Mitochondrial Protein Import: Biochemical and Genetic Evidence for Interaction of Matrix hsp70 and the Inner Membrane Protein MIM44

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Abstract. The import of preproteins into mitochondria involves translocation of the polypeptide chains through putative channels in the outer and inner membranes. Preprotein-binding proteins are needed to drive the unidirectional translocation of the precursor polypeptides. Two of these preprotein-binding proteins are the peripheral inner membrane protein MIM44 and the matrix heat shock protein hsp70. We report here that MIM44 is mainly exposed on the matrix side, and a fraction of mt-hsp70 is reversibly bound to the inner membrane. Mt-hsp70 binds to MIM44 in a 1:1 ratio, suggesting that mt-hsp70 is localizing to the membrane via its interaction with MIM44. Formation of the complex requires a functional ATPase domain of mt-hsp70. Addition of Mg-ATP leads to dissociation of the complex. Overexpression of mt-hsp70 rescues the protein import defect of mutants in MIM44; conversely, overexpression of MIM44 rescues protein import defects of mt-hsp70 mutants. In addition, yeast strains with conditional mutations in both MIM44 and mt-hsp70 are barely viable, showing a synthetic growth defect compared to strains carrying single mutations. We propose that MIM44 and mt-hsp70 cooperate in translocation of preproteins. By binding to MIM44, mt-hsp70 is recruited at the protein import sites of the inner membrane, and preproteins arriving at MIM44 may be directly handed over to mt-hsp70.

Import of preproteins into mitochondria is a complex process requiring a large number of components in distinct cellular subcompartments, the cytosol, outer membrane, inner membrane, and matrix. In the cytosol, molecular chaperones (70-kD heat shock protein [hsp70] and Ydjlp/Mas5p) and additional import stimulating factors (such as presequence-binding factor PBF and mitochondrial import stimulating factor MSF) support transfer of the preproteins to mitochondria (Chirico et al., 1988; Murakami et al., 1988; Murakami et al., 1988; Deshaies et al., 1988; Murakami and Mori, 1990; Hachiya et al., 1993; Cyr et al., 1994). The mitochondrial outer membrane contains a high molecular mass complex which includes several subunits constituting a general insertion pore and two import receptors (Söllner et al., 1992; Kassenbrock et al., 1993; Ramage et al., 1993). The mitochondrial inner membrane contains a high molecular mass complex which includes several subunits constituting a general insertion pore and two import receptors (Söllner et al., 1992; Kassenbrock et al., 1993; Ramage et al., 1993). Recently, three essential proteins of the mitochondrial inner membrane were identified that are involved in import of preproteins. MIM44 (formerly named Mpilp) is a peripheral membrane protein that binds preproteins (Maarse et al., 1992; Blom et al., 1993). The import site protein ISP45 was reported to be identical to MIM44 (Scherer et al., 1992; Horst et al., 1993). MIM23 (Mas6p) and MIM17 (Smslp) are integral membrane proteins that may constitute part of a translocation channel (Dekker et al., 1993; Emtage and Jensen, 1993; Maarse et al., 1994; Kübrich et al., 1994; Ryan et al., 1994). In the matrix, the heat shock protein hsp70 (Ssclp) is essential for translocation of preproteins and transfers the polypeptides to hsp60; partners of these heat shock proteins are mitochondrial GrpE, possibly mitochondrial DnaJ, and hspl0 (cpnl0) (Ostermann et al., 1989; Kang et al., 1990; Stuart et al., 1994a). Several specific processing enzymes operate in the matrix and inner membrane. Little is known how these components cooperate in the translocation of preproteins. In particular, it has not been understood so far if and how components of distinct subcompartments cooperate.

For this report we analyzed two essential yeast proteins that have been shown to function as binding proteins for preproteins during translocation across the mitochondrial inner membrane, MIM44 and mt-hsp70 (Kang et al., 1990; Manning-Krieg et al., 1991; Blom et al., 1993; Gambill et al., 1993). These two preprotein-binding proteins can both

1. Abbreviations used in this paper: DSS, disuccinimidyl suberate; hspl0, 70-kD heat shock protein; mt-hsp70, mitochondrial hsp70; MIM44, 44-kD protein of the mitochondrial inner membrane import machinery; SOD, superoxide dismutase; Su9-DHFR, fusion protein between presequence of Fo-ATPase subunit 9 and dihydrofolate reductase.

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be found at the matrix side of the mitochondrial inner membrane and interact in a nucleotide-sensitive manner. The functional interaction of mt-hsp70 and MIM44 was also demonstrated by genetic analysis. MIM44 and mt-hsp70 seem to directly cooperate in mitochondrial protein import, providing an interesting analogy to the interaction of BiP, the hsp70 of the endoplasmic reticulum lumen, and Sec63p in protein translocation into the endoplasmic reticulum. We propose that the reversible binding of mt-hsp70 to MIM44 is an important step in the efficient and ordered function of the mitochondrial protein import machinery.

