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

Joachim Rassow,* Ammy C. Maarse,‡ Elizabeth Krainer,§ Michael Kübrich,* Hanne Müller,* Michiel Meijer,‡ Elizabeth A. Craig,§ and Nikolaus Pfanner*

*Biochemisches Institut, Universität Freiburg, D-79104 Freiburg, Germany; ‡Institute for Molecular Cell Biology, BioCentrum Amsterdam, 1098 SM Amsterdam, The Netherlands; and §Department of Biomolecular Chemistry, University of Wisconsin-Madison, Wisconsin 53706

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][1] 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; 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). 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., 1994*a*). Several specific processing enzymes operate in the matrix and inner membrane.

Little is known how these import 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

Please address all correspondence to Dr. Nikolaus Pfanner, Biochemisches Institut, Universität Freiburg, Hermann-Herder-Straße 7, D-79104 Freiburg, Germany. Telephone: 49-761-203-5224; FAX: 49-761-203-5261.

1. *Abbreviations used in this paper*: DSS, disuccinimidyl suberate; hsp70, 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.

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 Kassenbrock et al. (1993) and with further modifications of the reaction-buffer (50 μM $MnCl_2$, 1.5 mM $MgCl_2$, 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 YEplac195::*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 with a temperature-sensitive phenotype, and, after a passage of *E. coli*, introduced 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*, YEplac181::*MIM44* and YEplac195::*MIM44* were isolated by cloning the 2.7-kb HindIII fragment containing the complete *MIM44* gene (Maarse et al., 1992) into YCplac111(*LEU2*), YEplac181-(*LEU2*), and YEplac195(*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::*SSC1* and YEplac181::*SSC1*, a 6-kb EcoRI fragment bearing the *SSC1* gene was liberated from a YEp13 derivative and cloned into YCplac111 and YEplac181.

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 reisolated by centrifugation (10 min 16,000 *g*). For sonication, 200 μg of isolated mitochondria were suspended in 450 μl SEM in the presence of pepstatin A (2 μg/ml). The samples were sonified (Branson Sonifier 250; Duty circle 50, output control 5) six times for 10 s, with intervals of 15 s for cooling on ice. Some samples received 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., T-9003).

Coimmunoprecipitations

^{35}S-labeled mitochondria (25 μg per sample) were suspended in 500 μl lysis-buffer (1% 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 I. S. cerevisiae Strains Used in This Study

Strain	Genotype	Source
PK82	MATα *his4-713 lys2 ura3-52 Δtrp1 leu2-3,112*	Gambill et al., 1993
PK83	MATα *ade2-101 lys2 ura3-52 Δtrp1 leu2-3,112 ssc1-3(LEU2)*	Gambill et al., 1993
MB6	*ade2-101 his3 leu2 lys2-801 trp1-289 ura3-52 mim44::LYS2* + YEplac195(*URA3*)::*MIM44*	this study
MB3	MATα *ade2-101 his3-Δ200 leu2-Δ1 lys2-801 ura3::LYS2*	Maarse et al., 1992
MB3-4	MATα *ade2-101 his3-Δ200 leu2-Δ1 lys2-801 ura3::LYS2 mim44-1*	Dekker et al., 1993
MB3-42	MATα *ade2-101 his3-Δ200 leu2-Δ1 lys2-801 ura3::LYS2 mim44-2*	Dekker et al., 1993
MB3-52	MATα *ade2-101 his3-Δ200 leu2-Δ1 lys2-801 ura3::LYS2 mim44-3*	Maarse et al., 1992
MB3-68	MATα *ade2-101 his3-Δ200 leu2-Δ1 lys2-801 ura3::LYS2 mim44-4*	Dekker et al., 1993
MB3-75	MATα *ade2-101 his3-Δ200 leu2-Δ1 lys2-801 ura3::LYS2 mim44-5*	Dekker et al., 1993
MB3-27	MATα *ade2-101 his3-Δ200 leu2-Δ1 lys2-801 ura3::LYS2 ssc1*	Dekker et al., 1993
MB3-43	MATα *ade2-101 his3-Δ200 leu2-Δ1 lys2-801 ura3::LYS2 ssc1*	Dekker et al., 1993
MB2-22	MATa/α *ADE2/ade2-101 his3/his3-Δ200 leu2/leu2-Δ1 lys2-801/lys2-801 TRP1/trp1-289 ura3-52/ura3-52 MIM44/mim44::LYS2*	Maarse et al., 1992
LK201	MATa *his3 leu2 lys2 trp1 ura3-52 mim44::LYS2* + YCplac22(*TRP1*)::*MIM44*	this study
LK208	MATa *his3 leu2 lys2 trp1 ura3-52 mim44::LYS2* + YCplac22(*TRP1*)::*mim44-6*	this study
LK209	MATa *his3 leu2 lys2 trp1 ura3-52 mim44::LYS2* + YCplac22(*TRP1*)::*mim44-7*	this study
LK215	MATa *leu2 lys2 trp1 ura3-52 ade2-201 mim44::LYS2 ssc1-3(LEU2)* + YCplac22(*TRP1*)::*mim44-6*	this study
LK218	MATa *leu2 lys2 trp1 ura3-52 ade2-201 mim44::LYS2 ssc1-3(LEU2)* + YCplac22(*TRP1*)::*mim44-7*	this study
LK221	MATa *leu2 lys2 trp1 ura3-52 ade2-201 mim44::LYS2 ssc1-3(LEU2)* + YCplac22(*TRP1*)::*MIM44*	this study

