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The polytopic mitochondrial inner membrane proteins MIM17 and MIM23 operate at the same preprotein import site

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Abstract

Three proteins of the mitochondrial inner membrane are known that are essential for the viability of yeast and seem to be involved in import of preproteins; the integral membrane proteins MIM17 and MIM23 and the peripheral membrane protein MIM44, MIM17 and MIM23 are homologous to each other in their hydrophobic domain, expose their termini to the intermembrane space, and span the inner membrane up to four times, each. A preprotein in transit across the mitochondrial membrane is specifically cross-linked to MIM17, MIM23, MIM44, and matrix hsp70. We conclude that MIM17 and MIM23 are integral parts of a preprotein translocation channel and cooperate with MIM44 and hsp70 at the same protein import site.

Key words: Mitochondrion; MIM17; MIM23; MIM44; hsp70; Protein translocation

1. Introduction

The majority of mitochondrial preproteins must be transported into or across the mitochondrial inner membrane to reach their functional destination [1–3]. Genetic screens for *Saccharomyces cerevisiae* mutants defective in mitochondrial protein import led to the identification of three essential inner membrane proteins, MIM44 (ISP45) [4], MIM23 (Mas6p) [5,6], and MIM17 [7]. MIM44 is a peripheral membrane protein [8], while MIM23 and MIM17 are integral proteins of the mitochondrial inner membrane [5–7]. By cross-linking to preproteins and use of inhibitory antibodies, evidence was provided that MIM44 and MIM23 are directly involved in the import of preproteins [6,8,9–11]. It was not known whether MIM44 and MIM23 cooperate in import of preproteins and if MIM17 is also directly involved in protein translocation.

For this report we characterized the membrane topology of MIM17 and MIM23 and found that both proteins, which are homologous in their hydrophobic domain, span the inner membrane up to four times. By specific cross-linking of a preprotein in transit, we provide evidence that MIM17 is in close proximity to the import site and that MIM17, MIM23, MIM44, and the matrix heat shock protein, hsp70, operate in the same import pathway of the inner membrane.

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Abbreviations: MIM17, MIM23, MIM44, mitochondrial inner membrane proteins of 17, 23 and 44 kDa, respectively; mt-hsp70, heat shock protein of 70 kDa in the mitochondrial matrix; *S. cerevisiae*, *Saccharomyces cerevisiae*.

2. Materials and methods

2.1. Yeast strains and DNA manipulations

The *S. cerevisiae* strains used are described in Table 1. Diploid MB2-15, harbouring one disrupted *MIM23* allele, was obtained by transforming MB2 with a 6 kb *Bgl*I fragment from YCplac111-*mim23::LYS2* and selection by Southern blot analysis of a Lys$^+$ transformant carrying the gene disruption. Similarly, haploid MB17 was obtained by sporulation and tetrad analysis of the heterozygous *MIM23* diploid MB2-15, which was transformed with plasmid YCplac111-*MIC23-c-myc*.

YCplac111-*MIM23::LYS2* was constructed by replacing the internal 514 bp *Bsu*36I–*Nco*I MIM23 fragment from YCplac111-*MIM23*(Sph) [5] with a blunt-ended *Hin*dIII *LYS2* fragment from pDP6.

The *MIM23-c-myc* was constructed by introduction of a *Bam*HI site at the 3′-terminus of *MIM23*. The entire coding sequence and 5′-flanking sequences of *MIM23* were amplified by the polymerase chain reaction (PCR) using plasmid YCplac111-*MIM23*(Sph) as template for the oligonucleotide 5′-GGGGATCCTTTTCAAGTAGTCTTTTC-3′ and the reverse M13 sequencing primer (Biolabs). After digestion with *Hin*dIII and *Bam*HI the amplified fragment was cloned into *Hin*dIII- and *Bam*HI-digested YCpmyc 111, creating YCplac111-*MIM23-c-myc*. DNA manipulations were done according to standard procedures (summarized in [7]).

2.2. Antibody production

Plasmid pQE13-*MIM23* were constructed by cloning the 210 bp *Nsi*I–*Pst*I fragment, encoding half of the hydrophilic amino-terminal domain and the first hydrophobic segment of MIM23, into the bacterial expression vector pQE 13 (Qiagen). The histidine-tagged MIM23 fusion protein was produced in *E. coli*, purified by Ni-nitrilotriacetic acid (NTA) affinity chromatography [12] and used for generation of antibodies (anti-MIM23) in rabbits. Antiserum was affinity-purified by MIM23 fusion protein coupled to CNBr-activated Sepharose (Pharmacia) and bound antibodies were eluted with 0.1 M glycine, pH 2.5.