Materials and Methods

Isolation of Temperature-Sensitive mim44 Mutants

Temperature-sensitive alleles of MIM44 (previously termed MPI1 [Maarse et al., 1992]) were generated by the low fidelity PCR technique as modified by Kassenbroek et al. (1993) and with further modifications of the reaction buffer (50 μM MnCl2, 1.5 mM MgCl2, pH 9). Four separate PCR reactions were performed and in each case the concentration of one dNTP was reduced fivefold with respect to the three others (25 μM vs 125 μM). MIM44 sequences were amplified with the M13 forward sequencing primer and the M13 reverse sequencing primer, using YCplac111::MIM44 as template DNA. After 30 cycles of 1 min at 94°C, 2 min at 50°C and 3 min at 74°C, the expected 2.8-kb product was obtained. The products from the four reactions were pooled and digested with EagI and EcoRI. The 1.8-kb EagI–EcoRI fragments containing the MIM44 coding region were purified by agarose gel electrophoresis and cloned into YCplac111::MIM44, cut with the same enzymes, thereby replacing most wild-type MIM44 sequences by potentially mutated sequences. The mutant DNA library, obtained by isolating plasmid DNA from about 1,000 E. coli transformants, was used to transform the mim44 deletion strain MB6 which harbors the URA3 marked plasmid YEplac95::MIM44. Leu-positive transformants were plated on solid medium containing 5-fluoroorotic acid (Boeke et al., 1987) and incubated at 23 or 35.4°C. Plasmid DNA was isolated from colonies and the M13 reverse sequencing primer, using E. coli, transformed into the heterozygous MIM44 diploid MB2-22 (Maarse et al., 1992). After random sporulation, haploid cells with a disrupted nuclear MIM44 gene and harboring the rescuing plasmid were also tested for temperature sensitivity. Temperature sensitivity could be established for several plasmid-borne mim44 alleles and two of them were designated mim44-6 and mim44-7 (Table I).

Construction of Plasmids

YCplac111::MIM44, YEpplac81::MIM44 and YEpplac95::MIM44 were isolated by cloning the 2.7-kb HindIII fragment containing the complete MIM44 gene (Maarse et al., 1992) into YCplac111(LEU2), YEpplac81-(LEU2), and YEpplac95(URA3), respectively (Gietz and Sugino, 1988). Centromeric plasmids containing either the wild-type MIM44 gene or the mim44-6 or mim44-7 alleles were constructed by cloning the 2.7-kb HindIII fragment into the HindIII site of YCplac22(TRP1) or YCplac33(URA3) (Gietz and Sugino, 1988). To generate YCplac111::SSCI and YEpplac81::SSCI, a 6-kb EcoRI fragment bearing the SSCI gene was liberated from a YEp3 derivative and cloned into YCplac111 and YEpplac81.

Covalent Coupling of Antibodies to Protein A–Sepharose

300 μl wet volume of protein A–Sepharose (Pharmacia LKB Biotechnology, Piscataway, NJ), 2 ml 100 mM potassium phosphate buffer, pH 7.5, and 1 ml antiserum were gently shaken for 1 h. After two times washing with 0.1 M sodium borate buffer, pH 9, the protein A–Sepharose was resuspended in 7 ml sodium borate buffer and 35 mg solid dimethyl pimelimidate (Sigma Chem. Co., St. Louis, MO; D-8388) were added. After an incubation of 30 min, the coupling reaction was stopped by washing and incubation for 2 h in 1 M Tris-HCl, pH 7.5. All steps were performed at room temperature. The Sepharose matrix with coupled antibodies was stored in 10 mM Tris-HCl, pH 7.5, 0.9% (wt/vol) NaCl at 4°C.

Submitochondrial Fractionation

Mitoplasts were generated by dilution of a suspension of mitochondria (1 mg/ml) in SEM (250 mM sucrose, 1 mM EDTA, 10 mM MOPS, pH 7.2) with a 10-fold excess of EM (1 mM EDTA, 10 mM MOPS, pH 7.2). After an incubation for 15 min on ice, the mitoplasts were resuspended by centrifugation (10 min 16,000 g). For sonication, 12 μg/ml trypsin before sonication and were subsequently incubated on ice for 20 min. The reaction was stopped by a 30-fold excess (wt/wt) of soybean trypsin inhibitor (Sigma Chem. Co., F-9003).

Commmunoprecipitations

35S-labeled mitochondria (25 μg per sample) were suspended in 500 μl lysis-buffer (% Triton X-100, 300 mM KCl, 10 mM Tris-HCl, pH 7.4, Pepstatin A (2 μg/ml)). The samples were shaken at 8°C for 10 min. After a