spin of 16,000 *g* for 10 min, the supernatants were added to antibodies prebound 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 $MgCl_2$ 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 (Harlow 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.

^{35}S-labeling of Mitochondria

^{35}S-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 Na_2SO_4 (for further components see Campbell and Duffus, 1988), and shaken at 30°C for 50 h, the final OD_{578} was 5.5. An aliquot was diluted into 50 ml of the medium at an OD_{578} of 0.04. After addition of 50 μl [^{35}S]sulfate (250 μCi; Amersham SJS1), this second preculture was grown for 43 h at 30°C to a final OD_{578} of 3.3. For the main culture, 300 ml medium were inoculated with 5 ml of the second preculture. 650 μl [^{35}S]sulfate (3.25 mCi) were added and the culture was shaken in a 1,000-ml flask for 24 h. At an OD_{578} 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 b_2 (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 b_2 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.

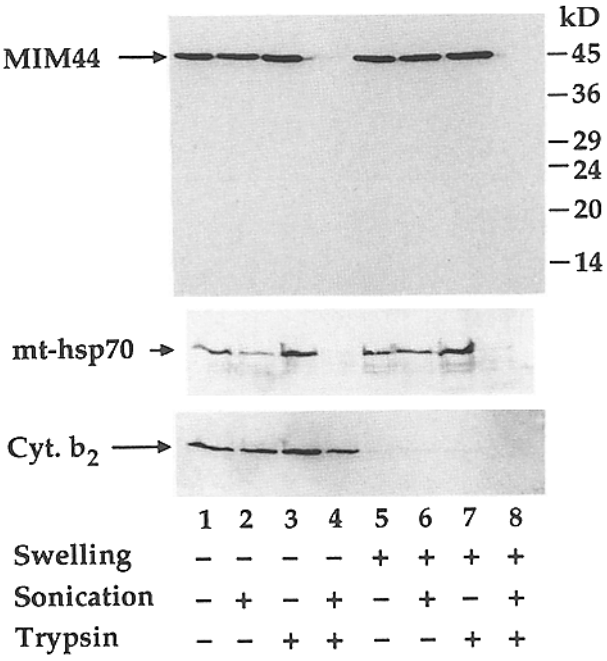

Figure 1. Topology of MIM44 in the mitochondrial inner membrane. Isolated yeast mitochondria from strain PK82 were subjected to swelling to form mitoplasts (samples *5–8*) and/or sonication (samples *2*, *4*, *6*, and *8*), and the accessibility of MIM44 for trypsin was monitored as indicated. Proteins 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. b_2*, cytochrome b_2 (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 floatation 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.

