were lysed with digitonin and subjected to coprecipitation with antibodies against Mom38 that had been covalently coupled to protein A-Sepharose (17, 31). The sample was split. Both halves were separated by urea-SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Immobilon P; Millipore). One half was stained with Coomassie brilliant blue R250 (lane 1). The other half was immunodecorated with antibodies generated against the C terminus of Isp6 (lane 2).

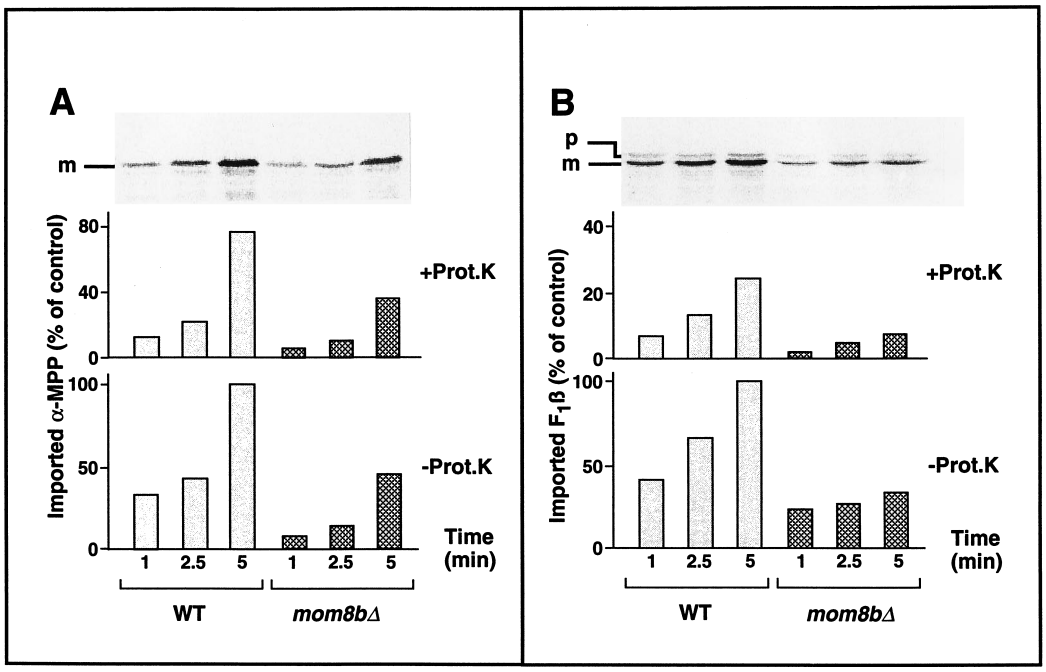

FIG. 2. Mitochondria lacking Mom8b (*mom8b*Δ) are impaired in import of cleavable preproteins. Rabbit reticulocyte lysates with radiolabeled precursors of α-MPP (A) and $F_1\beta$ (B) were incubated with isolated energized mitochondria at 25°C for the times indicated. The mitochondria were reisolated, and the sample was split into halves. One half was treated with proteinase K (Prot. K). The mitochondria were reisolated again and analyzed by SDS-PAGE and storage phosphor imaging technology. The amount of mature-size protein (m) in the samples without proteinase K treatment after a 5-min import was set at 100%. Results of an experiment typical of five independent experiments, all of which revealed similar degrees of import inhibition into *mom8b*Δ mitochondria, are shown. In the case of $F_1\beta$, a small amount of precursor form (p) was protected against proteinase K, representing imported but not yet processed preprotein (this is often observed for $F_1\beta$ with short import times). With *mom8b*Δ mitochondria, the amount of imported p-form protein was reduced, like that of the m form, compared with that in wild-type (WT) mitochondria.

amino acid residues of Isp6. We performed a large-scale coprecipitation of the receptor complex from unlabeled yeast mitochondria which allows preparation of sufficient quantities to detect the small subunits by staining with Coomassie blue (17, 31). By this method, yeast Mom7 can also be visualized (Fig. 1D, lane 1). The antiserum directed against Isp6 selectively recognized the band of Mom8b (Fig. 1D, lane 2). Moreover, the N-terminal amino acid residues of Mom8a and Mom7 could be determined from the purified receptor complex, proving that Mom8a and Mom7 are distinct proteins not related to Mom8b (16).

The combined evidence thus demonstrates that Isp6 is identical to Mom8b and therefore is a genuine component of the mitochondrial receptor complex. Since Mom8b was identified first and the "Mom" nomenclature is the only one used to describe the other two small subunits of the mitochondrial receptor complex, we will use Mom8b as the name.

Mom8b promotes efficient transfer of preproteins. Does a deletion of Mom8b affect the import of precursor proteins? Two typical mitochondrial preproteins, the α-subunit of the mitochondrial processing peptidase (α-MPP) and the β-subunit of the F_1-ATPase ($F_1\beta$), were synthesized in reticulocyte lysates in the presence of [^{35}S]methionine and incubated with isolated energized mitochondria. The import of preproteins was determined by the proteolytic removal of the presequence (formation of the mature-size form) and protection of the imported protein against externally added proteinase K. With both preproteins, the import into *mom8b*Δ mitochondria was not completely blocked but significantly reduced compared with that of wild-type mitochondria (Fig. 2).

The observation of a reduced efficiency of import into *mom8b*Δ mitochondria can be explained either by a specific effect of deletion of Mom8b on the function of the outer membrane receptor complex or by indirect effects on mitochondrial function, such as reduction of the Δψ across the inner membrane or reduced stability of the mitochondrial membranes. To exclude the latter two possibilities, we assessed the Δψ and tested the stability against externally added proteases of *mom8b*Δ and wild-type mitochondria. By use of the fluorescent dye $DiSC_3(5)$, we assessed the mitochondrial Δψ. A decrease in fluorescence quenching indicates the formation of a Δψ (7, 10, 46). The decreases in fluorescence quenching with *mom8b*Δ mitochondria and wild-type mitochondria were similar (Fig. 3), indicating that *mom8b*Δ mitochondria were not impaired in generation of a Δψ. The protease resistance of *mom8b*Δ mitochondria was indistinguishable from that of wild-type mitochondria, as determined with marker proteins for the mitochondrial subcompartments (data not shown). Moreover, we show below that the Mom proteins, such as the receptors Mom19 and Mom72 and the GIP component Mom38, contained by *mom8b*Δ and wild-type mitochondria were comparable.