Table 1. S. cerevisiae Strains Used in This Study

| Strain | Genotype | Source |
|--------|----------|--------|
| PK82   | MATa his4-713 lys2 ura3-52 Δtrp1 leu2-3,112 | Gamblill et al., 1993 |
| PK83   | MATa ade2-101 lys2 ura3-52 Δtrp1 leu2-3,112 ssc1-3(LEU2) | Gamblill et al., 1993 |
| MB6    | ade2-101 his3 leu2 lys2-801 trp1-289 ura3-52 mim44::LYS2 + YEpplac195(URA3)::MIM44 | this study |
| MB3    | ade2-101 his3Δ200 leu2-Δ1 lys2-801 ura3::LYS2 | Maarse et al., 1992 |
| MB3-4  | ade2-101 his3Δ200 leu2-Δ1 lys2-801 ura3::LYS2 mim44-1 | Dekker et al., 1993 |
| MB3-42 | ade2-101 his3Δ200 leu2-Δ1 lys2-801 ura3::LYS2 mim44-2 | Dekker et al., 1993 |
| MB3-52 | ade2-101 his3Δ200 leu2-Δ1 lys2-801 ura3::LYS2 mim44-3 | Maarse et al., 1992 |
| MB3-68 | ade2-101 his3Δ200 leu2-Δ1 lys2-801 ura3::LYS2 mim44-4 | Dekker et al., 1993 |
| MB3-75 | ade2-101 his3Δ200 leu2-Δ1 lys2-801 ura3::LYS2 mim44-5 | Dekker et al., 1993 |
| MB3-27 | ade2-101 his3Δ200 leu2-Δ1 lys2-801 ura3::LYS2 ssc1 | Dekker et al., 1993 |
| MB3-43 | ade2-101 his3Δ200 leu2-Δ1 lys2-801 ura3::LYS2 ssc1 | Dekker et al., 1993 |
| MB2-22 | MATaΔ ade2-101 his3Δ200 leu2-Δ1 lys2-801 ura3-52 ssc1-1(LEU2) ssc1-3(LEU2) TRP1-1298 ura3-52 mim44::LYS2 | Maarse et al., 1992 |
| LK201  | MATa his3 leu2 trp1 ura3-52 mim44::LYS2 + YCplac22(TRP1)::MIM44 | this study |
| LK208  | MATa his3 leu2 trp1 ura3-52 mim44::LYS2 + YCplac22(TRP1)::MIM44-6 | this study |
| LK209  | MATa his3 leu2 trp1 ura3-52 mim44::LYS2 + YCplac22(TRP1)::MIM44-7 | this study |
| LK215  | MATa leu2 lys2 trp1 ura3-52 ade2-201 mim44::LYS2 ssc1-1(LEU2) + YCplac22(TRP1)::MIM44-6 | this study |
| LK218  | MATa leu2 lys2 trp1 ura3-52 ade2-201 mim44::LYS2 ssc1-1(LEU2) + YCplac22(TRP1)::MIM44-7 | this study |
| LK221  | MATa leu2 lys2 trp1 ura3-52 ade2-201 mim44::LYS2 ssc1-1(LEU2) + YCplac22(TRP1)::MIM44 | this study |
spin of 16,000 g for 10 min, the supernatants were added to antibodies pre-bound to protein A-Sepharose, and shaken at 8°C for 35 min. Subsequently the protein A-Sepharose was washed three times with lysis-buffer. The coimmunoprecipitations were analyzed by SDS-PAGE and fluorography. Where indicated, 5 mM MgCl₂ and 4 mM ATP or 4 mM ATPγS were included in the lysis-buffer. The presence of additional protease inhibitors did not significantly change the pattern of the interaction of MIM44 with hsp70 in this assay. For a second immunoprecipitation under denaturing conditions, the immunocomplexes were dissociated in 20 μl 2% SDS, 60 mM Tris-HCl, pH 7.2, before dilution with a 30-fold excess of lysis-buffer.

Affinity-purified antibodies against MIM44 were obtained by elution of antibodies from a fragment of MIM44 (amino acid residues 68 to 345 with addition of six histidine residues at the amino terminus) expressed in E. coli (Blom et al., 1993). The MIM44 fragment was isolated by Ni-NTA affinity purification, and blotted on nitrocellulose from an SDS-polyacrylamide gel. Prebound antibodies were eluted from slices of the nitrocellulose by shaking with 100 mM glycine, pH 2.5, at 0°C for 10 min (Hartow and Lane, 1988). The pH was readjusted by addition of 1 M Tris-HCl, pH 8, and the antibodies were collected by protein A-Sepharose.

**35S-labeling of Mitochondria**

35S-labeling of yeast mitochondria followed in principle the procedure of Campbell and Duffus (1988). A first preculture of wild-type S. cerevisiae was diluted 400-fold with minimal medium, containing 0.1% (wt/vol) glucose and 2.2% (vol/vol) lactic acid (pH 5.5 with KOH) and 30 μM Na₂SO₄ (for further components see Campbell and Duffus, 1988), and shaken at 30°C for 50 h, the final OD₅₇₈ was 5.5. An aliquot was diluted into 50 ml of the medium at an OD₅₇₈ of 0.04. After addition of 50 μl [35S]sulfate (250 μCi; Amersham JSI), this second preculture was grown for 43 h at 30°C to a final OD₅₇₈ of 3.3. For the main culture, 300 ml medium were inoculated with 5 ml of the second preculture. 650 μl [35S]sulfate (3.25 mCi) were added and the culture was shaken in a 1,000-ml flask for 24 h. At an OD₅₇₈ of 1.1, cells were harvested and mitochondria were isolated as published previously (Daum et al., 1982; Hartl et al., 1987). The yield was 1.3 mg mitochondrial protein/300 ml culture, the specific radioactivity was 44,000 cpm/μg protein.

**Miscellaneous**

Synthesis of preproteins in rabbit reticulocyte lysate, cross-linking of mitochondrial proteins to Su9-DHFR by disuccinimidyl suberate (DSS) (Blom et al., 1993), TCA-precipitation, SDS-PAGE, immunodecoration, fluorography (Pfanner et al., 1987), and storage phosphor imaging technology (Molecular Dynamics, Sunnyvale, CA) were performed according to published procedures.

**Results**

**Localization of MIM44 and a Fraction of mt-hsp70 at the Matrix Side of the Inner Membrane**

While MIM44 has been previously determined to be localized to the inner membrane, its topology in the inner membrane remained ambiguous. MIM44 behaves as a peripheral membrane protein and contains no hydrophobic membrane anchor sequence suggesting that MIM44 is not an integral membrane protein. Antibodies directed against MIM44 (ISP45) were reported to inhibit protein import into mitochondria having a disrupted outer membrane (mitoplasts) (Scherer et al., 1992; Horst et al., 1993); in addition, a carboxy-terminal epitope tag was accessible to proteases in mitoplasts (Maarse et al., 1992). Authentic MIM44, however, was not cleaved by proteases added to mitoplasts even at high concentrations (Blom et al., 1993). These results can be explained if, (a) MIM44 is mainly located on the intermembrane space side of the inner membrane, but is folded in such a manner that no protease-accessible site is exposed on the intermembrane space side, or (b) if major portions of MIM44 are located on the matrix side and thus MIM44 has to span the inner membrane at least once.