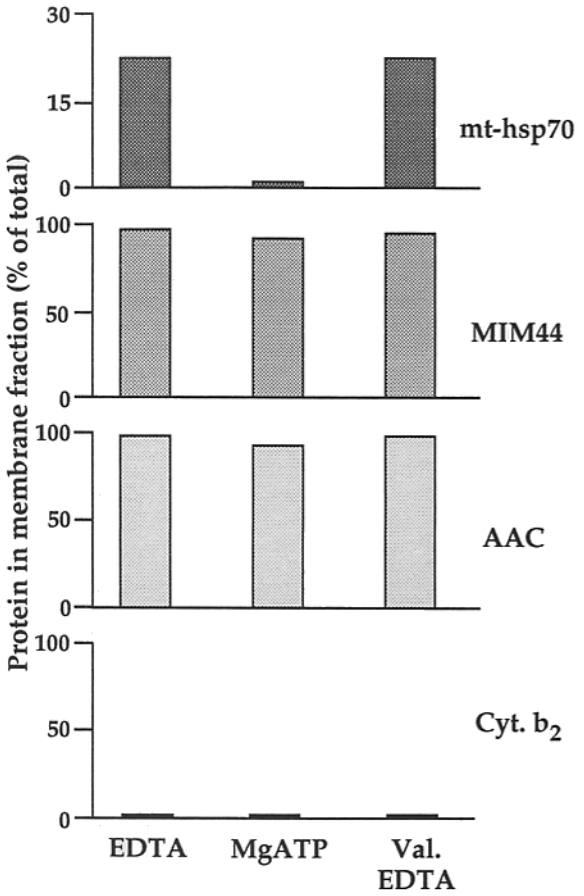

Figure 2. Partitioning of mitochondrial hsp70 between the inner membrane and the mitochondrial matrix. Mitochondria were sonicated in the presence of 10 mM MOPS pH 7.2, 100 mM KCl, and 1 mM EDTA or 4 mM MgATP and separated into membrane pellet and supernatant by centrifugation. Mitochondria of one sample were uncoupled by addition of 1 μM valinomycin (*Val.*) before sonication. Proteins were analyzed by SDS-PAGE, Western-blotting, and 2D-densitometry. *AAC*, ADP/ATP carrier (inner membrane).

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 *1*). 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 ATPγS 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*).

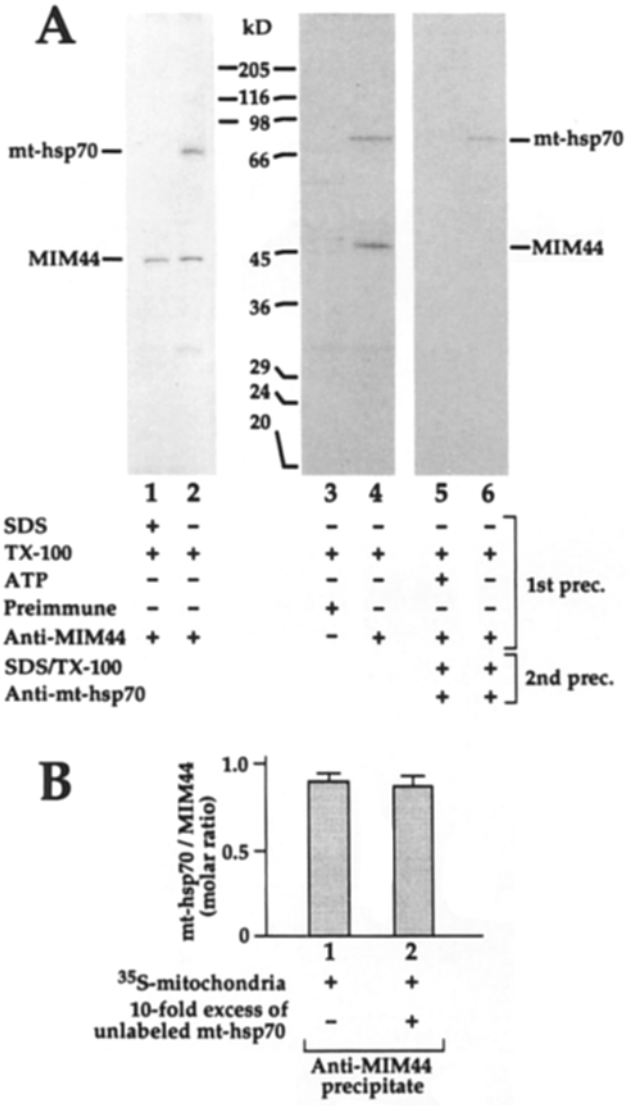

Figure 3. Identification of a complex between MIM44 and mitochondrial hsp70. (*A*) ^{35}S-labeled mitochondria were lysed in 1% Triton X-100, 300 mM KCl, 10 mM Tris-HCl, pH 7.2, and tested for coimmunoprecipitation of associated proteins by antibodies directed against MIM44 or antibodies from preimmune 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 (Ssc1p). 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.

