MOLECULAR AND CELLULAR BIOLOGY, Dec. 1993, p. 7364–7371
0270-7306/93/127364-08$02.00/0

Vol. 13, No. 12

The Essential Yeast Protein MIM44 (encoded by *MPI1*) Is Involved in an Early Step of Preprotein Translocation across the Mitochondrial Inner Membrane

JOLANDA BLOM,[1] MICHAEL KÜBRICH,[2] JOACHIM RASSOW,[2] WOLFGANG VOOS,[2] PETER J. T. DEKKER,[1] AMMY C. MAARSE,[1] MICHIEL MEIJER,[1] AND NIKOLAUS PFANNER[2]*

Section for Molecular Biology, Institute for Molecular Cell Biology, BioCentrum Amsterdam, 1098 SM Amsterdam, The Netherlands,[1] and Biochemisches Institut, Universität Freiburg, Hermann-Herder-Strasse 7, D-79104 Freiburg, Germany[2]

Received 28 June 1993/Returned for modification 30 July 1993/Accepted 31 August 1993

The essential yeast gene *MPI1* encodes a mitochondrial membrane protein that is possibly involved in protein import into the organelle (A. C. Maarse, J. Blom, L. A. Grivell, and M. Meijer, EMBO J. 11:3619–3628, 1992). For this report, we determined the submitochondrial location of the *MPI1* gene product and investigated whether it plays a direct role in the translocation of preproteins. By fractionation of mitochondria, the mature protein of 44 kDa was localized to the mitochondrial inner membrane and therefore termed MIM44. Import of the precursor of MIM44 required a membrane potential across the inner membrane and involved proteolytic processing of the precursor. A preprotein in transit across the mitochondrial membranes was cross-linked to MIM44, whereas preproteins arrested on the mitochondrial surface or fully imported proteins were not cross-linked. When preproteins were arrested at two distinct stages of translocation across the inner membrane, only preproteins at an early stage of translocation could be cross-linked to MIM44. Moreover, solubilized MIM44 was found to interact with in vitro-synthesized preproteins. We conclude that MIM44 is a component of the mitochondrial inner membrane import machinery and interacts with preproteins in an early step of translocation.

Only six essential genes encoding mitochondrial proteins are known in *Saccharomyces cerevisiae*. The products of four of these genes are located in the mitochondrial matrix: the two heat shock proteins hsp70 (6, 17) and hsp60 (5, 22, 35), and the two components of the mitochondrial processing peptidase (16, 30, 48, 49). ISP42, also termed MOM38, is located in the outer membrane and forms an essential part of the mitochondrial receptor complex that is responsible for the specific recognition and translocation of preproteins (2, 18, 20, 41, 43). All of the components mentioned were found to play a crucial role in the import pathways of cytosolically synthesized preproteins.

The sixth essential mitochondrial protein, encoded by *MPI1*, has also been implicated to be involved in import of preproteins (19). The gene *MPI1* was identified by its ability to rescue the genetic defect of a mutant impaired in mitochondrial protein import. The gene product was localized to the mitochondrial membranes by using a modified protein carrying an epitope tag. It was proposed that the protein was synthesized with an amino-terminal presequence and thus located in the inner membrane (19).

The identification and characterization of the inner membrane transport machinery is currently a major goal in the analysis of mitochondrial protein import. A demonstration that the *MPI1* gene product is located in the inner membrane and directly participates in the import of preproteins would be an important first step toward this goal.

Here we show that the *MPI1* gene product indeed represents a genuine component of the mitochondrial inner membrane import machinery (MIM) (27). The protein was localized to the inner membrane and termed MIM44. By a cross-link approach with distinct translocation intermediates of a preprotein, we demonstrate that MIM44 is in close proximity to a preprotein at an early stage of translocation across the inner membrane. Furthermore, we show that preproteins interact with solubilized MIM44.

MATERIALS AND METHODS

Raising of antibodies. The 833-bp internal *Pst*I-*Eco*RV fragment of the coding region of *MPI1* (amino acid residues 68 to 345) was cloned into the pQE-9 vector of the QIAexpress system (Qiagen) and expressed in *Escherichia coli*. The recombinant MIM44 fragment, containing six histidine residues, was purified by Ni-nitrilotriacetic acid (NTA) affinity chromatography (42) and used for generation of antibodies in rabbits.

Isolation and subfractionation of mitochondria. *S. cerevisiae* cells were grown, and mitochondria were isolated by differential centrifugation (7, 12). Mitoplasts were obtained either by diluting mitochondria (10 mg of protein per ml in sorbitol buffer [0.65 M sorbitol, 1 mM EDTA, 1 mM $MgCl_2$, 10 mM KP_i, pH 7.5]) 3.25-fold in 20 mM Tris-HCl (pH 7.4), followed by reisolation and resuspension in 1 mM EDTA–10 mM Tris-HCl (pH 7.4) (19), or, when the procedure performed after an in vitro import experiment, by diluting mitochondria (0.25 mg of protein per ml in SEM [250 mM sucrose, 1 mM EDTA, 10 mM morpholinepropanesulfonic acid {MOPS}, pH 7.2]) 10-fold in 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-KOH (pH 7.2).

Mitochondria or mitoplasts (0.2 mg/ml in sorbitol buffer) were sonicated six times for 5 s each time in the presence of

* Corresponding author.

1 mM phenylmethylsulfonyl fluoride (PMSF). Membranes and supernatant were then separated by centrifugation for 60 min at 165,000 × *g*.

For extraction at pH 11.5, mitochondria (0.2 mg of protein per ml) were incubated in 100 mM Na_2CO_3 for 45 min at 0°C and then centrifuged for 60 min at 165,000 × *g*.

Submitochondrial membrane vesicles were produced by swelling, shrinking, and sonication of mitochondria as described previously (31) and resuspended in 10 mM KCl–5 mM HEPES-KOH (pH 7.4)–1 mM PMSF. Then 0.25 ml of the vesicles (5 mg of protein; equivalent to 35 mg of protein of mitochondrial start material) was layered onto a linear sucrose gradient (12 ml; 0.85 to 1.6 M sucrose in 10 mM KCl–5 mM HEPES-KOH, pH 7.4) and centrifuged for 16 h at 100,000 × *g* and 4°C. Fractions were collected, and aliquots of 200 μl were subjected to precipitation with trichloroacetic acid and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and immunodecoration with antisera directed against marker proteins of outer membranes and inner membranes.