To distinguish between these possibilities, a more careful assessment of the protease sensitivity of MIM44 was undertaken. As expected MIM44 was resistant to protease digestion in intact mitochondria (Fig. 1, lane 3). In addition, MIM44 in mitoplasts, whose formation was carefully monitored by the release of the intermembrane space protein cytochrome b₂ (Fig. 1, lanes 5–8), was also protease inaccessible (Fig. 1, lane 7). However, MIM44 of mitochondria and mitoplasts sonicated to open the matrix space in the presence of low amounts of trypsin (Fig. 1, lanes 4 and 8) or proteinase K (not shown) was degraded. Thus MIM44 had the same protease accessibility as the hsp70 of the mitochondrial matrix. No immunodetectable fragments of MIM44 remained after this treatment. It should be noted that MIM44 was digested by very low concentrations of trypsin after opening of the matrix by sonication, which are not sufficient to fully degrade soluble cytochrome b₂ that is known to possess some endogenous protease resistance (Rassow and Pfanner, 1991). These results together with previous experiments (Maarse et al., 1992; Blom et al., 1993) suggest the following topology of MIM44. The major portion of the molecule is located on the matrix side of the inner membrane. The extreme carboxy terminus is exposed to the intermembrane space side, implying that a carboxy-terminal region of MIM44 crosses the inner membrane, probably in association with integral membrane proteins. MIM17 and MIM23 are possible candidates as anchor proteins for MIM44, however, such a function of the two smaller MIM proteins has not been demonstrated so far.

**Figure 1.** Topology of MIM44 in the mitochondrial inner membrane. Isolated yeast mitochondria from strain PK$2 were subjected to swelling to form mitoplasts (samples 5–8) and/or sonication (samples 2, 4, 6, and 8), and the accessibility of MIM44 for trypsin was monitored as indicated. Proteins were TCA precipitated and subjected to SDS-PAGE and Western blotting. MIM44 and marker proteins were analyzed by immunodecoration with specific antibodies. mt-hsp70, mitochondrial hsp70 (matrix); Cyt. b₂, cytochrome b₂ (intermembrane space).
We then determined the localization of another essential preprotein-binding protein of the mitochondrial import machinery, mt-hsp70. Mt-hsp70 had previously been shown to behave as a soluble protein of the mitochondrial matrix (Kang et al., 1990; Scherer et al., 1990). However, when a fractionation was performed in the absence of Mg-ATP, ~20% of mt-hsp70 was found in the membrane pellet after sonication of mitochondria (Fig. 2). This fraction of mt-hsp70 remained bound to the membrane vesicles after flotation in a sucrose density gradient (not shown), excluding that mt-hsp70 was found in the membrane pellet due to aggregation or association with ribosomes. Upon addition of Mg-ATP, mt-hsp70 was efficiently released from the membrane vesicles and recovered in the supernatant (Fig. 2). Dissipation of the membrane potential across the inner membrane by addition of the potassium ionophore valinomycin did not affect the membrane association of mt-hsp70. We conclude that a fraction of mt-hsp70 is reversibly bound to the mitochondrial inner membrane, independent of a membrane potential, and is released from the membranes by Mg-ATP.

**Immunoprecipitation of a Complex between MIM44 and mt-hsp70**

Since MIM44 and mt-hsp70 have at least in part a similar localization at the matrix side of the inner membrane, we asked if they interact with each other. $^{35}$S-labeled yeast mitochondria were lysed with the non-ionic detergent Triton X-100 and subjected to immunoprecipitations under non-denaturing conditions in the absence of Mg-ATP with antibodies directed against MIM44. The immunoprecipitates were analyzed by SDS-PAGE and autoradiography. Besides MIM44 itself, the most prominent band seen in the precipitate was a protein of ~70 kD (Fig. 3 A, lanes 2 and 4). Other bands seen in the immunoprecipitate were not significantly above the background level observed in a precipitation with preimmune serum (Fig. 3 A, lane 3). The 70-kD protein was not precipitated by antibodies against MIM44 under denaturing conditions (Fig. 3 A, lane 7). In a second immunoprecipitation with antibodies directed against mt-hsp70, carried out under denaturing conditions, the 70-kD protein was identified as mt-hsp70 (Fig. 3 A, lane 6). We conclude that the major protein associated with MIM44 in the presence of Triton X-100 is mt-hsp70. Considering the number of $^{35}$S-labeled amino acids in each protein, MIM44 and mt-hsp70 interact in a ratio that is close to 1:1 (Fig. 3 B).

It was possible that the binding of mt-hsp70 to MIM44 occurred after the lysis of mitochondria. In this case, unlabeled mt-hsp70 added during the lysis should compete with the $^{35}$S-labeled mt-hsp70 for binding to MIM44. To test this possibility, we included a 10-fold excess of unlabeled mt-hsp70 (compared to the amount of hsp70 present in the $^{35}$S-labeled mitochondria) in the Triton X-100 buffer (Fig. 3 B, column 2). However, the amount of labeled mt-hsp70 coprecipitated with anti-MIM44 antibodies was not reduced, demonstrating that the association between mt-hsp70 and MIM44 occurred in intact mitochondria.

By titrating the immunoreactivity of anti-MIM44 antibodies with mitochondria and purified expressed MIM44 on Western blots, MIM44 was found to represent ~0.25% of mitochondrial protein. Mt-hsp70 represents ~1% of mitochondrial protein. The abundance of MIM44 is thus sufficient to explain binding of ~20% of mt-hsp70 to the inner membrane in a ratio of MIM44:mt-hsp70 of 1:1.