A synthetic peptide corresponding to the 14 amino-terminal amino acid residues of MIM23 was coupled to keyhole limpet hemocyanin [13] and used for generation of antibodies in rabbits (anti-MIM23N).

2.3. Isolation of mitochondria, in vitro import of preproteins and cross-linking

Standard procedures were used for growth of *S. cerevisiae*; isolation of mitochondria; formation of mitoplasts; treatment of mitochondria at alkaline pH (pH 11.5); treatment with protease; synthesis of prepro-

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teins in rabbit reticulocyte lysate in the presence of [^{35}S]methionine; import of preproteins into isolated mitochondria; dissipation of the membrane potential by addition of valinomycin, antimycin A, and oligomycin (summarized in [7]); analysis by SDS-PAGE, Western blotting, autoradiography, fluorography, scanning densitometry [7], and storage phosphor imaging system (Molecular Dynamics).

To accumulate the membrane-spanning intermediate of Su9-DHFR, mitochondria were depleted of ATP by preincubation with apyrase (1 U/ml); import was performed for 30 min at 25°C in the presence of potassium ascorbate/TMPD, but in the absence of ATP [8]. For cross-linking, the mitochondria were re-isolated through a 500 mM sucrose cushion, washed in SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS, pH 7.2), resuspended in SEM, and incubated with 270 μM DSS for 20 min at 0°C. After the addition of 100 mM Tris, pH 7.2, a precipitation with trichloroacetic acid was performed [8,14]. 5–10% of a sample was directly analyzed by SDS-PAGE, while the remaining 90–95% were dissolved in SDS-containing buffer, diluted 20- to 40-fold in buffer containing 1% (w/v) Triton X-100 and subjected to immunoprecipitation [15] using protein A–Sepharose or protein G–Sepharose.

3. Results and discussion

A comparison of the deduced primary sequences of MIM17 [7] and MIM23 [5,6] reveals that a large region of MIM17 shows significant similarity to MIM23. The program BESTFIT (algorithm of Smith and Waterman [16]) shows that amino acid residues 2–132 of MIM17 and residues 80–219 of MIM23 have a similarity of 46%, including 24.4% identical residues (Fig. 1A). These regions have very similar hydrophobicity/hydrophilicity profiles and are predicted to contain four hydrophobic segments, each [17] (Fig. 1A). The central stretches of about 80 residues of the homologous regions, which include the hydrophobic segments II and III, show a higher similarity than the flanking regions. The hydrophobicity scores [18] of the hydrophobic segments in MIM17 and MIM23 indicate that they are probably not acting as independent membrane anchor sequences (with the exception of segment IV of MIM17), however, several membrane-spanning segments may cooperatively associate within the membrane [19,20]. This is not unusual for polytopic membrane proteins; examples are the mitochondrial inner membrane carrier proteins, such as the ADP/ATP carrier and the phosphate carrier [21]. The inclusion of polar residues in cooperating membrane-spanning sequences provides an explanation for why the ends of some homologous segments of MIM17 and MIM23, as predicted from the hydrophobicity values, do not exactly match, since polar residues located at the end of a hydrophobic segment are often excluded in the calculations. It is thus conceivable that, for example, segment III of MIM23 extends further to the carboxy-terminus, as is the case with segment III of MIM17.