We next examined whether specific functions of the receptor complex in the interaction with preproteins were impaired. With the precursor of the ADP-ATP carrier, a noncleavable preprotein with internal signal sequences, two distinct stages of interaction with the receptor complex can be distinguished (37, 39, 47, 48): (i) at low ATP concentrations and in the absence of a Δψ, the preprotein is preferentially accumulated at the surface receptors, and (ii) in the presence of ATP and the absence of a Δψ, the preprotein is inserted into the GIP and protected

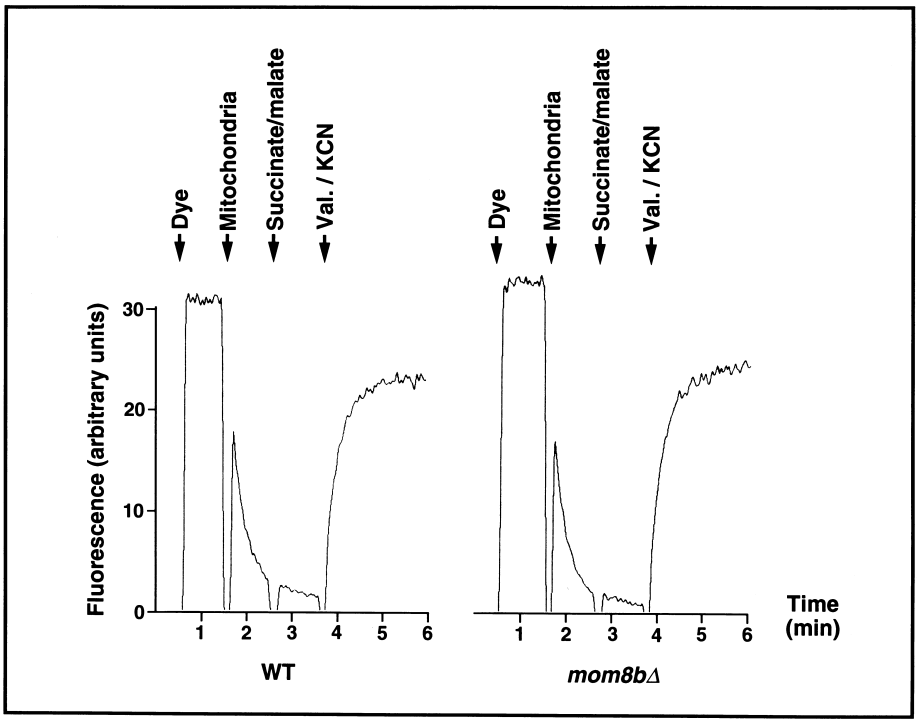

FIG. 3. Mitochondria lacking Mom8b are able to generate a $\Delta\psi$. The $\Delta\psi$ was assessed by fluorescence photometry with the potential-sensitive dye $DiSC_3(5)$. At the indicated times, mitochondria (100 μg of protein), 5 mM succinate plus 5 mM malate, and 1 μM valinomycin (Val.) plus 1 mM potassium cyanide (KCN) were added. The difference in fluorescence between the addition of mitochondria and substrates (generation of a $\Delta\psi$) and the addition of valinomycin and KCN (dissipation of $\Delta\psi$) provides an assessment of the $\Delta\psi$. WT, wild type.

against externally added trypsin (whereas the receptors are degraded by trypsin) (33, 36, 48).

We first tested whether the import of the ADP-ATP carrier into the inner membrane was impaired in *mom8b*Δ mitochondria and found a reduction of the import efficiency (Fig. 4A) similar to that observed for the cleavable preproteins α-MPP and $F_1\beta$. Then the precursor of the ADP-ATP carrier was accumulated at the two distinct outer membrane stages described above (receptor stage and GIP stage [Fig. 4B and C, respectively]). The mitochondria were lysed with detergent, and coprecipitations with antibodies directed against receptor Mom19 or Mom72 (Fig. 4B) or against Mom38 (Fig. 4C) were performed. Surprisingly, the fraction of total mitochondrion-associated preprotein that was found in the coprecipitate with anti-Mom19 or anti-Mom38 antibodies was significantly increased with *mom8b*Δ mitochondria compared with that for wild-type mitochondria (Fig. 4B and C, bars 3 and 4). Only a slight increase was observed in the coprecipitates with anti-Mom72 antibodies (Fig. 4B, bars 5 and 6). The efficiencies of the coimmunoprecipitations were comparable to those previously reported for interactions between preproteins and import components (30, 51, 53). Preimmune antibodies did not precipitate significant amounts of accumulated preproteins (Fig. 4B and C, bars 1 and 2), excluding that the increased yield of preproteins coprecipitated from *mom8b*Δ mitochondria was due to unspecific precipitation.

Moreover, the increased yields of coprecipitates from *mom8b*Δ mitochondria were not caused by a binding of preproteins to Mom proteins after lysis of mitochondria, since after addition of preproteins to lysed mitochondria, only background levels were coprecipitated with anti-Mom antibodies from both *mom8b*Δ and wild-type mitochondria (Fig. 4C, bars 7 and 8, and data not shown). Thus, the yields of preproteins associated with solubilized Mom proteins are so much smaller than those in intact mitochondria that they cannot contribute to the observed coprecipitable amounts of preprotein.

Since the coimmunoprecipitations include four washes of the antibody-bound protein complexes, relatively stable associations are preferentially detected, suggesting that the stability of association between preproteins and some components of the receptor complex, at least Mom19 and Mom38, is increased in *mom8b*Δ mitochondria.