**ATP Dependence of Interaction of MIM44 and mt-hsp70**

To quantitatively analyze the interaction of MIM44 and mt-hsp70, we prepared an affinity column with covalently coupled antibodies directed against MIM44. Unlabeled mitochondria were lysed with Triton X-100 and passed over the column. After several washing steps, bound proteins were eluted by lowering the pH to 2.5. Fig. 4 A shows that MIM44 quantitatively bound to the column (columns 6, 9, and 12), whereas about 15% of mt-hsp70 was found in the bound fraction (column 6). Addition of Mg-ATP or of the non-hydrolyzable analog ATP$_{y}$S led to efficient release of mt-hsp70 from MIM44 (Fig. 4 A, columns 9 and 12). ATP-dependent dissolution of the MIM44:mt-hsp70 complex detected by immunoprecipitation was also observed as addition of Mg-ATP promoted a quantitative release of mt-hsp70 from MIM44 in extracts of $^{35}$S-labeled mitochondria (Fig. 3 A, lane 5).
Identification of a complex between MIM44 and mitochondrial hsp70. (A) 35S-labeled mitochondria were lysed in 1% Triton X-100, 300 mM KCl, 10 mM Tris-HCl, pH 7.2, and tested for coimmunoprecipitation of associated proteins by antibodies directed against MIM44 or antibodies from preimmune serum. The mitochondria of sample 1 were denatured in SDS-containing buffer before dilution in Triton X-100 buffer. With sample 5, the first precipitation was performed in the presence of 4 mM Mg-ATP. With samples 5 and 6, the immunoprecipitated complex was dissociated by SDS and diluted in Triton X-100 buffer, followed by a second immunoprecipitation with antibodies directed against mt-hsp70 (Ssclp). Analysis was by SDS-PAGE and fluorography. A minor band corresponding to 32 kD (lanes 2 and 4) is probably unspecific since it also appeared with preimmune serum (lane 3).

(B) Coimmunoprecipitations of mt-hsp70 were performed in the presence and in the absence of a 10-fold excess of unlabeled mt-hsp70 (extracted from mitochondria by sonication). Indicated are the molar ratios of mt-hsp70 and MIM44 in the precipitated complexes. The standard errors of the means (SEM) were calculated from 5 (column 1) or 2 (column 2) independent experiments, respectively. The precipitable amount of a mitochondrial marker protein (such as AAC) was not influenced by the excess of unlabeled mt-hsp70.

Binding of mt-hsp70 to MIM44 is nucleotide-sensitive. (A) Mt-hsp70 stays bound to MIM44 in the presence of EDTA and is released in the presence of Mg-ATP. Antibodies of preimmune serum (columns 1-3) and antibodies directed against MIM44 (columns 4-12) were covalently coupled to protein A-Sepharose and tested for coimmunoprecipitations of mt-hsp70 from wild-type mitochondria in the presence of 1 mM EDTA (columns 1-6), 4 mM Mg-ATP (columns 7-9) or 4 mM Mg-ATPγS (columns 10-12). The immunocomplexes were analyzed by SDS-PAGE, immunoblotting, and 2D-densitometry. Preliminary results suggest that addition of Mg-ADP also led to some release of mt-hsp70 from MIM44. (B) Mt-hsp70 containing a point mutation in the ATP-binding domain (Sscl-3p) does not bind to MIM44. Mitochondria of wild-type PK82 (columns 1-3) and of the mutant sscI-3 (PK83) (columns 4-6) were incubated for 10 min at 37°C, cooled on ice, and then lysed and used for coimmunoprecipitations by covalently bound anti-MIM44 antibodies as described above.

We also tested the interaction of a temperature-sensitive mutant form of mitochondrial hsp70, sscI-3. As previously described, the mutant phenotype can be induced in vitro by preincubating isolated sscI-3 mitochondria at 37°C for 10 min (Gambill et al., 1993). The mitochondria were then lysed and passed over the anti-MIM44 affinity column. Whereas the binding of MIM44 to the column was un-
changed, the mutant Sscl-3p did not bind to the column (Fig. 4 B, column 6). A preincubation of wild-type mitochondria at 37°C did not change the binding of mt-hsp70 (Ssclp) to MIM44 (Fig. 4 B, column 3). When the ssc1-3 mitochondria were not preincubated at the non-permissive temperature, binding of Sscl-3p to MIM44 was not inhibited (data not shown). Since Sscl-3p has a mutation in the amino terminal ATPase domain, this result indicates that a functional ATPase domain of mt-hsp70 is needed for binding to MIM44.

Mitochondria with a Defective mt-hsp70 Accumulate Preproteins at MIM44

In the following, we tried to analyze how the interaction of preproteins with MIM44 and mt-hsp70 is affected by the association between MIM44 and mt-hsp70. We previously reported that mitochondria from the mutant ssc1-3 were unable to completely import preproteins into the mitochondrial matrix (Gambill et al., 1993). However, an amino-terminal portion of a preprotein was imported. This partial import was shown with a fusion protein between the presequence of Fo-ATPase subunit 9 and dihydrofolate reductase (Su9-DHFR) that is processed twice by the processing peptidase of the mitochondrial matrix. ssc1-3 mitochondria were able to process Su9-DHFR to the intermediate-sized form, which is 35 amino acid residues constituting the first half of the presequence were cleaved off. When the preprotein was denatured with urea before import, most accumulated as an intermediate that spanned across the inner membrane, with the major portion of the preprotein located in the intermembrane space and the presequence cleaved to the intermediate form (Gambill et al., 1993) (in addition, part of the preprotein was accumulated as the uncleaved precursor form) (Fig. 5 A). A similar intermediate was obtained when denatured Su9-DHFR was imported into wild-type mitochondria depleted of matrix ATP, probably due to the impairment of hsp70 function (Manning-Krieg et al., 1991; Gambill et al., 1993). Su9-DHFR accumulated in ATP-depleted wild-type mitochondria was efficiently cross-linked to MIM44 (Blom et al., 1993; Horst et al., 1993). With ATP-depleted wild-type and ssc1-3 mitochondria, chemical cross-linking should thus be an ideal procedure to test in organello if the presence or absence of association between MIM44 and mt-hsp70 influenced the interaction of preproteins with each of the two proteins.