Standard procedures were used for analysis by SDS-PAGE, transfer to nitrocellulose, immunodecoration, and fluorography (summarized in reference 40).

Import of preproteins into isolated mitochondria and cross-linking. The precursors of a fusion protein between the presequence of *Neurospora crassa* F_0-ATPase subunit 9 and the entire mouse dihydrofolate reductase sequence (Su9-DHFR), MIM44, and cytochrome c_1 were synthesized in rabbit reticulocyte lysates in the presence of [^{35}S]methionine (24, 28). Import into isolated mitochondria was performed in the presence of 1 mM ATP, 8 mM potassium ascorbate, and 0.2 mM *N,N,N',N'*-tetramethylphenylenediamine or 2 mM NADH and bovine serum albumin (BSA) buffer (3% [wt/vol] BSA, 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 10 mM MOPS, pH 7.2) (40) for 15 min at 25°C. For accumulation of membrane-spanning intermediates, mitochondria (in the presence of 20 μM oligomycin to inhibit the F_0F_1-ATPase) and reticulocyte lysate were depleted of ATP by a pretreatment with apyrase (10 U/ml) (26); the import was then performed in the presence of 20 μM oligomycin and without addition of ATP. For accumulation of precursor at the outer membrane, Su9-DHFR and mitochondria were incubated in the presence of 1 μm valinomycin and 20 μM oligomycin to dissipate the membrane potential across the inner membrane (40).

For cross-linking, the mitochondria were reisolated through a sucrose cushion (500 mM sucrose, 1 mM EDTA, 10 mM MOPS, pH 7.2), resuspended in SEM, swollen for 15 min at 0°C (formation of mitoplasts as described above), and incubated with 270 μM disuccinimidyl suberate (DSS; Pierce) for 20 min at 0°C; 100 mM Tris (pH 7.2) was then added (41). A precipitation with 7.5% trichloroacetic acid in the presence of 0.0125% deoxycholate was then performed. For immunoprecipitation, the samples were lysed in SDS-containing buffer prior to a 40-fold dilution in 1% (wt/vol) Triton X-100 buffer (18). Immunoprecipitation was performed in Triton X-100 buffer with protein A-Sepharose (25).

RESULTS

MIM44 is a peripheral membrane protein. An essential prerequisite for a clear localization of MIM44 and biochemical analysis of its function is the availability of a monospecific antibody that efficiently recognizes authentic MIM44. Several strategies for production of antibodies that we had successfully used with other proteins (4, 17, 39), including the use of peptides corresponding to various sequences of MIM44 and attempts to express complete MIM44 in *E. coli*, failed. Eventually we succeeded in production of a recombinant protein consisting of an N-terminal tag of six adjacent histidine residues and a 34-kDa fragment of MIM44 (residues 68 to 345) (42). The hybrid protein was purified via a Ni-metal chelate affinity column and used to generate antibodies in rabbits. The antiserum selectively recognized MIM44 from yeast mitochondria (Fig. 1A).

This antiserum was used for immunodecoration of MIM44 in mitochondrial subfractions. MIM44 remained associated with swollen mitochondria (mitoplasts) and was in the membrane pellet after sonication of the mitoplasts (Fig. 1B),

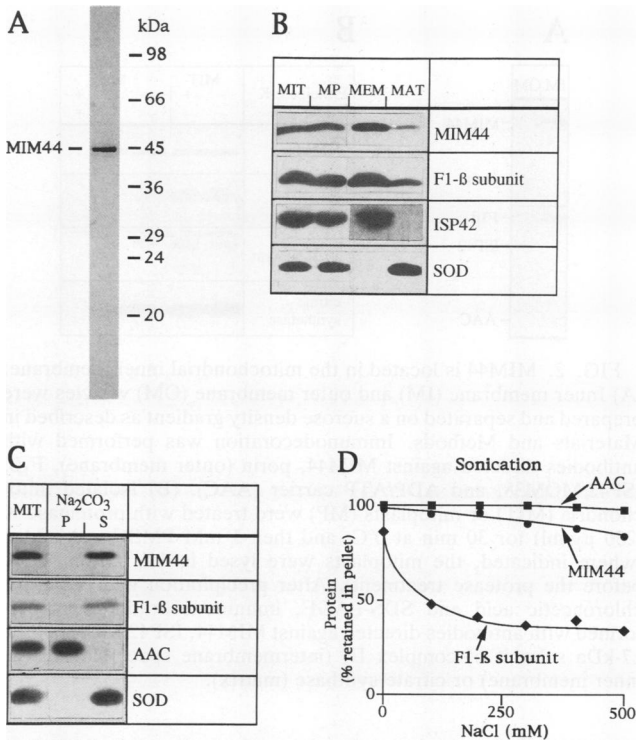

FIG. 1. MIM44 is a firmly associated peripheral membrane protein. (A) Antiserum directed against authentic MIM44. Isolated *S. cerevisiae* mitochondria (100 μg of protein) were resolved by SDS-PAGE, transferred to nitrocellulose, and immunodecorated with an antiserum prepared against a fragment of MIM44 (see Materials and Methods). (B) Fractionation of mitochondria. Mitochondria (MIT) were swollen to form mitoplasts (MP) and then separated into membranes (MEM) and matrix fraction (MAT) by sonication and centrifugation as described in Materials and Methods. Analysis was performed by immunodecoration with antibodies directed against MIM44, $F_1\beta$ (matrix side of the inner membrane), ISP42/MOM38 (outer membrane), and superoxide dismutase (SOD; matrix). (C) MIM44 is extracted from the membranes at pH 11.5. Mitochondria (MIT) were treated with 100 mM Na_2CO_3, and pellets (P) and supernatants (S) were separated as described in Materials and Methods. Immunodecoration was performed with antibodies directed against MIM44, $F_1\beta$, ADP/ATP carrier (AAC; inner membrane), and superoxide dismutase (SOD). (D) MIM44 is membrane associated upon sonication in the presence of salt. Mitoplasts were sonicated in the presence of NaCl as indicated, and pellets and supernatants were separated as described in Materials and Methods. Analysis was performed by SDS-PAGE and immunodecoration. The total amount of protein in a sample (pellet plus supernatant) was set to 100%. A similar result was obtained when mitochondria were subjected to sonication. AAC, ADP/ATP carrier.