Mom8b is required for the stability of the receptor complex. We wondered if the deletion of Mom8b affected the association of Mom proteins in the mitochondrial receptor complex. We performed coimmunoprecipitations of proteins from digitonin-lysed mitochondria with antibodies directed against Mom19, Mom72, or Mom38. The immunoprecipitates were decorated with specific antisera to visualize the content of Mom19 and Mom38 (Fig. 5A). The efficiency of coprecipitation of Mom19 from *mom8b*Δ mitochondria with both anti-Mom72 antibodies and anti-Mom38 antibodies was significantly decreased compared with that of wild-type mitochondria (Fig. 5A, lanes 5, 6, 9, and 10; Table 2). The efficiency of coprecipitation was further decreased when the mitochondria were preincubated at 37°C (Fig. 5A, lanes 7, 8, 11, and 12; Table 2). Similarly, the efficiency of coprecipitation of Mom38 was decreased with anti-Mom19 and anti-Mom72 antibodies (Fig. 5A, lanes 2, 4, 6, and 8; and Table 2). The lower efficiency of coprecipitation was not caused by a lower mitochondrial content of those complex subunits, as Western blotting (immunoblotting) indicated that they were present in comparable amounts in wild-type and *mom8b*Δ mitochondria (Fig. 5B). Moreover, the antibodies precipitated comparable amounts of

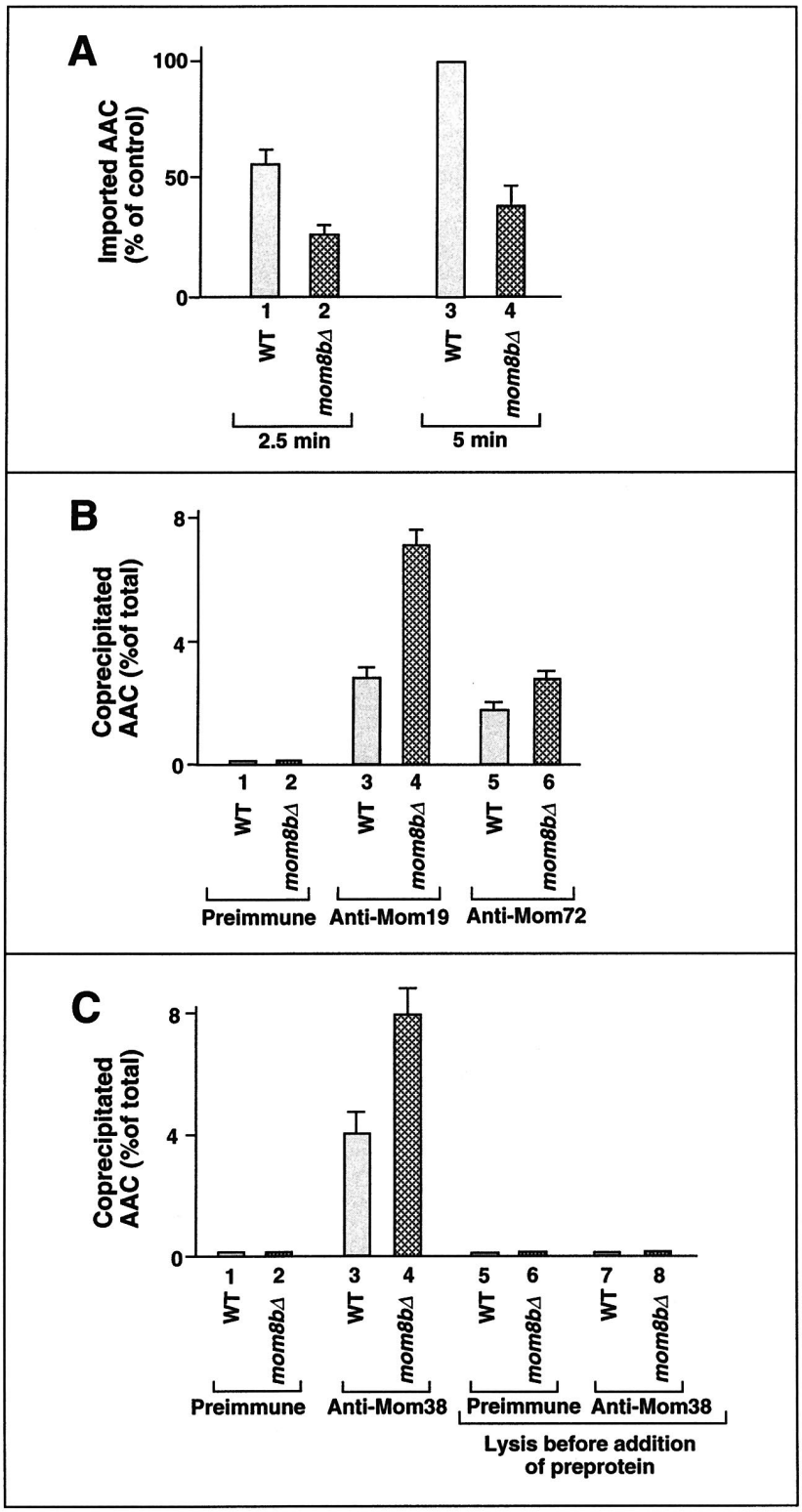

FIG. 4. Deletion of Mom8b impairs import of the precursor of the ADP-ATP carrier (AAC) and stabilizes interaction of the precursor with Mom proteins. (A) Mitochondria lacking Mom8b are impaired in the import of the AAC. The experiment was performed as described in the legend to Fig. 2 except that ^{35}S-labeled precursor of AAC was used and all samples were treated with proteinase K after the import. (B and C) Increased yields of coprecipitated preproteins from *mom8b*Δ mitochondria. The precursor of the AAC was accumulated at the receptor stage in the absence of a $\Delta\psi$ and at low ATP concentrations (B) or was accumulated at the GIP stage in the absence of a $\Delta\psi$ but in the presence of ATP (C) (samples 1 to 4) (see Materials and Methods). The mitochondria were reisolated, lysed with digitonin, and subjected to coimmunoprecipitations with antibodies directed against Mom19, Mom72, or Mom38 or preimmune antibodies as indicated. For samples 5 to 8 of panel C, ^{35}S-labeled precursor of AAC was added to digitonin-lysed mitochondria, and then coprecipitations were done as indicated. The total amount of AAC precursor that was immunoprecipitated by antiserum directed against AAC was set at 100% for each condition. The values shown (with standard errors of the means of three or four independent determinations) indicate the fraction of coprecipitable AAC precursor at each stage. WT, wild type.