35S-labeled Su9-DHFR was accumulated in mitochondrial import sites at low ATP (Fig. 5 A). Cross-linking with the homobifunctional amino-reactive cross-linking reagent DSS (Blom et al., 1993) was performed, and cross-linked proteins were identified by immunoprecipitation under denaturing conditions. Su9-DHFR was efficiently cross-linked to MIM44 in both wild-type and ssc1-3 mitochondria (Fig. 5 B, columns 1 and 2), demonstrating that the preprotein is translocated to MIM44 even in the absence of mt-hsp70 binding to MIM44. As previously described, Su9-DHFR yields two cross-link products with MIM44 of slightly different gel mobility that possibly represent cross-linking to the precursor- and the intermediate-forms of Su9-DHFR (Blom et al., 1993).

The cross-linking of Su9-DHFR to mt-hsp70, however, was strongly decreased in the ssc1-3 mitochondria (Fig. 5 B, lane 4). In wild-type mitochondria, the cross-link product of 170 kD is of a size consistent with Su9-DHFR and a monomer of mt-hsp70 (Fig. 5 B, lane 3). The cross-link product of 170 kD probably includes a dimer of mt-hsp70. In addition, a high molecular mass aggregate is precipitated with the anti-mt-hsp70 antibodies. All three mt-hsp70 containing cross-linking bands are strongly reduced in ssc1-3 mitochondria. Previously we were unable to show an association between accumulated Su9-DHFR and mt-hsp70 using coimmunoprecipitation experiments starting from ATP-depleted wild-type mitochondria or ssc1-3 mitochondria, indicating that ATP and a functional ATPase domain of mt-
ever, by cross-linking we now demonstrate that mt-hsp70 in hsp70 were needed to obtain a binding of the preprotein to mt-hsp70 that was stable enough to survive the coimmunoprecipitation procedure (Gambill et al., 1993). However, by cross-linking we now demonstrate that mt-hsp70 in ATP-depleted wild-type mitochondria is in close proximity to the preprotein in transit. Since only a short portion of the presequence of accumulated Su9-DHFR is located on the matrix side of the inner membrane, this cross-linking provides independent evidence that mt-hsp70 is in very close proximity to the protein import site, that is to MIM44.

Genetic Evidence for Interaction of MIM44 and mt-hsp70

We applied two genetic approaches to obtain independent evidence for an interaction of MIM44 and mt-hsp70, multicopy suppression and synthetic lethal analysis.

We previously constructed a test plasmid encoding the URA3 gene product orotidine 5'-phosphate decarboxylase (OMP decarboxylase) with the amino-terminal mitochondrial-targeting sequence of superoxide dismutase (SOD). When this test plasmid was introduced into the yeast strain MB3, carrying a deletion of the chromosomal URA3 coding region, the fusion protein was efficiently imported into mitochondria and the cells remained inviable in uracil-free medium due to lack of OMP decarboxylase activity in the cytosol (Maarse et al., 1992). The mim44 mutants MB3-4, MB3-42, MB3-52, MB3-68, and MB3-75 are at least partially blocked in mitochondrial import of the SOD-OMP decarboxylase fusion protein, which allows them to grow in the absence of exogenously added uracil (Maarse et al., 1992; Dekker et al., 1993). The dependence of growth on the addition of uracil can thus be taken as an indication of the efficiency of the import of the test protein into mitochondria in vivo. To test the effects of overexpression of mt-hsp70 on the growth of mim44 mutants in the absence of uracil, the mim44 mutants carrying the test plasmid were transformed with the mt-hsp70 gene SSCI, cloned on either the centromeric vector YCplac111 or the multi-copy vector YEplac181. Double transformants in which mt-hsp70 was expressed from the multi-copy plasmid showed a greatly diminished growth on uracil-free medium (Table II). This decrease in growth demonstrates that overexpression of mt-hsp70 can at least partially suppress the import defect of all five mim44 mutants. Overexpression of mt-hsp70, however, did not rescue the lethal phenotype of a mim44 deletion mutant (not shown), indicating that mt-hsp70 cannot fully replace the function of MIM44.

In a similar way we tested whether overexpression of MIM44 will suppress the ura-positive mutant phenotype of the ssc1 mutants MB3-27 and MB3-43 isolated in the manner described for the mim44 mutants (Dekker et al., 1993). Expression of MIM44 from the multi-copy vector YEplac181, but not from the centromeric vector YCplac111, reduced growth of double transformed strains on medium lacking uracil (Table II). Thus, overexpression of MIM44 can partially relieve the import defect of the SOD-OMP decarboxylase fusion protein in both mt-hsp70 mutant strains, again indicating a genetic interaction of MIM44 and mt-hsp70.