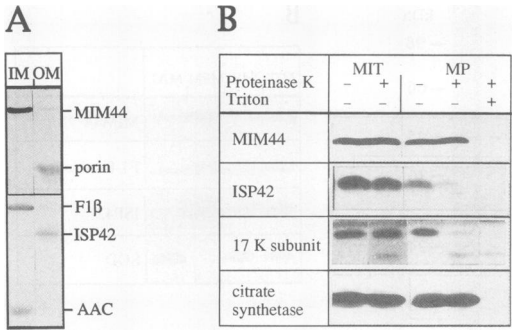

FIG. 2. MIM44 is located in the mitochondrial inner membrane. (A) Inner membrane (IM) and outer membrane (OM) vesicles were prepared and separated on a sucrose density gradient as described in Materials and Methods. Immunodecoration was performed with antibodies directed against MIM44, porin (outer membrane), $F_1\beta$, ISP42/MOM38, and ADP/ATP carrier (AAC). (B) Isolated mitochondria (MIT) or mitoplasts (MP) were treated with proteinase K (250 μg/ml) for 30 min at 0°C, and then 1 mM PMSF was added (where indicated, the mitoplasts were lysed in 1% Triton X-100 before the protease treatment). After precipitation with 7.5% trichloroacetic acid and SDS-PAGE, immunodecoration was performed with antibodies directed against MIM44, ISP42/MOM38, the 17-kDa subunit of complex III (intermembrane space side of the inner membrane) or citrate synthase (matrix).

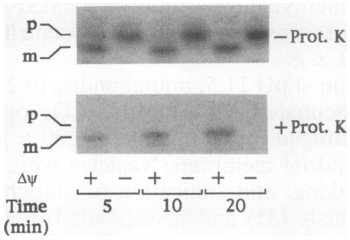

FIG. 3. Import of the precursor of MIM44 into mitochondria requires a membrane potential and includes proteolytic processing. Reticulocyte lysate containing ^{35}S-labeled precursor of MIM44 was incubated with isolated mitochondria in the presence of a membrane potential ΔΨ (addition of NADH) or absence of ΔΨ (addition of valinomycin and oligomycin) for the indicated times at 25°C. Then a treatment with proteinase K (Prot. K; 75 μg/ml) was performed, and the mitochondria were reisolated. Analysis was by SDS-PAGE and fluorography. p, precursor MIM44; m, mature MIM44.

indicating that MIM44, like ISP42/MOM38, is a mitochondrial membrane protein. Soluble proteins such as the matrix protein superoxide dismutase were found solely in the matrix fraction, and a protein peripherally bound to the matrix side of the inner membrane (F_1-ATPase subunit β [$F_1\beta$]) was partially found in the matrix fraction (Fig. 1B).

Mitochondria were treated with carbonate at pH 11.5 (10), leading to an extraction of soluble proteins (superoxide dismutase) or peripheral membrane proteins ($F_1\beta$), while integral membrane proteins (ADP/ATP carrier) were not extracted (Fig. 1C). MIM44 was extracted from the membranes at pH 11.5 (Fig. 1C), suggesting that it was not embedded in the lipid phase of the membrane. This finding is consistent with the primary protein sequence that does not contain a typical hydrophobic membrane anchor sequence (19). We then examined how stably MIM44 was associated with the membranes by sonication at different salt concentrations (Fig. 1D). MIM44 was found predominantly in the membrane pellet. At high concentrations of NaCl, a minor fraction of MIM44 was released from the membranes (Fig. 1D). It should be noted that soluble proteins such as superoxide dismutase were completely released to the supernatant in the absence of NaCl (Fig. 1B), and a major fraction of the peripheral membrane protein $F_1\beta$ was released to the supernatant at low concentrations of NaCl (Fig. 1D). When the membranes were lysed with detergent, MIM44 was quantitatively found in the supernatant (not shown). We conclude that MIM44 is a firmly associated peripheral mitochondrial membrane protein.

MIM44 is located in the mitochondrial inner membrane. We then investigated whether MIM44 was located in the mitochondrial outer membrane or inner membrane. Submitochondrial vesicles were prepared by a combination of swelling, shrinking, and sonication of mitochondria (31), and inner and outer membrane vesicles were separated by sucrose density gradient centrifugation (Fig. 2A). Immunodecoration with antisera against marker proteins of the inner membrane (ADP/ATP carrier and $F_1\beta$) and outer membrane (porin and ISP42/MOM38) demonstrates the high degree of separation of inner membrane and outer membrane vesicles (the outer membrane fraction contained a small amount of inner membrane markers, about 5% of that present in the inner membrane fraction). Immunodecoration with antiserum directed against MIM44 showed a localization of MIM44 in the mitochondrial inner membrane (Fig. 2A).

For Fig. 2B, a subfractionation of mitochondria was combined with a protease treatment. MIM44 was not digested by proteinase K in intact mitochondria and upon opening of the intermembrane space (formation of mitoplasts) (Fig. 2B). The outer membrane protein ISP42/MOM38 and a protein peripherally associated with the intermembrane space side of the inner membrane (the 17-kDa subunit of complex III of the respiratory chain) (23) were accessible to the protease. MIM44 was digested by the protease only after opening of the mitochondrial matrix by lysis of the membranes with detergent (Fig. 2B). This finding suggests that MIM44 is an inner membrane protein that does not expose a protease-sensitive site to the intermembrane space.

Import of the precursor of MIM44 requires a membrane potential. The precursor of MIM44 was synthesized in vitro in rabbit reticulocyte lysates in the presence of [^{35}S]methionine and incubated with isolated energized yeast mitochondria. The preprotein was proteolytically processed to the mature form of 44 kDa, and this mature form was not degraded by externally added protease (Fig. 3). After dissipation of the membrane potential ΔΨ across the inner membrane, the processing and transport to a protease-protected location were blocked (Fig. 3). The import of MIM44 thus shows two characteristics, ΔΨ dependence and proteolytic processing, that are not found with outer membrane proteins (3, 9, 13, 29) but are consistent with a location of MIM44 in the inner membrane.

A preprotein in transit across the mitochondrial membranes is in proximity to MIM44. Is MIM44 part of a putative protein translocation channel of the mitochondrial inner membrane (19, 27)? To answer this question, we tested whether a preprotein traversing the mitochondrial membranes was in proximity to MIM44.