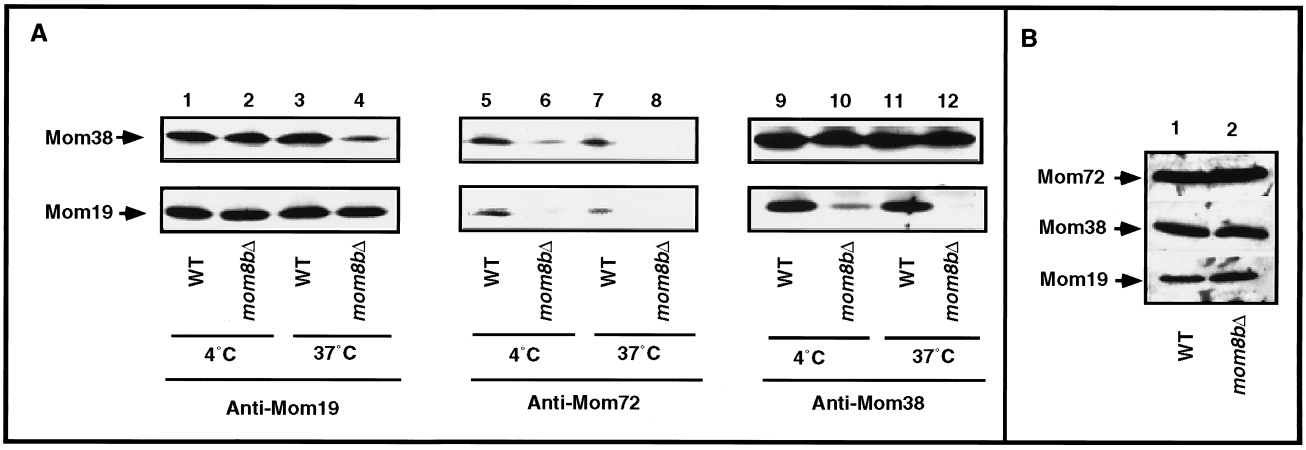

FIG. 5. Deletion of Mom8b destabilizes interactions of Mom38, Mom19, and Mom72. (A) Coprecipitations of Mom19, Mom72, and Mom38. Isolated mitochondria (nonradiolabeled) were preincubated for 5 min at 37 or 4°C, lysed with digitonin, and subjected to coimmunoprecipitations with anti-Mom19 (lanes 1 to 4), anti-Mom72 (lanes 5 to 8), and anti-Mom38 (lanes 9 to 12) antibodies (which had been covalently bound to protein A-Sepharose). The immunoprecipitates were analyzed by Western blotting with antisera directed against Mom38 or Mom19. The lower panel of lanes 5 to 8 was exposed twice as long as the other panels. (B) Deletion of Mom8b does not reduce the total amounts of Mom19, Mom38, and Mom72 present in mitochondria. Wild-type mitochondria and *mom8b*Δ mitochondria (10 μg of protein each) were subjected to Western blotting with antisera directed against Mom72, Mom38, and Mom19. WT, wild type.

the genuine antigen from wild-type and *mom8b*Δ mitochondria under nondenaturing conditions (digitonin extraction) (Fig. 5A; Table 2), excluding the possibility that in *mom8b*Δ mitochondria, these complex subunits had a tendency for aggregation or denaturation that prevented their solubilization by the digitonin treatment. Moreover, anti-Mom38 antibodies coprecipitated Mom8a with wild-type efficiency (Fig. 1C, lanes 1 and 2; Table 2), whereas anti-Mom19 antibodies precipitated smaller amounts of Mom8a (Table 2).

We conclude that the total mitochondrial amounts of the analyzed components of the receptor complex (Mom19, Mom72, Mom38, and Mom8a) are not decreased by a deletion of Mom8b; however, their association in a complex is destabilized. While Mom8a remains stably bound to Mom38, the association of Mom19 and Mom72 with Mom38-Mom8a is weakened. The dissociation of the complex is enhanced at high temperatures.

TABLE 2. Deletion of Mom8b decreases the stability of association of Mom19, Mom38, and Mom72[a]

Mom protein	Precipitated protein	*mom8b*Δ/wild-type ratio	
		4°C	37°C
Total	Mom19	1.1	0.9
	Mom38	1.0	0.9
	Mom72	1.0	1.1
Coprecipitated	Mom19 with anti-Mom38	0.3	0.1
	Mom19 with anti-Mom72	0.4	0.3
	Mom38 with anti-Mom72	0.4	0.1
	Mom8a with anti-Mom19	0.4	ND
	Mom8a with anti-Mom38	1.0	ND

[a] The experiment was performed as described in the legend to Fig. 5A. Shown are the ratio between *mom8b*Δ and wild-type mitochondria of the total immunoprecipitable amounts of Mom proteins (with antiserum specific for each Mom protein) and the *mom8b*Δ/wild-type ratio of the coprecipitated amounts of the Mom proteins. For determination of the values for Mom8a, the experiment was performed as described in the legend to Fig. 1C. Values are the means of three to five independent experiments with standard errors of the means of ≤0.1. ND, not determined.

Synthetic phenotypes of double deletions *mom19*Δ *mom8b*Δ and *mom72*Δ *mom8b*Δ. Synthetic phenotypes have been found to be of high value to characterize functional cooperation of components by genetic means. That is, mutations in two components (or deletions) lead to a phenotype not observed with mutation or deletion of a single component and thereby provide indication for a functional relationship between two components (18, 21, 41). The biochemical results obtained suggest a functional relationship of Mom8b to the import receptors. We examined whether genetic evidence for a functional relationship between Mom19 or Mom72 and Mom8b could be obtained.