The phenomenon of mutations in different genes producing a severe growth defect or lethality can be indicative of functional interaction between gene products (Huffaker et al., 1987; Kaiser and Schekman, 1990; Scidmore et al., 1993). To determine if strains carrying both ssc1-3 and mim44 temperature-sensitive alleles are viable, a strain containing ssc1-3 and a deletion of the MIM44, rescued by a wild-type MIM44 gene on a low copy number plasmid also containing the URA3 gene was constructed. The strain was then transformed with plasmids containing the TRP1 gene and either the wild-type MIM44 gene, the mim44-6 allele, or the mim44-7 allele. All three strains were able to lose the URA3-containing plasmid, as evidenced by growth on media containing 5-fluoro-orotic acid. However, while patches of strains containing the wild-type plasmid grew well, those with the temperature-sensitive alleles grew very poorly. At 23°C the strains containing the temperature-sensitive MIM44 alleles could only form very small colonies which were extremely difficult to propagate. At 30°C, very little growth was observed, whereas the individual ssc1-3 and mim44 ts mutants grew reasonably well compared to wild-type (Fig. 6). The strongly pronounced temperature sensitivity of the ssc1 and mim44 double mutants indicates a synthetic effect which argues in favor of a genetic interaction of MIM44 and mt-hsp70.

| Table II. Multi-Copy Suppression of mim44 Mutants by SSCI and ssc1 Mutants by MIM44 |
|---------------------------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| Strain containing test-plasmid             | Additional plasmid        |                          |                          |                          |
|                                            | None                      | YCplac111::MIM44         | YEplac181::MIM44         | YCplac111::SSCI          | YEplac181::SSCI          |
| MB3                                         |                          |                          |                          |                          |                          |
| mim44 mutants                               |                          |                          |                          |                          |                          |
| MB3-4                                       | +++                      | -                        | -                        | +                        | +                        |
| MB3-42                                      | +++                      | -                        | -                        | +                        | +                        |
| MB3-52                                      | +++                      | -                        | -                        | +                        | +                        |
| MB3-68                                      | +++                      | -                        | -                        | +                        | +                        |
| MB3-75                                      | +++                      | -                        | -                        | +                        | +                        |
| ssc1 mutants                                |                          |                          |                          |                          |                          |
| MB3-27                                      | +++                      | +                        | -                        | -                        | -                        |
| MB3-43                                      | +++                      | +                        | -                        | -                        | -                        |

- indicates no growth after 6 d at 23°C on selective minimal medium plates lacking uracil (i.e., complementation of mutants); + indicates very slow growth after 4-5 d at 23°C on selective minimal medium plates lacking uracil (partial complementation); +++ indicates growth after 2-3 d at 23°C on selective minimal medium plates lacking uracil (no complementation). All transformants grow well (+ + +) after 2-3 d at 23°C on selective minimal medium plates containing 40 μg/ml uracil.

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Figure 6. Synthetic growth defects of mim44 and ssc1 temperature-sensitive mutations. Strains containing temperature-sensitive mutations in MIM44 and SSC1 were streaked on plates containing rich media and incubated at 30°C for 3 d. WT (LK201), mim44-6 (LK208), mim44-7 (LK209), ssc1-3 (LK221), mim44-6 ssc1-3 (LK215), and mim44-7 ssc1-3 (LK218).

In summary, we conclude that these genetic data strongly support the biochemical evidence for an interaction of MIM44 and mt-hsp70.

Discussion

We report a new partner for the hsp70 of the mitochondrial matrix, the peripheral inner membrane protein MIM44. Both proteins were previously shown to be required for translocation of preproteins across the mitochondrial membranes and to function as binding proteins for the precursor polypeptides. It has not been anticipated, however, that these two import components directly cooperate, since mt-hsp70 was considered as a soluble protein of the mitochondrial matrix, while several reports indicated a location of MIM44 on the intermembrane space side of the inner membrane. However, we found major portions of MIM44 exposed to the matrix side of the inner membrane and ~20% of mt-hsp70 associated with the inner membrane in the absence of ATP.

This binding of mt-hsp70 was transient and relieved by addition of Mg-ATP.

Coimmunoprecipitations showed that MIM44 and mt-hsp70 associate in a complex in a ratio of about 1:1. We cannot exclude that other proteins are present in this complex, yet analysis from 35S-labeled mitochondria indicates that the abundance of other putative components in the complex would be below the stoichiometric level in the presence of Triton X-100. The abundance of MIM44 in mitochondria is ~20-25% of that of mt-hsp70, such that MIM44 could provide enough binding sites for the nucleotide-dependent membrane association of mt-hsp70.

We consider the interaction between MIM44 and mt-hsp70 as specific for the following reasons. (a) The association was shown by two distinct procedures, coimmunoprecipitation and affinity chromatography, and control experiments indicate that the interaction occurs inside mitochondria. (b) The association requires a functional ATPase domain of mt-hsp70 and is dissociated by addition of Mg-ATP. A functional ATPase domain thus seems to be required for both binding to and release from MIM44. (c) The molar ratios of MIM44 to mt-hsp70 in the complex and in total mitochondria are consistent with the degree of membrane association of mt-hsp70. (d) In vivo, overexpression of mt-hsp70 rescues the protein translocation defect of MIM44 mutants and vice versa. (e) Double mutants between temperature-sensitive alleles of mt-hsp70 and MIM44 show a synthetic growth defect. The combined biochemical and genetic findings thus provide strong indication for a cooperation of MIM44 and mt-hsp70 in mitochondrial biogenesis and function.