We used a fusion protein, Su9-DHFR, that is cleaved twice by the processing peptidase upon import into the mitochondrial matrix (24, 28). The preprotein was synthesized in rabbit reticulocyte lysate in the presence of [^{35}S]methionine and was accumulated as a translocation intermedi-

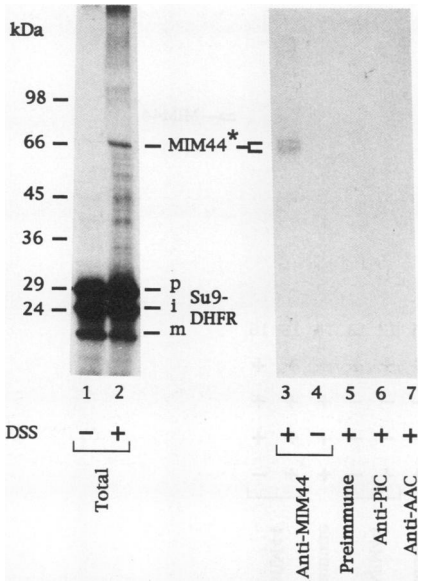

FIG. 4. Cross-linking of a preprotein in transit to MIM44. Reticulocyte lysate containing ^{35}S-labeled precursor of Su9-DHFR was incubated with isolated yeast mitochondria at low ATP (see Materials and Methods) and thus accumulated in the mitochondrial membranes. Where indicated, the reisolated mitochondria were subjected to cross-linking with 270 μM DSS as described in Materials and Methods. Samples 1 and 2 were directly subjected to SDS-PAGE and fluorography, while the mitochondria of samples 3 to 7 (each representing double the amount of sample 1 or 2) were dissociated in SDS-containing buffer, diluted 40-fold in Triton X-100 buffer, and subjected to immunoprecipitation with antiserum against MIM44, preimmune serum, or antiserum against the phosphate carrier (PiC) or the ADP/ATP carrier (AAC). The immunoprecipitates were then analyzed by SDS-PAGE and fluorography. p, i, and m, precursor-, intermediate-, and mature-size forms of Su9-DHFR, respectively; MIM44*, cross-link product between Su9-DHFR and MIM44. MIM44* runs as a double band on the gels; in the samples in which total mitochondrial proteins were applied to the gel, the two bands are very close together since they are running directly below the BSA band.

ate spanning both mitochondrial membranes by incubating it with energized mitochondria at low levels of ATP. Thereby the presequence was partially translocated into the mitochondrial matrix such that the first half was cleaved off by the processing peptidase (Fig. 4). About half of the accumulated intermediate Su9-DHFR molecules were accessible to externally added protease, indicating that they spanned both mitochondrial membranes (see below). The mitochondria were reisolated, incubated in swelling buffer to open the intermembrane space, and treated with the homobifunctional noncleavable cross-linking reagent DSS. After quenching of the cross-linker, the mitochondria were lysed, and the proteins were analyzed by SDS-PAGE and fluorography. Lane 2 of Figure 4 shows that several cross-link products were formed. In parallel samples, the mitochondria were denatured in SDS and then immunoprecipitated in Triton X-100 buffer. Antiserum against MIM44 precipitated a major cross-link product of about 65 kDa that actually consisted of a double band (Fig. 4, lane 3). Neither preimmune serum nor antisera directed against the abundant inner membrane proteins ADP/ATP carrier and phosphate carrier precipitated cross-link products (Fig. 4, lanes 5 to 7), excluding a nonspecific cross-linking to major components of the mitochondrial inner membrane.

Su9-DHFR was also accumulated at the outer membrane in the absence of a membrane potential (Fig. 5, lanes 1 and 2) or was completely imported into the mitochondrial matrix in the presence of ATP and $\Delta\Psi$ (Fig. 5, lanes 5 and 6). Under none of these conditions did we observe a cross-linking of the protein to MIM44 (Fig. 5, lanes 9 and 15), demonstrating that the preprotein had to be arrested in a membrane-spanning location to be in proximity to MIM44.

MIM44 is involved in an early step of preprotein translocation across the inner membrane. Preprotein translocation across the mitochondrial inner membrane can be divided into two steps (Fig. 6A). In the first stage, the preprotein spans both outer and inner membranes (translocation contact site intermediate) (28, 38, 44). In the second stage, the preprotein is completely translocated across the outer membrane and extends from the intermembrane space into the matrix (intermembrane space intermediate) (15, 34). At low ATP, the intermediate Su9-DHFR accumulated at both stages. About half of the molecules were in the two-membrane-spanning location, as they were processed to the intermediate-size form in the matrix and digested by protease added to the isolated mitochondria (Fig. 6B). The rest of the intermediate Su9-DHFR molecules was in the one-membrane-spanning location: the processed protein was protected against protease in intact mitochondria yet became accessible to protease after opening of the intermembrane space (Fig. 6B).

In Fig. 6C, we tested which of the translocation intermediates of Su9-DHFR gave rise to the cross-link product with MIM44. The major cross-link product was found only when the preprotein was accumulated in the two-membrane-spanning fashion (lanes 2 and 6). When the mitochondria were treated with protease, leaving the intermembrane space intermediate (and of course MIM44; see Fig. 2) intact, only a very small amount of the cross-link product was observed (Fig. 6C, lanes 4 and 8). A quantitation revealed that about 4 to 5% of Su9-DHFR at intermediate stage b was cross-linked to MIM44. This represents a high efficiency of cross-linking of preproteins in an organelle (11). We conclude that MIM44 is in close proximity to a preprotein in an early stage of translocation across the mitochondrial inner membrane, i.e., in a stage in which part of the preprotein is still in the cytosol.

The two bands forming the cross-link product with MIM44 differ in size by about 4 kDa (Fig. 4, 5, and 6C), the same difference that is found between precursor- and intermediate-size forms of Su9-DHFR. It is thus conceivable that the cross-link products with MIM44 were formed with both the precursor- and intermediate-size forms of Su9-DHFR.