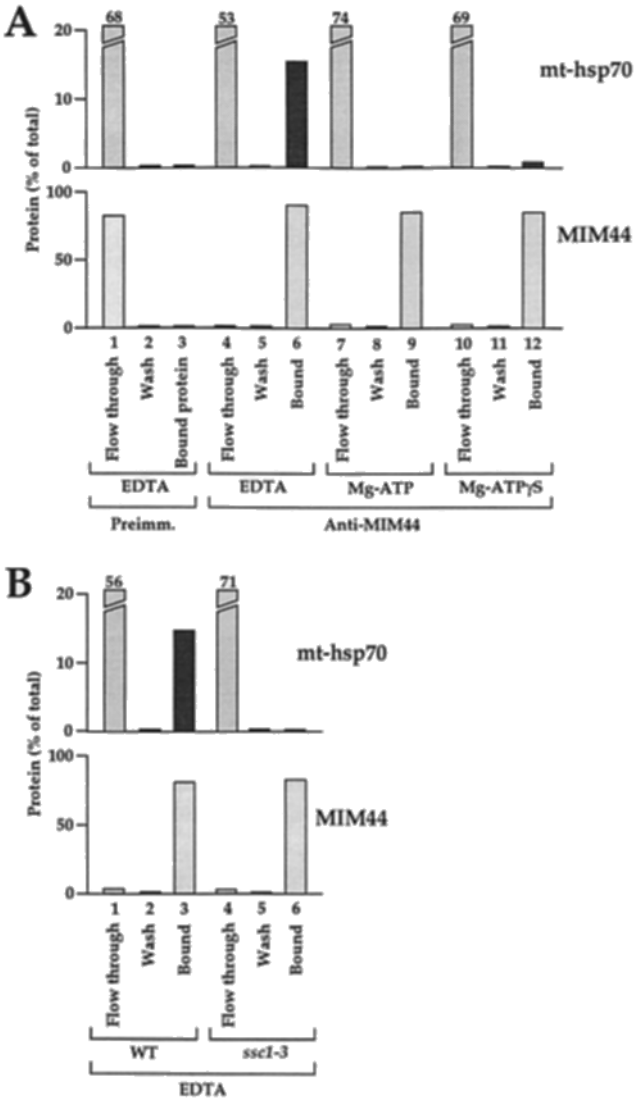

Figure 4. Binding of mt-hsp70 to MIM44 is nucleotide-sensitive. (*A*) Mt-hsp70 stays bound to MIM44 in the presence of EDTA and is released in the presence of Mg-ATP. Antibodies of preimmune serum (columns *1–3*) and antibodies directed against MIM44 (columns *4–12*) were covalently coupled to protein 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 (Ssc1-3p) does not bind to MIM44. Mitochondria of wild-type PK82 (columns *1–3*) and of the mutant *ssc1-3* (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, *ssc1-3*. As previously described, the mutant phenotype can be induced in vitro by preincubating isolated *ssc1-3* 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 *sscl-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 *sscl-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 F_o-ATPase subunit 9 and dihydrofolate reductase (Su9-DHFR) that is processed twice by the processing peptidase of the mitochondrial matrix. *sscl-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 *sscl-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.

^{35}S-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 *sscl-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 *sscl-3* mitochondria (Fig. 5 *B*, lane *4*). In wild-type mitochondria, the cross-link product of 100 kD is of a size consistent with Su9-DHFR and a