Yeast cells lacking either Mom8b or Mom72 are viable on fermentable and nonfermentable carbon sources at both 30 and 37°C (22, 32, 40, 50), although growth of the single mutants on nonfermentable medium at 37°C is reduced (Fig. 6A, sectors 2 and 3). By crossing of the *mom8b*Δ strain and the *mom72*Δ strain and sporulation of the resulting diploid, we generated strain MM310, which lacked both Mom8b and Mom72 (Table 1). The doubling times for the double mutant at 30°C on fermentable and nonfermentable media were about twofold less than that of the single mutants (growth of the single mutants at 30°C is indistinguishable from that of wild-type cells on both media). At 37°C on nonfermentable medium, the double mutant was inviable (Fig. 6A, sector 4). When Mom8b was expressed from a plasmid, growth of the *mom8b*Δ strain on nonfermentable medium at 37°C was fully restored, and growth of the *mom72*Δ *mom8b*Δ strain was like that of the *mom72*Δ strain (including viability at 37°C on nonfermentable medium). These results argue for a synthetic growth defect of the double mutant lacking Mom8b and Mom72.

Similarly, we tried to obtain a *mom19*Δ *mom8b*Δ double mutant. We crossed the *mom8b*Δ strain with a *mom19*Δ strain (which is viable on fermentable medium [32]) and sporulated the resulting heterozygous diploid (MM415; Table 1). On fermentable medium at 30°C, viable and inviable spores were obtained. Figure 6B shows representative tetrads, a nonparental-ditype tetrad and a tetratype tetrad. Analysis of the genotypes of the viable spores showed that inviability was due to a lack of both genes. The doubly deficient spores germinated but

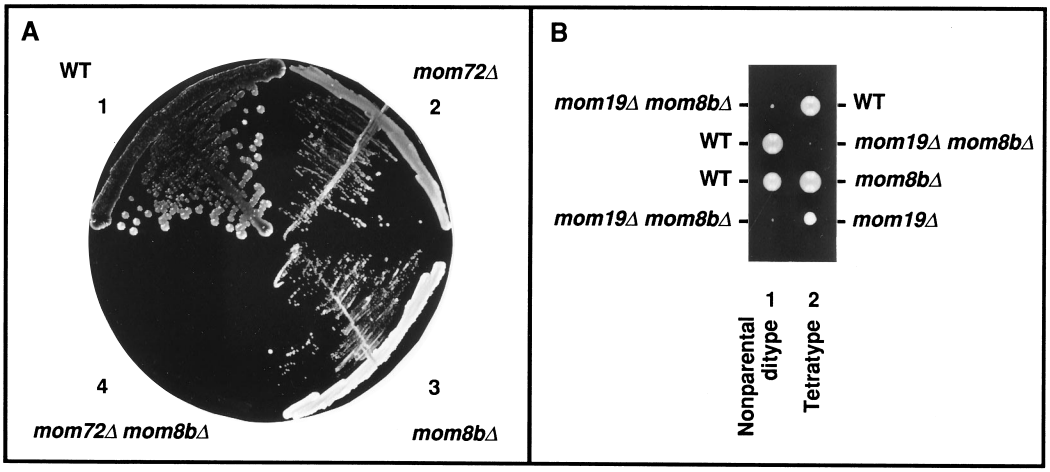

FIG. 6. Synthetic growth defects of *S. cerevisiae* double mutants lacking Mom8b and either Mom72 or Mom19. (A) Strains YPH500 (wild type [WT]), MM208 (*mom72*Δ), MM307 (*mom8b*Δ), and MM310 (*mom72*Δ *mom8b*Δ) were grown on a YPG plate at 37°C. (B) The heterozygous diploid strain MM415, containing one disrupted allele of the *MOM19* gene and one disrupted allele of the *MOM8b* gene, was sporulated, and the asci were dissected. The phenotypes of the viable spores were analyzed to assign the genotypes of the spores as indicated. A total of 55 tetrads were analyzed, including 14 tetrads of the parental ditype, 12 tetrads of the nonparental ditype, and 29 tetratype tetrads. Results are shown for one nonparental-ditype tetrad (lane 1) and one tetratype tetrad (lane 2) grown on YPD medium at 30°C.

stopped growth after ~12 divisions, even when transferred to new plates. As a control, strain MM415 was transformed with a plasmid expressing Mom8b (strain MM415-A). Sporulation of these cells yielded viable spores which could be deduced to contain disrupted chromosomal copies of *mom19* and *mom8b*, and all of these cells carried the plasmid marker. At the lower temperature (23°C), *mom19*Δ *mom8b*Δ cells were found to grow also in the absence of the plasmid, yet extremely slowly. We conclude that the deletion of both Mom8b and Mom19 leads to a strong synthetic growth defect.

DISCUSSION

We report that Isp6 is identical to Mom8b of the receptor complex in the yeast mitochondrial outer membrane and promotes cooperation between receptors and the GIP and thereby supports efficient transfer of preproteins. Mom8b/Isp6 is thus the first small subunit of the receptor complex that can be functionally characterized by biochemical and genetic means.

Kassenbrock et al. (22) had identified Isp6 as a high-copy-number suppressor of a mutation in Mom38. On the basis of its gel mobility, they concluded that Isp6 was not any of the known components of the receptor complex. By analyzing the mobility in different gel systems, we surprisingly found that Isp6 imported into isolated mitochondria had the same gel mobility as the previously identified small subunit Mom8b. A yeast mutant with a disrupted *ISP6* gene selectively lacked Mom8b of the receptor complex, and antibodies generated against a C-terminal peptide of Isp6 specifically recognized Mom8b. By determination of peptide sequences, the other two small subunits of the complex, Mom8a and Mom7, were shown not to be related to Isp6. We conclude that Isp6 is identical to Mom8b. It is quite satisfying that two fundamentally different approaches (coprecipitation [17, 31] and genetic suppression [22]) led to the identification of the same small subunit of the receptor complex, providing the strongest evidence so far for a significance of the presence of small subunits in preparations of the mitochondrial receptor complex.