Interaction of preproteins with solubilized MIM44. We examined whether MIM44 has a binding activity for polypeptide chains. Mitochondria were lysed with nonionic detergent and incubated with ^{35}S-labeled precursor proteins that had been synthesized in rabbit reticulocyte lysate. Then immunoprecipitations were performed under nondenaturing conditions with antiserum directed against MIM44 and, as controls, with preimmune serum or unrelated antisera (against proteins not involved in protein import) (Fig. 7A). We used the precursor of the outer membrane protein porin and precursor proteins that were transported into the inner membrane (cytochrome c_1; Fe/S protein of the bc_1 complex) or across the inner membrane ($F_1\beta$). A significant association with MIM44 after the immunoprecipitation was found with the precursors of cytochrome c_1, Fe/S protein, and $F_1\beta$ but not porin (Fig. 7A). The association took place both in the presence and in the absence of ATP (not shown). We

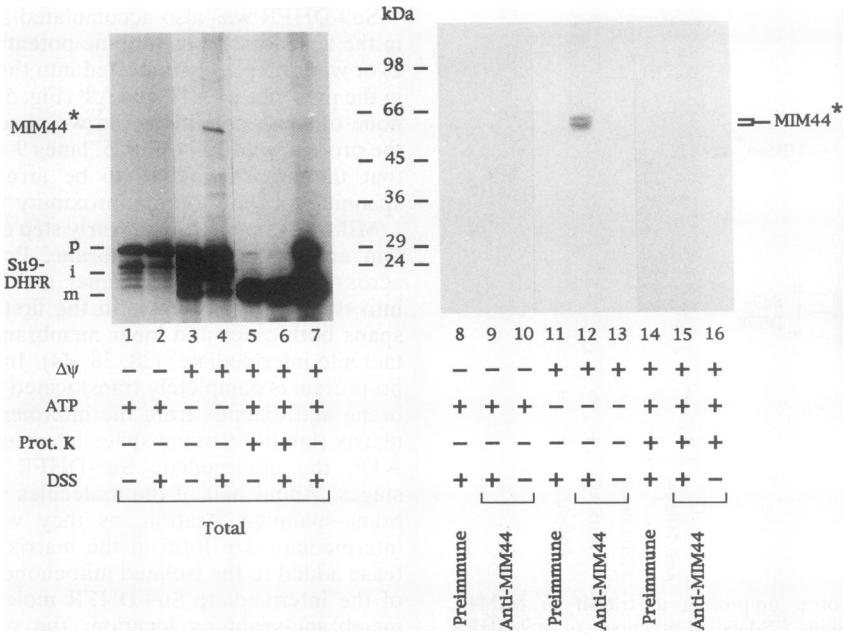

FIG. 5. Preproteins on the mitochondrial surface and fully imported proteins are not cross-linked to MIM44. ^{35}S-labeled Su9-DHFR was incubated with isolated mitochondria in the absence of a membrane potential ΔΨ (lanes 1, 2, and 8 to 10), at low levels of ATP (and the presence of ΔΨ) (lanes 3, 4, and 11 to 13), or in the presence of ΔΨ and ATP (lanes 5 to 7 and 14 to 16) (see Materials and Methods). Then the mitochondria were subjected to cross-linking (for lanes 5, 6, and 14 to 16, the swollen mitochondria were treated with 30 μg proteinase K [Prot. K] per ml prior to cross-linking). The mitochondrial proteins were either directly analyzed by SDS-PAGE and fluorography (lanes 1 to 7) or subjected to immunoprecipitation as indicated and as described in the legend to Fig. 4 (lanes 8 to 16 were exposed four times longer than lanes 1 to 7). Other designations are as for Fig. 4.

conclude that MIM44 is able to form a complex with preproteins.

The precursor of cytochrome c_1 carries a bipartite presequence. The amino-terminal portion is hydrophilic and positively charged and is required for translocation into the matrix, while the carboxyl-terminal portion of the presequence contains a hydrophobic stretch and is needed for sorting of the protein to the intermembrane space (13). For Fig. 7B, the precursor of cytochrome c_1 was partially processed to the intermediate-size form that lacks the matrix-targeting sequence (lane 1) or further processed to the intermediate- and mature-size forms by isolated mitochondria (lane 4). The mitochondria were lysed, and immunoprecipitations with anti-MIM44 serum (lanes 3 and 6) or preimmune serum (lanes 2 and 5) were performed under nondenaturing conditions. Lane 3 of Fig. 7B shows that removal of the matrix-targeting sequence strongly decreased the binding efficiency of cytochrome c_1 to MIM44. Practically no binding was observed with the mature-size cytochrome c_1 (Fig. 7B, lane 6). This finding supports the conclusion drawn from cross-linking of the translocation intermediate that MIM44 plays a direct role in an early step of preprotein import.

DISCUSSION

MPI1 is the first essential gene which can be demonstrated to encode a component of the protein import machinery in the mitochondrial inner membrane. We report that MIM44, the product of the *MPI1* gene, shows the characteristics of a mitochondrial inner membrane protein and is in close proximity to a precursor protein at a distinct step of translocation across the inner membrane.

MIM44 was found to behave as a peripheral membrane protein firmly associated with the mitochondrial inner membrane. Import of the precursor of MIM44 required a membrane potential ΔΨ across the inner membrane and involved proteolytic processing of the protein, in agreement with a location in the inner membrane. MIM44 was not accessible to protease from the intermembrane space side but became protease accessible after disruption of the inner membrane, indicating that major portions of MIM44 are located in the inner membrane or at the matrix face of the membrane. We previously found that a modified MIM44 carrying a carboxyl-terminal epitope tag was accessible to protease after opening of the intermembrane space (19). It may thus be speculated that the extreme carboxyl terminus of MIM44 is oriented toward the intermembrane space side.

MIM44 is in close proximity to a preprotein in transit across the inner membrane, as it can be efficiently cross-linked to it, suggesting that MIM44 is part of the inner membrane import machinery. The specificity of the cross-linking approach is underscored by the lack of cross-linking of abundant inner membrane proteins to the preprotein. We analyzed four distinct stages of preprotein translocation into mitochondria (Fig. 6A): (a) binding to the outer membrane; (b) an early step of translocation across the inner membrane; (c) a late step of translocation across the inner membrane; and (d) complete import into the matrix. The cross-link product with MIM44 was virtually found only with intermediate b, demonstrating that MIM44 is in proximity to a preprotein in an early step of transfer across the inner membrane. This stage-specific cross-linking provides further evidence for the specificity of the cross-link approach used. Although it does not exclude that MIM44 is also involved in a later step of translocation, it indicates that MIM44 acts in