MIM23 is 64 amino acid residues larger than MIM17. It contains an amino-terminal hydrophilic domain not present in MIM17, while MIM17 contains only a short hydrophilic segment at the carboxy-terminus that is not present in MIM23 (Fig. 1A). The precursors of MIM17 [7] and MIM23 [5] were imported into mitochondria. As expected, the imported proteins were not digested by treatment of mitochondria with protease (Fig. 1B, lanes 1). Upon opening of the intermembrane space (to form mitoplasts), about 7 kDa were cleaved off from MIM23 by treatment with trypsin, whereas MIM17 remained intact (Fig. 1B, lanes 2). The remaining fragment MIM23′ was slightly smaller than MIM17. Only after lysis of the inner membrane were MIM17 and the fragment MIM23′ digested (Fig. 1B, lanes 3). The primary sequence comparison predicted that the amino-terminal domain of MIM23 would be cleaved off from the intermembrane space side. To test whether the cleaved domain of MIM23 is located at the amino-terminus, we raised an antiserum against a synthetic peptide corresponding to the amino-terminal 14 amino acid residues of MIM23. On immunoblots, the anti-MIM23N serum reacted with MIM23 from mitochondria and mitoplasts (Fig. 1B, lanes 8–10), but did not recognize the fragment MIM23′ generated by trypsin treatment of mitoplasts (Fig. 1B, lane 11), indicating that the amino-terminal region of MIM23 was removed by trypsin. Immunoglobulins G prepared from the anti-MIM23N serum reduced the efficiency of preprotein import across the inner membrane of mitoplasts (Fig. 1D, column 6) (the import was not completely blocked; in agreement, deletion of the amino- terminal region of MIM23 was found to reduce, but not to abolish, MIM23 function; (P.D., unpublished). Controls show that preimmune antibodies did not inhibit protein import into mitoplasts, and that the anti- MIM23N antibodies did not inhibit protein import into intact mitochondria (Fig. 1D). We conclude that the amino-terminus of MIM23 is located on the intermembrane space side of the inner membrane.

The fragment MIM23′ was resistant to extraction at alkaline pH (Fig. 1C, column 4). That is, the fragment MIM23′ behaved as an integral membrane protein, like

Table 1
S. cerevisiae strains used in this study

Strain	Genotype	Source
MB2	*MATa/α ade2-101/ADE2 his3/his3-Δ200 leu2/leu2-Δ1 lys2-801/lys2-801 trp1-289/TRP1 ura3-52/ura3-52* rho$^+$	[4]
MB2-15	*MATa/α ade2-101/ADE2 his3/his3-Δ200 leu2/leu2-Δ1 lys2-801/lys2-801 trp1-289/TRP1 ura3-52/ura3-52 mim23::LYS2/MIM23* rho$^+$	This study
MB16	*ade2-101 his3 leu2 lys2-801 trp1-289 ura3-52 mim17::LYS2* + YCplac111-*MIM17-c-myc (LEU2)* rho$^+$	[7]
MB17	*ade2-101 his3 leu2 lys2-801 trp1-289 ura3-52 mim23::LYS2* + YCplac111-*MIM23-c-myc (LEU2)* rho$^+$	This study

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MIM23   1              MSWLFGDKTPTDDANAAVGGQDTTKPKELSLKQSLGFEPNINNIISGPGGMHVDTARLHPLAGLDK 66

MIM23  67 GVEYLDLEEEQLSSLEGSQGLIPSRGWTDDLCYGTGAVYLLGLGIGGFSGMMQGLQNIPPNSPGKLQLNTVLNHITKR 144
                                                  I
MIM17   1              MSADHSRDPCPIVILND:::::FGGAFAMGAIGGVVWHGIKGFRNSPLGERG....SGAMSAIKAR 57

MIM23 145 GPFLGNNAGILALSYNIINSTIDALRGKHDTAGSIGAGALTGALFKSSKGLKPMGYSSAMVAAACAVWCSVKKRLLEK 222
MIM17  58 APVLGGNFGVWGGLFSTFDCAVKAVRKREDPWNAIIAGFFTGGALAVRGGWRHTRNSSITCACLLGVIEGVGLMFQRY 135
                II                    III                  IV