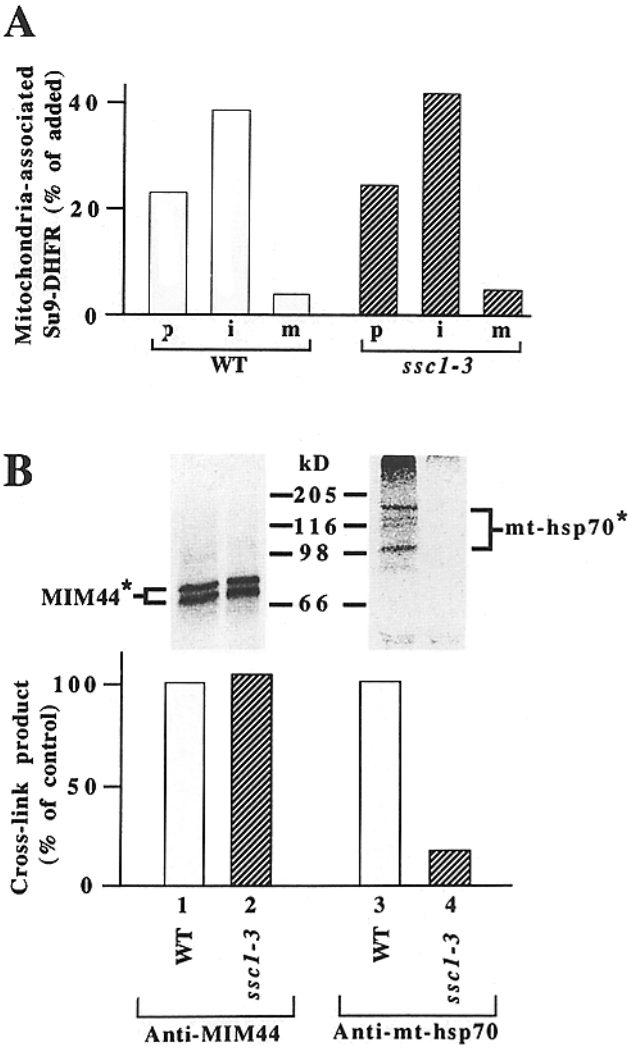

Figure 5. Cross-linking of MIM44 and hsp70 to a preprotein arrested in translocation across the mitochondrial membranes. (*A*) Su9-DHFR accumulates in translocation sites of wild-type (PK82) and *sscl-3* (PK83) mitochondria with similar efficiency. The preprotein Su9-DHFR was synthesized in rabbit reticulocyte lysate in the presence of [^{35}S]methionine and imported into ATP-depleted mitochondria of the mt-hsp70 mutant *sscl-3* and of the corresponding wild-type (*WT*). *p*, precursor protein; *i*, processing intermediate (cleavage of the first part of the presequence); *m*, mature protein. (*B*) Cross-linking of Su9-DHFR. The translocation intermediates of Su9-DHFR were cross-linked by disuccinimidyl suberate (DSS). The products of the reaction were analyzed by immunoprecipitations with antibodies against MIM44 (lanes *1* and *2*) or mt-hsp70 (lanes *3* and *4*), respectively. *MIM44**, *mt-hsp70**, cross-linking products between Su9-DHFR and MIM44 or mt-hsp70, respectively.

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 *sscl-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 *sscl-3* mitochondria, indicating that ATP and a functional ATPase domain of mt-

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 *SSC1*, 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-fluoroorotic 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 SSC1 and ssc1 Mutants by MIM44

Strain containing test-plasmid	Additional plasmid				
	None	YCplac111::*MIM44*	YEplac181::*MIM44*	YCplac111::*SSC1*	YEplac181::*SSC1*
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.