The import of preproteins was two- to threefold delayed in *mom8b*Δ mitochondria compared with that in wild-type mitochondria. This was found for typical preproteins with amino-terminal presequences which are imported mainly via the Mom19 pathway and for a preprotein with internal signal sequences which preferentially uses the Mom72 pathway. A role of Mom8b in protein import is thus not restricted to only one of the import pathways. The generation of a $\Delta\psi$ and the stability of the membranes of *mom8b*Δ mitochondria were indistinguishable from those of wild-type mitochondria, excluding these indirect effects as a cause for the reduced rate of protein import. When the association of preproteins with Mom proteins was studied, we made a surprising observation. The yield of outer membrane-accumulated preproteins coprecipitated with Mom19 and Mom38 from *mom8b*Δ mitochondria was significantly higher than that from wild-type mitochondria (i.e., the fraction of accumulated preprotein that was found in the coprecipitate after several washing steps). This suggests that a deletion of Mom8b increases the stability of interaction of preproteins with Mom19 and Mom38.

In contrast, a deletion of Mom8b decreased the stability of interaction of receptors Mom19 and Mom72 with Mom38 without changing the total mitochondrial amounts of these Mom proteins. The association between Mom38 and Mom8a was not affected. The dissociation of the receptors from Mom38-Mom8a was enhanced at the higher temperature. We conclude that Mom8b has a stabilizing effect on the receptor complex and favors the association of receptors Mom19 and Mom72 with Mom38-Mom8a. It was previously shown that a large fraction (at least 70 to 80%) of all Mom19 molecules is present in the affinity-purified receptor complex but that the interaction between Mom72 and the receptor complex is quite transient, i.e., only ~15 to 20% of the Mom72 molecules are found in the affinity-purified complex in the presence of Mom8b (23, 31); the fraction is further decreased upon deletion of Mom8b. Since only the small fraction of Mom72 which can be detected in association with the complex should be influenced by a deletion of Mom8b, this provides an explanation for the only minor increase in the coprecipitation of accumulated preproteins with Mom72 from *mom8b*Δ mitochondria (Fig. 4B).

Stabilizing effects of small subunits in multisubunit membrane complexes of mitochondria have been reported for com-

plexes of the respiratory chain. The best example is the deletion of the 8.5-kDa component of the yeast bc_1 complex (3). This impaired the association of other subunits with the complex without affecting the total mitochondrial levels of the other subunits. Maarse et al. (29) similarly reported destabilizing effects of deletion of a small subunit on other complex subunits, though in that case, the cellular steady-state levels of some subunits were decreased.

A functional relationship of Mom8b to both receptors is also suggested by genetic evidence for interaction of Mom8b with Mom19 and Mom72 (synthetic growth defects of double deletion mutants). Upon demonstration of the identity of Isp6 with Mom8b, the genetic and biochemical evidence for interaction of Isp6 with Mom38 provided by Kassenbrock et al. (22) can be set in context. Together, the genetic data suggest a functional interaction of Mom8b with (at least) three other Mom proteins, receptors Mom19 and Mom72 and the essential protein Mom38 of the GIP. This agrees well with the biochemically observed linker function of Mom8b in stabilizing the interactions between receptors and the GIP.

Chemical cross-linking with preproteins arrested at the outer membrane showed one or two small proteins (about 5 to 10 kDa) which were close to a preprotein at the GIP (49). Cross-linking experiments with the *mom8b*Δ mitochondria indicate that Mom8b is not among these cross-linking products, suggesting Mom8a and Mom7 as likely candidates for the small proteins interacting with preproteins (25).

We propose the following working model for the function of Mom8b in the mitochondrial receptor complex. Preproteins are initially recognized and bound by the import receptors Mom19 and Mom72, possibly in cooperation with partner proteins, such as Mom22 and Mas37 (11, 17, 23, 34, 38). Mom8b stabilizes the interaction of import receptors with the components of the GIP (Mom38) and thereby promotes the release of preproteins from the receptors for transfer to the GIP. In addition, Mom8b may promote the release of preproteins from Mom38 and thus also support preprotein transfer from the GIP to further components of the mitochondrial import machinery; possible explanations are the general stabilizing effect of Mom8b on the receptor complex and/or modulatory effects on the function of Mom38. An influence of Mom8b on the function of Mom38 is in agreement with the genetic characterization of interaction of the two components (multicopy suppression and synthetic lethality [22]). In summary, Mom8b seems to function as a linker between receptors and the GIP and to thereby promote an efficient flow of preproteins (probably without direct interaction with preproteins).

A small subunit (<10 kDa) has also been found in the protein translocation complex of the endoplasmic reticulum. The essential protein Sss1p (Sec61γ) (9, 15) shows similarity to SecE, a subunit of the preprotein translocase of bacteria (4, 35, 44). In analogy to the interaction of Mom8b with the core subunit of the mitochondrial GIP (Mom38), Sss1p-Sec61γ-SecE genetically and physically interacts with the core membrane subunit of the translocase (Sec61p-Sec61α of the endoplasmic reticulum and SecY of bacteria) (4, 9, 15, 35, 44), yet no evidence for direct interaction of the small subunit with preproteins could be detected (20). It is tempting to speculate that small subunits in a translocation complex have important modulatory effects on the functions of other (larger) subunits of the transport machinery and the stability of the complex.

ACKNOWLEDGMENTS

We are grateful to G. Igloi for oligonucleotide synthesis and to J. Rassow and W. Voos for constructive comments on the manuscript. We thank N. Zufall for expert technical assistance.

This study was supported by the Deutsche Forschungsgemeinschaft, the Fonds der Chemischen Industrie, and an EMBO long-term fellowship (to A.A.).

REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1989. Current protocols in molecular biology. John Wiley and Sons Inc., New York.
2. **Baker, K. P., and G. Schatz.** 1991. Mitochondrial proteins essential for viability mediate protein import into yeast mitochondria. Nature (London) **349:**205–208.
3. **Brandt, U., S. Uribe, H. Schägger, and B. L. Trumpower.** 1994. Isolation and characterization of *QCR10*, the nuclear gene encoding the 8.5-kDa subunit 10 of the *Saccharomyces cerevisiae* cytochrome bc_1 complex. J. Biol. Chem. **269:**12947–12953.
4. **Brundage, L., J. P. Hendrick, E. Schiebel, A. J. M. Driessen, and W. Wickner.** 1990. The purified E. coli integral membrane protein SecY/E is sufficient for reconstitution of SecA-dependent precursor protein translocation. Cell **62:**649–657.
5. **Cao, W., and M. G. Douglas.** 1995. Biogenesis of ISP6, a small carboxyl-terminal anchored protein of the receptor complex of the mitochondrial outer membrane. J. Biol. Chem. **270:**5674–5679.
6. **Daum, G., P. C. Böhni, and G. Schatz.** 1982. Import of proteins into mitochondria: cytochrome b_2 and cytochrome c peroxidase are located in the intermembrane space of yeast mitochondria. J. Biol. Chem. **257:**13028–13033.
7. **Eilers, M., W. Oppliger, and G. Schatz.** 1987. Both ATP and an energized inner membrane are required to import a purified precursor protein into mitochondria. EMBO J. **6:**1073–1077.
8. **Elble, R.** 1992. A simple and efficient procedure for transformation of yeasts. BioTechniques **13:**18–20.
9. **Esnault, Y., M.-O. Blondel, R. J. Deshaies, R. Schekman, and F. Képès.** 1993. The yeast *SSS1* gene is essential for secretory protein translocation and encodes a conserved protein of the endoplasmic reticulum. EMBO J. **12:**4083–4093.
10. **Gärtner, F., W. Voos, A. Querol, B. Miller, E. A. Craig, M. G. Cumsky, and N. Pfanner.** 1995. Mitochondrial import of subunit Va of cytochrome *c* oxidase characterized with yeast mutants: independence from receptors, but requirement for matrix hsp70 translocase function. J. Biol. Chem. **270:**3788–3795.
11. **Gratzer, S., T. Lithgow, R. E. Bauer, E. Lamping, F. Paltauf, S. D. Kohlwein, V. Haucke, T. Junne, G. Schatz, and M. Horst.** 1995. Mas37p, a novel receptor subunit for protein import into mitochondria. J. Cell Biol. **129:**25–34.
12. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
13. **Hartl, F.-U., J. Ostermann, B. Guiard, and W. Neupert.** 1987. Successive translocation into and out of the mitochondrial matrix: targeting of proteins to the intermembrane space by a bipartite signal peptide. Cell **51:**1027–1037.
14. **Hartl, F.-U., N. Pfanner, D. W. Nicholson, and W. Neupert.** 1989. Mitochondrial protein import. Biochim. Biophys. Acta **988:**1–45.
15. **Hartmann, E., T. Sommer, S. Prehn, D. Görlich, S. Jentsch, and T. A. Rapoport.** 1994. Evolutionary conservation of components of the protein translocation complex. Nature (London) **367:**654–657.
16. **Hönlinger, A., C. Eckerskorn, and N. Pfanner.** Unpublished data.
17. **Hönlinger, A., M. Kübrich, M. Moczko, F. Gärtner, L. Mallet, F. Bussereau, C. Eckerskorn, F. Lottspeich, K. Dietmeier, M. Jacquet, and N. Pfanner.** 1995. The mitochondrial receptor complex: Mom22 is essential for cell viability and directly interacts with preproteins. Mol. Cell. Biol. **15:**3382–3389.
18. **Huffaker, T. C., M. A. Hoyt, and D. Botstein.** 1987. Genetic analysis of the yeast cytoskeleton. Annu. Rev. Genet. **21:**259–284.
19. **Ito, K., T. Date, and W. Wickner.** 1980. Synthesis, assembly into the cytoplasmic membrane, and proteolytic processing of the precursor of coliphage M13 coat protein. J. Biol. Chem. **255:**2123–2130.
20. **Joly, J. C., and W. Wickner.** 1993. The SecA and SecY subunits of translocase are the nearest neighbors of a translocating preprotein, shielding it from phospholipids. EMBO J. **12:**255–263.
21. **Kaiser, C., and R. Schekman.** 1990. Distinct sets of SEC genes govern transport vesicle formation and fusion early in the secretory pathway. Cell **61:**723–733.
22. **Kassenbrock, C. K., W. Cao, and M. G. Douglas.** 1993. Genetic and biochemical characterization of ISP6, a small mitochondrial outer membrane protein associated with the protein translocation complex. EMBO J. **12:**3023–3034.
23. **Kiebler, M., P. Keil, H. Schneider, I. J. van der Klei, N. Pfanner, and W. Neupert.** 1993. The mitochondrial receptor complex: a central role of MOM22 in mediating preprotein transfer from receptors to the general insertion pore. Cell **74:**483–492.
24. **Kiebler, M., R. Pfaller, T. Söllner, G. Griffiths, H. Horstmann, N. Pfanner, and W. Neupert.** 1990. Identification of a mitochondrial receptor complex required for recognition and membrane insertion of precursor proteins. Nature (London) **348:**610–616.