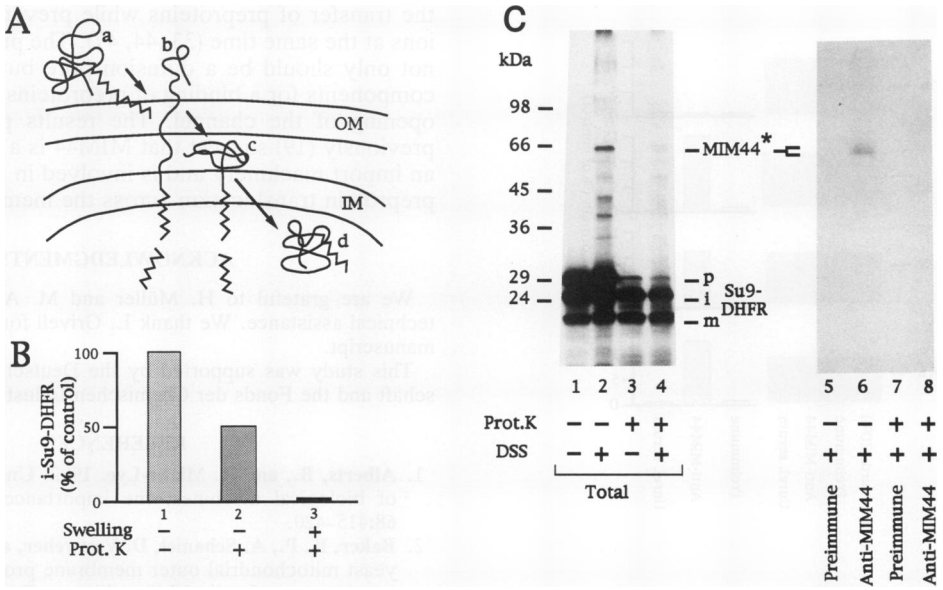

FIG. 6. Preproteins in an early stage of translocation across the inner membrane are cross-linked to MIM44. (A) Import steps of Su9-DHFR (modified from references 15, 24, 28, 34, and 37). IM, inner membrane; OM, outer membrane. (B) Su9-DHFR was accumulated in the mitochondrial membranes by performing import at low ATP and in the presence of $\Delta\Psi$ as described in the legend to Fig. 4. The mitochondria of sample 2 were treated with proteinase K (Prot. K). The mitochondria of sample 3 were swollen and then treated with proteinase K. Analysis was performed by SDS-PAGE, fluorography, and laser densitometry. i-Su9-DHFR, intermediate Su9-DHFR. (C) Su9-DHFR was accumulated at low ATP and in the presence of $\Delta\Psi$ as described above. The mitochondria of samples 3, 4, 7, and 8 were then treated with proteinase K (Prot.K). Then cross-linking and immunoprecipitations were performed as indicated and as described in Materials and Methods and the legend to Fig. 4. Proteins are designated as for Fig. 4.

an early translocation step. The involvement of MIM44 in an early step is also suggested by the observation that, as discussed below, solubilized MIM44 preferentially interacts with the precursor form of a preprotein. According to the dynamic model of mitochondrial protein import (27, 34), the protein import machineries of the mitochondrial outer and inner membranes are not permanently associated but may be transiently connected by a traversing polypeptide chain. While at intermediate stage b, the two import machineries are necessarily in close contact, they may be dissociated when a preprotein has reached intermediate stage c. It is thus possible that a dissociation of the machineries leads to a rearrangement of the components of the inner membrane machinery such that MIM44 is no more in close contact with the traversing polypeptide chain.

A role of MIM44 in protein import is also supported by the observation that preproteins transported into or across the inner membrane interact with solubilized MIM44. Since the specificity of mitochondrial protein import is determined by the import machinery of the outer membrane, in particular the surface receptors (3, 29), it may be assumed that components of the inner membrane import machinery such as MIM44 have a relatively broad specificity. They could, for example, have chaperone-like functions in mediating the translocation of preproteins. However, a relatively specific interaction of polypeptides with MIM44 is suggested by the lack of interaction of MIM44 with the precursor of the outer membrane protein porin and the observation that removal of the presequence strongly decreased the binding efficiency of a preprotein to MIM44. A detailed characterization of the mechanism and specificity of interaction of preproteins and MIM44 awaits purification of the native protein.

Scherer et al. (37) reported that a protein with a size of about 45 kDa (ISP45) was part of the protein import site of the yeast mitochondrial inner membrane. ISP45 was cross-linked to a preprotein accumulated at the late step of translocation across the inner membrane (corresponding to stage c shown in Fig. 6A). Very recently, it was proposed that ISP45 was encoded by *MPI1*, i.e., was identical to MIM44 (14). Several findings, however, are in conflict with the proposed identity of ISP45 and MIM44. Though ISP45 was initially suggested to behave as a peripheral membrane protein (37), Horst et al. have convincingly shown that ISP45 is not extracted at all from the membranes at alkaline pH (11.5) and thus concluded that ISP45 is an integral membrane protein (14). This is in contrast to the quantitative extraction of MIM44 at alkaline pH shown here; also, the protein sequence of MIM44 does not contain a hydrophobic membrane anchor sequence (19). Moreover, ISP45 was found to be rapidly degraded to a smaller fragment (14), while we never observed such a fragmentation of MIM44 in mitochondria or mitochondrial extracts.

It is becoming increasingly evident that a large number of cellular processes are performed not by individual proteins but by protein complexes (for an overview, see reference 1). This has also been found for the various processes of protein translocation into and across membranes (8, 21, 32, 36, 45, 46). In mitochondria, a seven-subunit protein complex of the outer membrane that performs the tasks of specific recognition and membrane translocation of preproteins was identified (18, 20, 41). It is thus reasonable to assume that MIM44 is not the only component of the mitochondrial inner membrane import machinery. Since MIM44 behaves like a peripheral membrane protein, it may be speculated that the association of MIM44 with the inner membrane occurs via interaction with yet to be identified integral membrane proteins. It can be predicted that a translocation channel of the inner membrane must be tightly regulated, as it allows