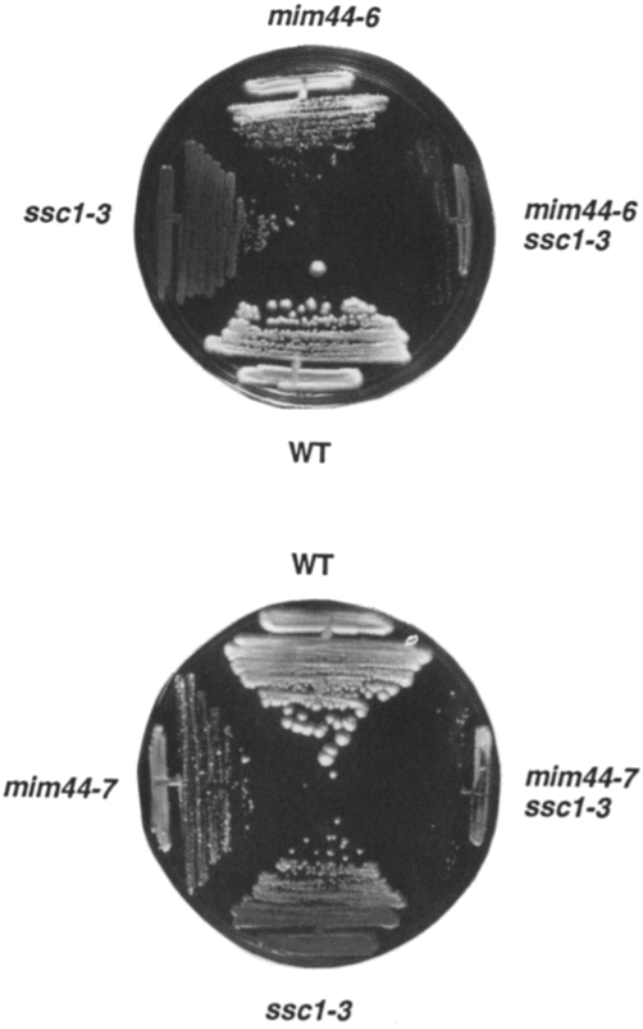

Figure 6. Synthetic growth defects of *mim44* and *sscl* temperature-sensitive mutations. Strains containing temperature-sensitive mutations in *MIM44* and *SSCl* were streaked on plates containing rich media and incubated at 30°C for 3 d. WT (LK201), *mim44-6* (LK208), *mim44-7* (LK209), *sscl-3* (LK221), *mim44-6 sscl-3* (LK215), and *mim44-7 sscl-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 ^{35}S-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 $\Delta\psi$-mediated import of the presequence, hsp70 could trap the preprotein by immediate binding and initiate the $\Delta\psi$-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., 1994*a*). 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., 1994*b*; 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 lumenal 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.

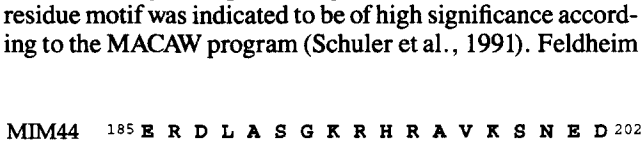

Figure 7. A short motif of MIM44 with sequence similarity to Sec63p and DnaJ in a triple alignment. Vertical lines indicate identical residues and double dots indicate isofunctional residues in all three proteins. Sequences were adopted from Ohki et al. (1986) (*E. coli* DnaJ), Sadler et al. (1989) (*S. cerevisiae* Sec63p), and Maarse et al. (1992) (*S. cerevisiae* MIM44).

We wish to thank Drs. L. Grivell (Amsterdam), P. Keil, and W. Voos (Freiburg) for helpful discussions and technical advice and Dr. B. Guiard (Gif-sur-Yvette) for providing an antiserum against yeast cytochrome b_2.

The work was supported by the Deutsche Forschungsgemeinschaft, the Fonds der Chemischen Industrie and Public Health Services grant R01 GM27870.

Received for publication 25 July 1994 and in revised form 5 September 1994.

References

Blom, J., M. Kübrich, J. Rassow, W. Voos, P. J. T. Dekker, A. C. Maarse, M. Meijer, and N. Pfanner. 1993. The essential yeast protein MIM44 (encoded by MPI1) is involved in an early step of preprotein translocation across the mitochondrial inner membrane. *Mol. Cell. Biol.* 13:7364–7371.

Boeke, J. D., J. Trueheart, G. Natsoulis, and G. R. Fink. 1987. 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. *Methods Enzymol.* 154:164–175.

Brodsky, J. L., and R. Schekman. 1993. A Sec63p-BiP complex from yeast is required for protein translocation in a reconstituted proteoliposome. *J. Cell Biol.* 123:1355–1363.

Campbell, J., and J. H. Duffus. 1988. Yeast, a practical approach. IRL Press Limited, Oxford. 289 pp.

Chirico, W. J., M. G. Waters, and G. Blobel. 1988. 70K heat shock related proteins stimulate protein translocation into microsomes. *Nature (Lond.).* 332:805–810.

Cyr, D. M., X. Lu, and M. Douglas. 1992. Regulation of hsp70 function by a eukaryotic DnaJ homolog. *J. Biol. Chem.* 267:20927–20931.

Cyr, D. M., T. Langer, and M. G. Douglas. 1994. DnaJ-like proteins: molecular chaperones and specific regulators of hsp70. *Trends Biochem. Sci.* 19: 176–181.

Daum, G., P. C. Böhni, and G. Schatz. 1982. Import of proteins into mitochondria: cytochrome b_2 and cytochrome c peroxidase are located in the intermembrane space of yeast mitochondria. *J. Biol. Chem.* 257:13028–13033.

Dekker, P. J. T., P. Keil, J. Rassow, A. C. Maarse, and M. Meijer. 1993. Identification of MIM23, a putative component of the protein import machinery of the mitochondrial inner membrane. *FEBS (Fed. Eur. Biochem.*

Soc.) Lett. 330:66–70.