25. **Kübrich, M., and N. Pfanner.** Unpublished data.
26. **Lithgow, T., B. S. Glick, and G. Schatz.** 1995. The protein import receptor of mitochondria. Trends Biochem. Sci. **20:**98–101.
27. **Lithgow, T., T. Junne, K. Suda, S. Gratzer, and G. Schatz.** 1994. The mitochondrial outer membrane protein Mas22p is essential for protein import and viability of yeast. Proc. Natl. Acad. Sci. USA **91:**11973–11977.
28. **Lithgow, T., T. Junne, C. Wachter, and G. Schatz.** 1994. Yeast mitochondria lacking the two import receptors Mas20p and Mas70p can efficiently and specifically import precursor proteins. J. Biol. Chem. **269:**15325–15330.
29. **Maarse, A. C., M. de Haan, P. J. Schoppink, J. A. Berden, and L. A. Grivell.** 1988. Inactivation of the gene encoding the 11-kDa subunit VIII of the ubiquinol-cytochrome-*c* oxidoreductase in *Saccharomyces cerevisiae*. Eur. J. Biochem. **172:**179–184.
30. **Manning-Krieg, U. C., P. E. Scherer, and G. Schatz.** 1991. Sequential action of mitochondrial chaperones in protein import into the matrix. EMBO J. **10:**3273–3280.
31. **Moczko, M., K. Dietmeier, T. Söllner, B. Segui, H. F. Steger, W. Neupert, and N. Pfanner.** 1992. Identification of the mitochondrial receptor complex in *Saccharomyces cerevisiae*. FEBS Lett. **310:**265–268.
32. **Moczko, M., B. Ehmann, F. Gärtner, A. Hönlinger, E. Schäfer, and N. Pfanner.** 1994. Deletion of the receptor MOM19 strongly impairs import of cleavable preproteins into *Saccharomyces cerevisiae* mitochondria. J. Biol. Chem. **269:**9045–9051.
33. **Moczko, M., F. Gärtner, and N. Pfanner.** 1993. The protein import receptor MOM19 of yeast mitochondria. FEBS Lett. **326:**251–254.
34. **Nargang, F. E., K.-P. Künkele, A. Mayer, R. G. Ritzel, W. Neupert, and R. Lill.** 1995. "Sheltered disruption" of *Neurospora crassa* MOM22, an essential component of the mitochondrial protein import complex. EMBO J. **14:**1099–1108.
35. **Nishiyama, K.-I., S. Mizushima, and H. Tokuda.** 1992. The carboxyl-terminal region of SecE interacts with SecY and is functional in the reconstitution of protein translocation activity in *Escherichia coli*. J. Biol. Chem. **267:**7170–7176.
36. **Pfaller, R., H. F. Steger, J. Rassow, N. Pfanner, and W. Neupert.** 1988. Import pathways of precursor proteins into mitochondria: multiple receptor sites are followed by a common membrane insertion site. J. Cell Biol. **107:**2483–2490.
37. **Pfanner, N., and W. Neupert.** 1987. Distinct steps in the import of ADP/ATP carrier into mitochondria. J. Biol. Chem. **262:**7528–7536.
38. **Pfanner, N., T. Söllner, and W. Neupert.** 1991. Mitochondrial import receptors for precursor proteins. Trends Biochem. Sci. **16:**63–67.
39. **Pfanner, N., M. Tropschug, and W. Neupert.** 1987. Mitochondrial protein import: nucleoside triphosphates are involved in conferring import-competence to precursors. Cell **49:**815–823.
40. **Ramage, L., T. Junne, K. Hahne, T. Lithgow, and G. Schatz.** 1993. Functional cooperation of mitochondrial protein import receptors in yeast. EMBO J. **12:**4115–4123.
41. **Rassow, J., A. C. Maarse, E. Krainer, M. Kübrich, H. Müller, M. Meijer, E. A. Craig, and N. Pfanner.** 1994. Mitochondrial protein import: biochemical and genetic evidence for interaction of matrix hsp70 and the inner membrane protein MIM44. J. Cell Biol. **127:**1547–1556.
42. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
43. **Schägger, H., and G. von Jagow.** 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. **166:**368–379.
44. **Schatz, P. J., K. L. Bieker, K. M. Ottemann, T. J. Silhavy, and J. Beckwith.** 1991. One of the three transmembrane stretches is sufficient for the functioning of the SecE protein, a membrane component of the *E. coli* secretion machinery. EMBO J. **10:**1749–1757.
45. **Sikorski, R. S., and P. Hieter.** 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:**19–27.
46. **Sims, P. J., A. S. Waggoner, C.-H. Wang, and J. F. Hoffman.** 1974. Studies on the mechanism by which cyanine dyes measure membrane potential in red blood cells and phosphatidylcholine vesicles. Biochemistry **13:**3315–3330.
47. **Söllner, T., R. Pfaller, G. Griffiths, N. Pfanner, and W. Neupert.** 1990. A mitochondrial import receptor for the ADP/ATP carrier. Cell **62:**107–115.
48. **Söllner, T., J. Rassow, and N. Pfanner.** 1991. Analysis of mitochondrial protein import using translocation intermediates and specific antibodies. Methods Cell Biol. **34:**345–358.
49. **Söllner, T., J. Rassow, M. Wiedmann, J. Schlossmann, P. Keil, W. Neupert, and N. Pfanner.** 1992. Mapping of the protein import machinery in the mitochondrial outer membrane by crosslinking of translocation intermediates. Nature (London) **355:**84–87.
50. **Steger, H. F., T. Söllner, M. Kiebler, K. A. Dietmeier, R. Pfaller, K. S. Trülzsch, M. Tropschug, W. Neupert, and N. Pfanner.** 1990. Import of ADP/ATP carrier into mitochondria: two receptors act in parallel. J. Cell Biol. **111:**2353–2363.
51. **Ungermann, C., W. Neupert, and D. M. Cyr.** 1994. The role of hsp70 in conferring unidirectionality on protein translocation into mitochondria. Science **266:**1250–1253.
52. **Vestweber, D., J. Brunner, A. Baker, and G. Schatz.** 1989. A 42K outer-membrane protein is a component of the yeast mitochondrial protein import site. Nature (London) **341:**205–209.
53. **Wagner, I., H. Arlt, L. van Dyck, T. Langer, and W. Neupert.** 1994. Molecular chaperones cooperate with PIM1 protease in the degradation of misfolded proteins in mitochondria. EMBO J. **13:**5135–5145.