MIM17 136 AAWQAKPMAPPLPEAPSSQPLQA                                                    158
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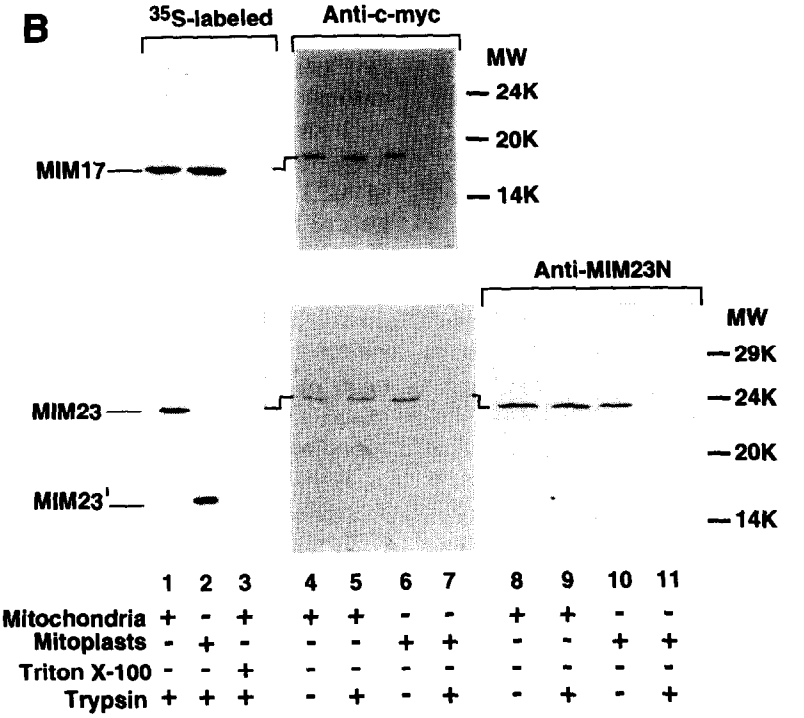

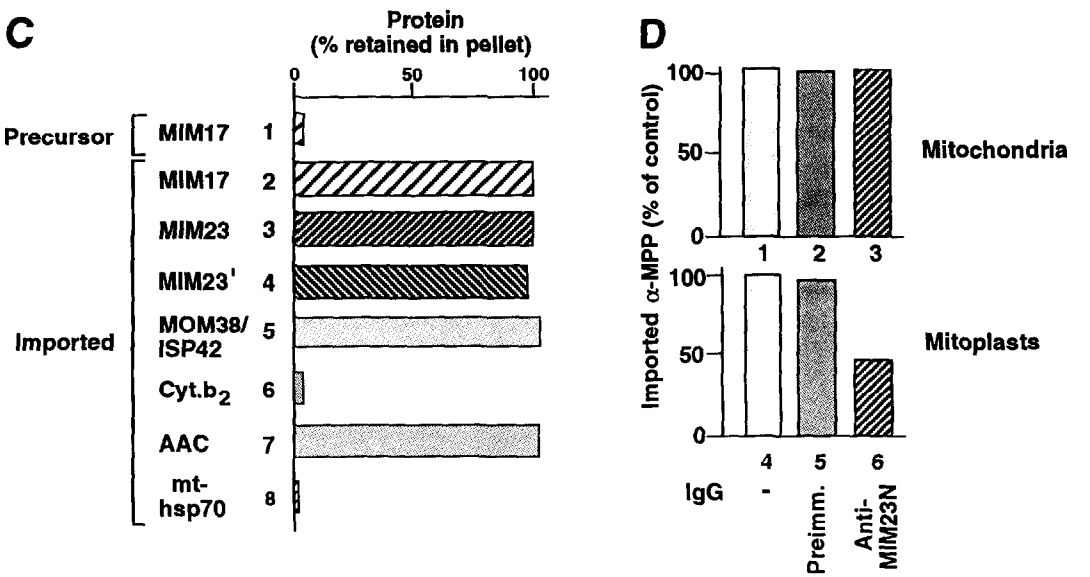

the full-length MIM23 and MIM17 (Fig. 1C, columns 2 and 3). Controls show that the integral membrane proteins MOM38 (ISP42) of the outer membrane and ADP/ATP carrier of the inner membrane are resistant to extraction at pH 11.5 (Fig. 1C, columns 5 and 7), while the soluble proteins cytochrome b_2 and mt-hsp70 are extracted (Fig. 1C, columns 6 and 8). The precursor proteins in the reticulocyte lysate are also soluble at alkaline pH [22], as shown here for MIM17 (Fig. 1C, column 1). We conclude that the 17 kDa fragment of MIM23, which is resistant to trypsin in mitoplasts, includes the hydrophobic domain.