Deshaies, R. J., B. D. Koch, M. Werner-Washburne, E. A. Craig, and R. Schekman. 1988. A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. *Nature (Lond.).* 332: 800–805.

Emtage, J. L. T., and R. E. Jensen. 1993. MAS6 encodes an essential inner membrane component of the yeast mitochondrial protein import pathway. *J. Cell Biol.* 122:1003–1012.

Feldheim, D., J. Rothblatt, and R. Schekman. 1992. Topology and functional domains of Sec63p, an ER membrane protein required for secretory protein translocation. *Mol. Cell. Biol.* 12:3288–3296.

Gambill, D., W. Voos, P. J. Kang, B. Miao, T. Langer, E. A. Craig, and N. Pfanner. 1993. A dual role for mitochondrial heat shock protein 70 in membrane translocation of preproteins. *J. Cell Biol.* 123:109–117.

Gietz, R. D., and A. Sugino. 1988. New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast lacking six-base pair restriction sites. *Gene (Amst.).* 74:527–534.

Hachiya N., R. Alam, Y. Sakasegawa, M. Sakaguchi, K. Mihara, and T. Omura. 1993. A mitochondrial import factor purified from rat liver cytosol is an ATP-dependent conformational modulator for precursor proteins. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:1579–1586.

Harlow, E., and D. Lane. 1988. Antibodies-A laboratory manual. Cold Spring Harbor Laboratory, New York. 511–553.

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.

Hartl, F.-U., R. Hlodan, and T. Langer. 1994. Molecular chaperones in protein folding: the art of avoiding sticky situations. *Trends Biochem. Sci.* 19:20–25.

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 (Eur. Mol. Biol. Organ.) J.* 12:3035–3041.

Huffaker, T. C., M. A. Hoyt, and D. Botstein. 1987. Genetic analysis of the yeast cytoskeleton. *Annu. Rev. Genet.* 21:259–284.

Kaiser, C., and R. Schekman. 1990. Distinct sets of SEC genes govern transport vesicle formation and fusion early in the secretory pathway. *Cell.* 61:723–733.

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(Lond.).* 348:137–142.

Kassenbrock, C. K., W. Cao, and M. G. Douglas. 1993. Genetic and biochemical characterization of ISP6, a small mitochondrial outer membrane protein associated with the protein translocation complex. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:3023–3034.

Kübrich, M., P. Keil, J. Rassow, P. J. T. Dekker, J. Blom, M. Meijer, and N. Pfanner. 1994. The polytopic mitochondrial inner membrane proteins MIM17 and MIM23 operate at the same preprotein import site. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 349:222–228.

Landry, S. J., R. Jordan, R. McMacken, and L. M. Gierasch. 1992. Different conformations for the same polypeptide bound to chaperones DnaK and GroEL. *Nature (Lond.).* 355:455–457.

Langer, T., C. Lu, H. Echols, J. Flanagan, M. K. Hayer, and F.-U. Hartl. 1992. Successive action of DnaK, DnaJ and GroEL along the pathway of chaperone-mediated protein folding. *Nature (Lond.).* 356:683–689.

Liberek, K., J. Marszalek, D. Ang, and C. Georgopoulos. 1991. Escherichia coli DnaJ and GrpE heat shock proteins jointly stimulate ATPase activity of DnaK. *Proc. Natl. Acad. Sci. USA.* 88:2847–2878.

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 (Eur. Mol. Biol. Organ.) J.* 11:3619–3628.

Maarse, A. C., J. Blom, P. Keil, N. Pfanner, and M. Meijer. 1994. Identification of the essential yeast protein MIM17, an integral mitochondrial inner membrane protein involved in protein import. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 349:215–221.

Manning-Krieg, U. C., P. E. Scherer, and G. Schatz. 1991. Sequencial action of mitochondrial chaperones in protein import into the matrix. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:3273–3280.

Murakami, K., and M. Mori. 1990. Purified presequence binding factor (PBF) forms an import-competent complex with a purified mitochondrial precursor protein. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:3201–3208.

Murakami, H., D. Pain, and G. Blobel. 1988. 70 kD heat shock-related protein is one of at least two cytosolic factors stimulating protein import into mitochondria. *J. Cell Biol.* 107:2051–2057.