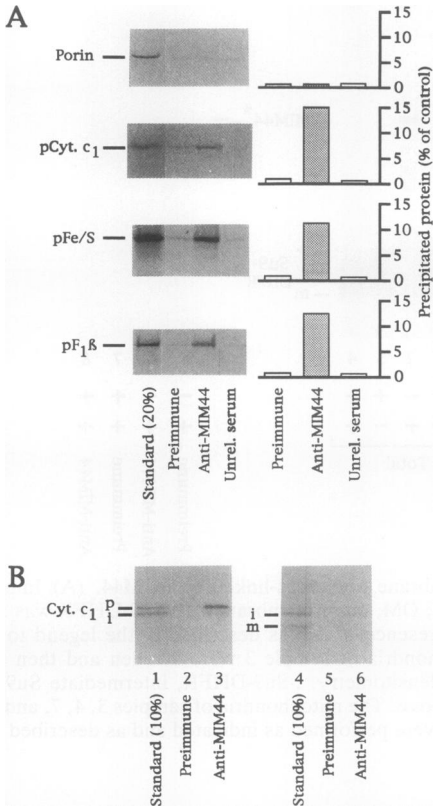

FIG. 7. Coimmunoprecipitation of in vitro-synthesized preproteins with solubilized MIM44. (A) The precursors of porin, cytochrome c_1 (pCyt. c_1), Fe/S protein (pFe/S), and $F_1\beta$ ($pF_1\beta$) were synthesized in rabbit reticulocyte lysates in the presence of [^{35}S]methionine. Isolated yeast mitochondria (20 μg) were lysed in 250 μl of 3.7% (wt/vol) octanoyl-*N*-methylglucamide (MEGA-8; Boehringer Mannheim)–200 mM KCl–20 mM MOPS (pH 7.2)–0.5 mM PMSF–12.5 μg of soybean trypsin inhibitor per ml–2.5 μg of leupeptin per ml–12.5 μg of aprotinin per ml and, after a clarifying spin (17,000 × *g* for 7 min), incubated with the reticulocyte lysate (2 μl) under gentle shaking for 30 min at 4°C. Then immunoprecipitations with antiserum against the respective protein (anti-porin, anti-cytochrome c_1, anti-Fe/S protein, or anti-$F_1\beta$), preimmune antiserum, antiserum against MIM44, or unrelated (Unrel.) antisera (anti-$F_1\beta$ with the precursors of porin, cytochrome c_1, and Fe/S protein; against a major 14-kDa outer membrane protein in case of $pF_1\beta$) were performed by using protein A-Sepharose (furthermore, immunoprecipitation with anti-14-kDa protein antiserum yielded only background levels also for the other three preproteins; not shown). The immunoprecipitates were washed three times in the MEGA-8 buffer described above (without protease inhibitors) and analyzed by SDS-PAGE, fluorography, and laser densitometry. The amount of preprotein immunoprecipitated with the antibodies directed against the respective protein was set to 100% (control). Standard represents 20% of the control immunoprecipitation. No immunoprecipitation of preproteins with anti-MIM44 antibodies was observed when the samples were dissolved in SDS-containing buffer (18) prior to dilution in MEGA-8 buffer. (B) The precursor of cytochrome c_1 (Cyt. c_1) was incubated with energized yeast mitochondria for 10 min at 8°C (samples 1 to 3) or 25°C and then treated with proteinase K (40 μg/ml) (samples 4 to 6) (40). The mitochondria were lysed in the MEGA-8 buffer described above. Immunoprecipitations and analysis by SDS-PAGE and fluorography were performed as described for panel A. Standard represents 10% of the control immunoprecipitation (with antibodies against cytochrome c_1). p, i, and m, precursor-, intermediate-, and mature-size cytochrome c_1, respectively.

the transfer of preproteins while preventing the leakage of ions at the same time (33, 44, 47). The putative channel thus not only should be a diffusion pore but also must contain components for a binding of preproteins that may trigger an opening of the channel. The results presented here and previously (19) suggest that MIM44 is a component of such an import machinery and is involved in an early function of preprotein translocation across the membrane.

ACKNOWLEDGMENTS

We are grateful to H. Müller and M. A. de Jong for expert technical assistance. We thank L. Grivell for critically reading the manuscript.

This study was supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

REFERENCES

1. **Alberts, B., and R. Miake-Lye.** 1992. Unscrambling the puzzle of biological machines: the importance of the details. Cell **68:**415–420.
2. **Baker, K. P., A. Schaniel, D. Vestweber, and G. Schatz.** 1990. A yeast mitochondrial outer membrane protein essential for protein import and cell viability. Nature (London) **348:**605–609.
3. **Baker, K. P., and G. Schatz.** 1991. Mitochondrial proteins essential for viability mediate protein import into yeast mitochondria. Nature (London) **349:**205–208.
4. **Becker, K., B. Guiard, J. Rassow, T. Söllner, and N. Pfanner.** 1992. Targeting of a chemically pure preprotein to mitochondria does not require the addition of a cytosolic signal recognition factor. J. Biol. Chem. **267:**5637–5643.
5. **Cheng, M. Y., F.-U. Hartl, J. Martin, R. A. Pollock, F. Kalousek, W. Neupert, E. M. Hallberg, R. L. Hallberg, and A. L. Horwich.** 1989. Mitochondrial heat-shock protein hsp60 is essential for assembly of proteins imported into yeast mitochondria. Nature (London) **337:**620–625.
6. **Craig, E. A., J. Kramer, J. Shilling, M. Werner-Washburne, S. Holmes, J. Kosic-Smithers, and C. M. Nicolet.** 1989. *SSC1*, an essential member of the yeast HSP70 multigene family, encodes a mitochondrial protein. Mol. Cell. Biol. **9:**3000–3008.
7. **Daum, G., S. M. Gasser, and G. Schatz.** 1982. Import of proteins into mitochondria: energy-dependent, two-step processing of the intermembrane space enzyme cytochrome b_2 by isolated yeast mitochondria. J. Biol. Chem. **257:**13075–13080.
8. **Dingwall, C., and R. Laskey.** 1992. The nuclear membrane. Science **258:**942–947.
9. **Douglas, M. G., M. McCammon, and A. Vassarotti.** 1986. Targeting proteins into mitochondria. Microbiol. Rev. **50:**166–178.
10. **Fujiki, Y., A. L. Hubbard, S. Fowler, and P. B. Lazarow.** 1982. Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. J. Cell Biol. **93:**97–102.
11. **Görlich, D., T. V. Kurzchalia, M. Wiedmann, and T. A. Rapoport.** 1991. Probing the molecular environment of translocating polypeptide chains by cross-linking. Methods Cell. Biol. **34:** 241–262.
12. **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.
13. **Hartl, F.-U., N. Pfanner, D. W. Nicholson, and W. Neupert.** 1989. Mitochondrial protein import. Biochim. Biophys. Acta **988:**1–45.
14. **Horst, M., P. Jenö, N. G. Kronidou, L. Bolliger, W. Oppliger, P. Scherer, U. Manning-Krieg, T. Jascur, and G. Schatz.** 1993. Protein import into yeast mitochondria: the inner membrane import site protein ISP45 is the *MPI1* gene product. EMBO J. **12:**3035–3041.
15. **Hwang, S. T., C. Wachter, and G. Schatz.** 1991. Protein import into the yeast mitochondrial matrix: a new translocation intermediate between the two mitochondrial membranes. J. Biol.

Chem. **266**:21083–21089.