The similarity of the hydrophobic domains of MIM17 and MIM23 suggests that both proteins possess a similar membrane topology with four hydrophobic segments each. This would imply that the carboxy-termini of the two MIM proteins face the intermembrane space. Since a direct determination of the location of the carboxy-termini was not possible due to the lack of appropriate proteolytic fragments, we employed epitope-tagged forms of the proteins. We used the yeast strain MB16 which expresses MIM17 with a c-Myc epitope at the carboxy-terminus [7] and constructed a disruption mutant of *MIM23* (previously termed *MPI3* [5]) rescued by the expression of a fusion protein between MIM23 and a c-Myc epitope at the carboxy-terminus (strain MB17). On immunoblots, anti-c-Myc antibodies recognized the epitope-tagged MIM17 and MIM23 from mitochondria and mitoplasts (Fig. 1B, lanes 4–6). Treatment of mitoplasts with protease removed the c-Myc epitope of both proteins (Fig. 1B, lanes 7), demonstrating that MIM17 and MIM23 expose their extreme carboxy-termini to the intermembrane space.

In summary, the amino-terminus of MIM23 and the carboxy-termini of MIM17 and MIM23 were found to be located on the intermembrane space side of the inner membrane. In view of the homology of the hydrophobic domains, it is very likely that each protein exposes both termini to the intermembrane space and consequently must span the inner membrane at least twice. The number of predicted hydrophobic segments suggests that each protein spans the membrane four times.

MIM17 and MIM23 cannot substitute for each other, since a deletion of either gene is lethal [6,7]. We tested if over-expression of one protein could rescue defects in the other protein. Over-expression of MIM17 from the multicopy vector YEplac181 [23] neither suppressed the import defect of *mim23* mutants [5] nor rescued the lethality of a *mim23* null mutant. Consistently, over-expression of MIM23 from YEplac181 did not suppress the transport defect of mutant MB3-33 and other *mim17* mutants [7] and could not rescue the lethality of a *mim17* null mutants. This indicates that both MIM17 and MIM23 are required for the function of the inner membrane import machinery.

Import of a fusion protein between the presequence of F_0-ATPase subunit 9 and the entire dihydrofolate reductase (Su9-DHFR) into the mitochondrial matrix requires ATP [24]. At low levels of ATP, Su9-DHFR is accumulated as a translocation intermediate spanning the mitochondrial inner membrane and was successfully used for cross-linking to MIM44/ISP45 [8,9] and MIM23/Mas6p [11]. We accumulated ^{35}S-labeled Su9-DHFR as a translocation intermediate at low ATP, re-isolated the mitochondria and added the amino-reactive homobifunctional cross-linking reagent disuccinimidyl suberate (DSS). After quenching of DSS, the mitochondrial proteins were analyzed by SDS-PAGE. Fig. 2A, lane 2, shows that several cross-linking products were generated. After denaturation of the mitochondria in SDS, mitochondrial proteins present in the cross-linking products were identified by immunoprecipitation. A product of about 45 kDa contained MIM17, in good agreement with the sum of the molecular masses of MIM17 and

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Fig. 1. Orientation of MIM17 and MIM23 in the mitochondrial inner membrane. (A) Comparison of the primary sequences of MIM17 and MIM23. Identical residues are indicated by a vertical line. Double dots and single dots indicate residues of high and low similarity, respectively (program BESTFIT, algorithm of Smith and Waterman [16]). Predicted hydrophobic segments (I, II, III and IV) are indicated by a horizontal line. (B) Accessibility to proteases after opening of the intermembrane space. Samples 1–3: ^{35}S-labeled MIM17 and MIM23 were imported into isolated wild-type yeast mitochondria. Samples 4–7: mitochondria were isolated from yeast strain MB16, containing MIM17 with a c-Myc epitope (upper panel), or from yeast strain MB17, containing MIM23 with a c-Myc epitope (lower panel) (the proteins carrying the c-Myc epitope have a slightly higher molecular weight). Samples 8–11: mitochondria were isolated from wild-type yeast. Where indicated, the mitochondria were swollen to form mitoplasts (the formation of mitoplasts was confirmed by the analysis of marker proteins as previously reported [32,33]) or lysed with 1% (w/v) Triton X-100. Then a treatment with trypsin (50 μg/ml) was performed as indicated. Analysis was by SDS-PAGE and fluorography (lanes 1–3) or immunodecoration with antibodies directed against c-Myc (lanes 4–7) or antibodies prepared against the amino-terminal 14 amino acid residues of MIM23 (anti-MIM23N) (lanes 8–11). MIM23′, fragment of MIM23 formed by trypsin activity in mitoplasts. (C) Treatment at alkaline pH. Reticulocyte lysate containing the precursor of MIM17 (sample 1) or mitochondria containing imported MIM17 or MIM23 (samples 2 and 3) or mitoplasts with MIM23′ (sample 4) were treated with sodium carbonate. Pellets and supernatants were separated. Shown is the fraction of the protein retained in the pellets. Marker proteins are MOM38/ISP42 (outer membrane), cytochrome b_2 (Cyt. b_2, intermembrane space), ADP/ATP carrier (AAC, inner membrane), and mitochondrial hsp70 (mt-hsp70, matrix). (D) Anti-MIM23N antibodies reduce protein import into mitoplasts. Isolated mitochondria (10 μg mitochondrial protein) or mitoplasts were preincubated with 100 μg immunoglobulins G (IgG) prepared from preimmune serum or anti-MIM23N serum [34]. The precursor of the α-subunit of the mitochondrial processing peptidase (α-MPP) was synthesized in rabbit reticulocyte lysate and imported into the energized mitochondria or mitoplasts for 5 min at 25°C. Then a treatment with proteinase K (50 μg/ml) was performed. Analysis was by SDS-PAGE, fluorography, and densitometry [35]. The amount of import into mitochondria or mitoplasts not preincubated with IgGs was set to 100%.