Ohki, M., F. Tamura, S. Nishimura, and H. Uchida. 1986. Nucleotide sequence of the *Escherichia coli dnaJ* gene and purification of the gene product. *J. Biol. Chem.* 261:1778–1781.

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 (Lond.).* 341:125–130.

Pfanner, N., M. Tropschug, and W. Neupert. 1987. Mitochondrial protein import: nucleotide triphosphates are involved in conferring import-competence to precursors. *Cell.* 49:815–823.

Ramage, L., T. Junne, K. Hahne, T. Lithgow, and G. Schatz. 1993. Functional cooperation of mitochondrial protein import receptors in yeast. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:4115–4123.

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 (Fed. Eur. Biochem. Soc.) Lett.* 293:85–88.

Rassow, J., F.-U. Hartl, B. Guiard, N. Pfanner, and W. Neupert. 1990. Polypeptides traverse the mitochondrial envelope in an extended state. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 275:190–194.

Rowley, N., C. Prip-Buus, B. Westermann, C. Brown, E. Schwarz, B. Barrell, and W. Neupert. 1994. Mdj1p, a novel chaperone of the DnaJ family, is involved in mitochondrial biogenesis and protein folding. *Cell.* 77:249–259.

Ryan, K. R., M. M. Menold, S. Garrett, and R. E. Jensen. 1994. *SMS1*, a high-copy suppressor of the yeast *mas6* mutant, encodes an essential inner membrane protein required for mitochondrial protein import. *Mol. Biol. Cell.* 5:529–538.

Sadler, I., A. Chiang, T. Kurihara, J. Rothblatt, J. Way, and P. Silver. 1989. A yeast gene important for protein assembly into the endoplasmic reticulum and the nucleus has homology to DnaJ, an Escherichia coli heat shock protein. *J. Cell Biol.* 109:2665–2675.

Sanders, S. L., K. M. Whitfield, J. P. Voger, M. D. Rose, and R. Schekman. 1992. Sec61p and BiP directly facilitate polypeptide translocation into the endoplasmic reticulum. *Cell.* 69:353–365.

Scherer, P. E., U. C. Krieg, S. T. Hwang, D. Vestweber, and G. Schatz. 1990. A precursor protein partly translocated into yeast mitochondria is bound to a 70 kD mitochondrial stress protein. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:4315–4322.

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.* 89:11931–11934.

Schuler, G. D., S. F. Altschul, and D. J. Lipman. 1991. A workbench for multiple alignment construction and analysis. *Proteins Struct. Funct. Genet.* 9:180–190.

Scidmore, M. A., H. H. Okamura, and M. D. Rose. 1993. Genetic interactions between *KAR2* and *SEC63*, encoding eukaryotic homologues of DnaK and DnaJ in the endoplasmic reticulum. *Mol. Biol. Cell.* 4:1145–1159.

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 (Lond.).* 355:84–87.

Stuart, R. A., D. M. Cyr, E. A. Craig, and W. Neupert. 1994*a*. Mitochondrial molecular chaperones: their role in protein translocation. *Trends Biochem. Sci.* 19:87–92.

Stuart, R. A., A. Gruhler, I. van der Klei, B. Guiard, H. Koll, and W. Neupert. 1994*b*. The requirement of matrix ATP for the import of precursor proteins into the mitochondrial matrix and intermembrane space. *Eur. J. Biochem.* 220:9–18.

Voos, W., D. Gambill, B. Guiard, N. Pfanner, and E. A. Craig. 1993. Presequence and mature part of preproteins strongly influence the dependence of mitochondrial protein import in heat shock protein 70 in the matrix. *J. Cell Biol.* 123:119–126.

Wachter, C., G. Schatz, and B. S. Glick. 1994. Protein import into mitochondria: the requirement for external ATP is precursor-specific whereas intramitochondrial ATP is universally needed for translocation into the matrix. *Mol. Biol. Cell.* 5:465–474.

Wickner, S., J. Hoskins, and K. McKenny. 1991. Function of DnaJ and DnaK as chaperones in origin-specific DNA binding by Rep A. *Nature (Lond.).* 350:165–167.

Wickner, S., D. Skowyra, J. Hoskins, and K. McKenny. 1992. DnaJ, DnaK, and GrpE heat shock proteins are required in oriP1 DNA replication solely at the RepA monomerization step. *Proc. Natl. Acad. Sci. USA.* 89:10345–10349.