16. **Jensen, R. E., and M. P. Yaffe.** 1988. Import of proteins into yeast mitochondria: the nuclear *MAS2* gene encodes a component of the processing protease that is homologous to the *MAS1*-encoded subunit. EMBO J. **7**:3863–3871.
17. **Kang, P. J., J. Ostermann, J. Shilling, W. Neupert, E. A. Craig, and N. Pfanner.** 1990. Requirement for hsp70 in the mitochondrial matrix for translocation and folding of precursor proteins. Nature (London) **348**:137–143.
18. **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.
19. **Maarse, A. C., J. Blom, L. A. Grivell, and M. Meijer.** 1992. *MPI1*, an essential gene encoding a mitochondrial membrane protein, is possibly involved in protein import into yeast mitochondria. EMBO J. **11**:3619–3628.
20. **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.
21. **Nunnari, J., and P. Walter.** 1992. Protein targeting to and translocation across the membrane of the endoplasmic reticulum. Curr. Opin. Cell Biol. **4**:573–580.
22. **Ostermann, J., A. L. Horwich, W. Neupert, and F.-U. Hartl.** 1989. Protein folding in mitochondria requires complex formation with hsp60 and ATP hydrolysis. Nature (London) **341**:125–130.
23. **Oudshoorn, P.** 1991. Import of proteins into yeast mitochondria. Ph.D. thesis. University of Amsterdam, Amsterdam, The Netherlands.
24. **Pfanner, N., H. Müller, M. A. Harmey, and W. Neupert.** 1987. Mitochondrial protein import: involvement of the mature part of a cleavable precursor protein in the binding to receptor sites. EMBO J. **6**:3449–3454.
25. **Pfanner, N., and W. Neupert.** 1985. Transport of proteins into mitochondria: a potassium diffusion potential is able to drive the import of ADP/ATP carrier. EMBO J. **4**:2819–2825.
26. **Pfanner, N., and W. Neupert.** 1986. Transport of F_1-ATPase subunit β into mitochondria depends on both a membrane potential and nucleoside triphosphates. FEBS Lett. **209**:152–156.
27. **Pfanner, N., J. Rassow, I. J. van der Klei, and W. Neupert.** 1992. A dynamic model of the mitochondrial protein import machinery. Cell **68**:999–1002.
28. **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.
29. **Pfanner, N., T. Söllner, and W. Neupert.** 1991. Mitochondrial import receptors for precursor proteins. Trends Biochem. Sci. **16**:63–67.
30. **Pollock, R. A., F.-U. Hartl, M. Y. Cheng, J. Ostermann, A. Horwich, and W. Neupert.** 1988. The processing peptidase of yeast mitochondria: the two co-operating components MPP and PEP are structurally related. EMBO J. **7**:3493–3500.
31. **Pon, L., T. Moll, D. Vestweber, B. Marshallsay, and G. Schatz.** 1989. Protein import into mitochondria: ATP-dependent protein translocation activity in a submitochondrial fraction enriched in membrane contact sites and specific proteins. J. Cell Biol. **109**:2603–2616.
32. **Rapoport, T. A.** 1992. Transport of proteins across the endoplasmic reticulum membrane. Science **258**:931–936.
33. **Rassow, J., B. Guiard, U. Wienhues, V. Herzog, F.-U. Hartl, and W. Neupert.** 1989. Translocation arrest by reversible folding of a precursor protein imported into mitochondria. A means to quantitate translocation contact sites. J. Cell Biol. **109**:1421–1428.
34. **Rassow, J., and N. Pfanner.** 1991. Mitochondrial preproteins en route from the outer membrane to the inner membrane are exposed to the intermembrane space. FEBS Lett. **293**:85–88.
35. **Reading, D. S., R. L. Hallberg, and A. M. Myers.** 1989. Characterization of the yeast *HSP60* gene coding for a mitochondrial assembly factor. Nature (London) **337**:655–659.
36. **Sanders, S. L., and R. Schekman.** 1992. Polypeptide translocation across the endoplasmic reticulum membrane. J. Biol. Chem. **267**:13791–13794.
37. **Scherer, P. E., U. C. Manning-Krieg, P. Jenö, G. Schatz, and M. Horst.** 1992. Identification of a 45-kDa protein at the protein import site of the yeast mitochondrial inner membrane. Proc. Natl. Acad. Sci. USA **89**:11930–11934.
38. **Schleyer, M., and W. Neupert.** 1985. Transport of proteins into mitochondria: translocational intermediates spanning contact sites between outer and inner membranes. Cell **43**:339–350.
39. **Schneider, H., T. Söllner, K. Dietmeier, C. Eckerskorn, F. Lottspeich, B. Trülzsch, W. Neupert, and N. Pfanner.** 1991. Targeting of the master receptor MOM19 to mitochondria. Science **254**:1659–1662.
40. **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.
41. **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.
42. **Stüber, D., H. Matile, and G. Garotta.** 1990. System for high-level production in Escherichia coli and rapid purification of recombinant proteins: application to epitope mapping, preparation of antibodies, and structure-function analysis. Immunol. Methods **4**:121–152.
43. **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.
44. **Vestweber, D., and G. Schatz.** 1988. A chimeric mitochondrial precursor protein with internal disulfide bridges blocks import of authentic precursors into mitochondria and allows quantitation of import sites. J. Cell Biol. **107**:2037–2043.
45. **Waegemann, K., and J. Soll.** 1991. Characterization of the protein import apparatus in isolated outer envelopes of chloroplasts. Plant J. **1**:149–158.
46. **Wickner, W., A. J. M. Driessen, and F.-U. Hartl.** 1991. The enzymology of protein translocation across the *Escherichia coli* plasma membrane. Annu. Rev. Biochem. **60**:101–124.
47. **Wienhues, U., K. Becker, M. Schleyer, B. Guiard, M. Tropschug, A. L. Horwich, N. Pfanner, and W. Neupert.** 1991. Protein folding causes an arrest of preprotein translocation into mitochondria in vivo. J. Cell Biol. **115**:1601–1609.
48. **Witte, C., R. E. Jensen, M. P. Yaffe, and G. Schatz.** 1988. *MAS1*, a gene essential for yeast mitochondrial assembly, encodes a subunit of the mitochondrial processing protease. EMBO J. **7**:1439–1447.
49. **Yang, M., R. E. Jensen, M. P. Yaffe, W. Oppliger, and G. Schatz.** 1988. Import of proteins into yeast mitochondria: the purified matrix processing protease contains two subunits which are encoded by the nuclear *MAS1* and *MAS2* genes. EMBO J. **7**:3857–3862.