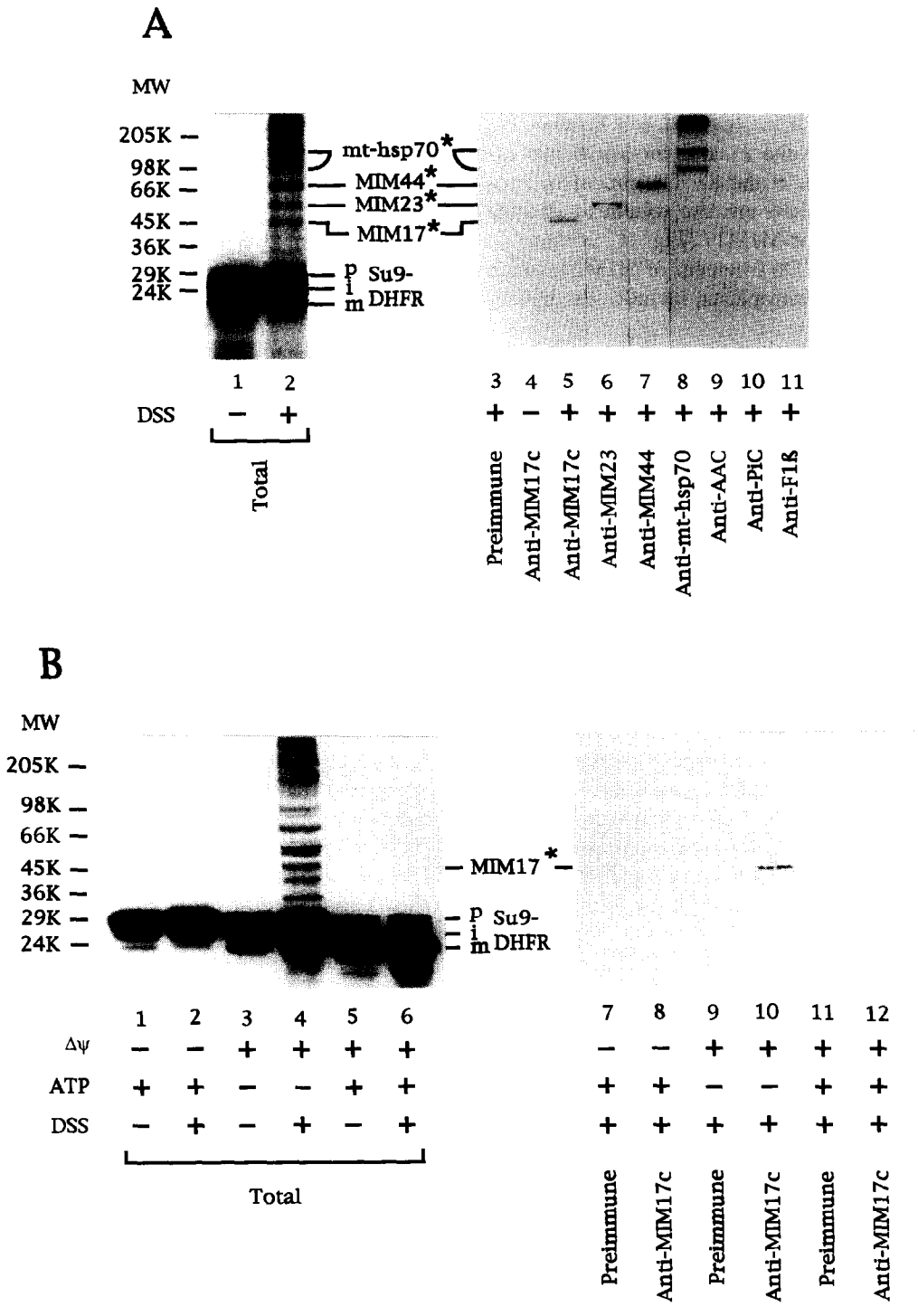

Fig. 2. Cross-linking of MIM17, MIM23, MIM44, and mitochondrial hsp70 to a preprotein spanning the mitochondrial membranes. (A) Cross-linking of a membrane-spanning intermediate of Su9-DHFR. The ^{35}S-labeled preprotein Su9-DHFR was accumulated as an intermediate in isolated mitochondria from strain MB16 (containing MIM17 with a c-Myc epitope) at low ATP as described in section 2 [8]. Where indicated, the re-isolated mitochondria were treated with the cross-linking reagent disuccinimidyl suberate (DSS). For lanes 1 and 2, the mitochondria were directly analyzed by SDS-PAGE. About 55% of mitochondria-associated Su9-DHFR was in the intermediate-sized (i) form. For lanes 3–11, the SDS-denatured mitochondrial proteins were subjected to immunoprecipitation with the indicated antibodies (anti-MIM17c, antibodies against c-Myc) as described in section 2. The cross-linking products between Su9-DHFR and MIM-proteins or hsp70 are indicated by a star. P_iC, phosphate carrier; p, i, m, precursor-, intermediate- and mature-sized forms of Su9-DHFR. (B) Su9-DHFR accumulated at the mitochondrial outer membrane and fully imported Su9-DHFR are not cross-linked to MIM17. The experiment was performed as described above except that in samples 1 and 2 the membrane potential ($\Delta\Psi$) was dissipated (and ATP was present) and that in samples 5 and 6 a membrane potential and ATP were present (full import; treatment with proteinase K after the import reaction). As previously described [8], Su9-DHFR accumulated as the precursor (p) form in lanes 1 and 2 (more than 90% of mitochondria-associated Su9-DHFR), and as the mature (m) form in lanes 5 and 6 (about 90% of mitochondria-associated Su9-DHFR).