We propose that the cooperation of these two essential components of the mitochondrial protein import machinery is facilitated by direct binding to each other. In a nucleotide-dependent manner, matrix hsp70 cycles between a soluble state and a membrane bound state. A fraction of mt-hsp70 thus stays in a "stand by" modus in closest vicinity to the translocation site. After the Δψ-mediated import of the presequence, hsp70 could trap the preprotein by immediate binding and initiate the Δψ-independent translocation of the mature protein. Some data indicate an extended structure of the polypeptide in transit across the mitochondrial membranes (Rassow et al., 1990). In this conformation the preprotein is an ideal substrate for hsp70 binding (Landry et al., 1992). Besides trapping the preprotein in the translocation site, hsp70 is necessary to prevent the hydrophobic collapse of the translocating protein and facilitate binding of additional hsp70 molecules to the preprotein (Stuart et al., 1994a). Hsp70 localized directly at the exit of the translocation channel would have a chance to interfere with a process such as a hydrophobic collapse which takes only milliseconds. Because of this time scale a direct interaction of hsp70 with MIM44 may be essential, although the general concentration of hsp70 and possibly of other chaperone proteins in the mitochondrial matrix is high.

It was previously proposed that mt-hsp70 has a dual role in membrane translocation of preproteins, not only facilitating the unfolding of preproteins (unfoldase function), but also actively driving unfolded polypeptide chains across the inner membrane (translocase function) (Gambill et al., 1993; Voos et al., 1993; Stuart et al., 1994b; Wachter et al., 1994). We speculate that the ATP-dependent release of mt-hsp70 from MIM44, possibly accompanied by conforma-
tional changes of MIM44 and mt-hsp70, adds to the force driving polypeptide chains across the mitochondrial inner membrane. In support of this view, a stable association between mt-hsp70 and a preprotein in transit (stable enough to survive a coimmunoprecipitation) is only obtained in the presence of ATP (Manning-Krieg et al., 1991; Gambill et al., 1993), while cross-linking under ATP-depleted conditions in organello indicates that mt-hsp70 is already in close proximity to the preprotein when it is bound to MIM44. We suggest that an ATP-dependent reaction cycle of mt-hsp70 (Hartl et al., 1994), which includes interaction with MIM44 and preproteins, is an essential step in protein import into the mitochondrial matrix.

Our results do not exclude a function of MIM44 in initial steps of translocation across the inner membrane in addition to its function at the matrix side of the membrane. MIM44 seems to funnel preproteins by a $\Delta\psi$-dependent step into the mitochondrial hsp70 system. Cross-linking studies suggest that MIM44 is a major component of the translocation site (Blom et al., 1993), and antibodies bound to MIM44 at the outer surface of the inner membrane apparently shield the translocation sites from preproteins (Scherer et al., 1992). We assume that a small carboxy-terminal portion of MIM44 is sufficient to mediate the effect of the antibodies.

Interestingly, Brodsky and Schekman (1993) reported that a fraction of BiP (Kar2p), the hsp70 of the endoplasmic reticulum (ER), interacts with the membrane protein Sec63p in yeast in an ATP-dependent manner. Sec63p is part of the protein import machinery of the ER and contains a domain of about 70 amino acid residues in its luminal part that is homologous to DnaJ, termed J-domain (Sadler et al., 1989; Feldheim et al., 1992; Cyr et al., 1994). Prokaryotic DnaJ and eukaryotic homologs have been shown to function as binding partners of hsp70 (Wickner et al., 1991, 1992; Liberek et al., 1991; Langer et al., 1992; Cyr et al., 1992, 1994). The transient interaction of BiP and Sec63p was proposed to occur via the J-domain and to be part of a reaction cycle required for protein translocation into the ER (Sanders et al., 1992; Brodsky and Schekman, 1993; Scidmore et al., 1993). MIM44 does not reveal a significant overall homology to known chaperones or other proteins, indicating that MIM44 is a new partner of hsp70. However, a comparison of the sequences of Sec63p and MIM44 revealed a motif of 18 amino acid residues with similarity between Sec63p (residues 138–155) and MIM44 (residues 185–202) (7 identical and 5 isofunctional residues). The motif is located in the J-domain of Sec63p. When MIM44, Sec63p, and $E. coli$ DnaJ were aligned together (Fig. 7), the similarity of the 18-residue motif was indicated to be of high significance according to the MACAW program (Schuler et al., 1991). Feldheim et al. (1992) demonstrated that the highly conserved aspartate (the last residue of the motif found here) is required for the function of Sec63p. However, in MIM44 only this aspartate is present from the conserved HPD motif (histidine, proline, aspartate) that is found in all DnaJ-like proteins analyzed so far, and the sequence similarity of MIM44 to the DnaJ-like proteins is over a much more limited region than was described between Sec63p and DnaJ (Sadler et al., 1989). Thus MIM44 cannot be seen as a DnaJ-like protein, yet future studies will have to address the possibility if this short conserved motif is involved in the interaction of MIM44 and mt-hsp70. It should be noted that, in contrast to MIM44, a direct interaction between Sec63p and preproteins in transit has not been found so far. The available evidence thus suggests an interesting analogy of association of the luminal hsp70 of both mitochondria and ER with a membrane-bound component of the translocation complex, although mechanistic details may be distinct for each organelle. Very recently, a mitochondrial homolog of DnaJ (MDJ1) was identified that is located on the matrix side of the inner membrane (Rowley et al., 1994). While MDJ1 is involved in folding of imported proteins, it has not been possible so far to demonstrate an interaction of MDJ1 and mt-hsp70 (Cyr et al., 1994). More importantly in the context of this study, a deletion of MDJ1 does not affect the translocation of preproteins into mitochondria, making it very unlikely that MDJ1 plays a critical role in polypeptide translocation and the translocase function of mt-hsp70. Instead, we propose that MIM44 is responsible for accumulation of mt-hsp70 at the import sites, thus linking the $\Delta\psi$-dependent translocation machinery of the inner membrane to the ATP-driven motor of mitochondrial protein translocation.

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