intermediate-sized Su9-DHFR (about 24 kDa) (Fig. 2A, lane 5). As a control for the specificity of the cross-linking approach we show that cross-linking was not observed with the abundant inner membrane proteins ADP/ATP carrier, phosphate carrier, and $F_1\beta$ (Fig. 2A, lanes 9–11). Moreover, Su9-DHFR accumulated at the mitochondrial outer membrane in the absence of a membrane potential (Fig. 2B, lanes 1 and 2) and Su9-DHFR fully imported into the mitochondrial matrix (Fig. 2B, lanes 5 and 6) did not yield a cross-linking product with MIM17 (Fig. 2B, lanes 8 and 12). We conclude that a preprotein in transit across the mitochondrial inner membrane is in close contact with MIM17.

In further immunoprecipitations, the cross-linking products of 51 and 68 kDa were shown to contain MIM23 and MIM44, respectively (Fig. 2A, lanes 6 and 7). Cross-linking products of about 95 and 160 kDa were selectively recognized by antibodies directed against mitochondrial hsp70 (Fig. 2A, lane 8), indicating that a monomer and possibly a dimer of hsp70 were linked to the preprotein (in addition, high molecular weight cross-linking products were efficiently recognized by antibodies against mt-hsp70, but not by the other antibodies, in agreement with the view that oligomeric complexes containing mt-hsp70 interact with preproteins in transit [25–27]). Most of the major cross-linking products formed by DSS with the membrane-spanning intermediate of Su9-DHFR could thus be identified.

Previously reported cross-linkings of preproteins to MIM23/Mas6p and MIM44/ISP45 were done in individual studies, and the various conditions for accumulation of the preproteins and cross-linking were not directly comparable. Here we show in one experiment that MIM17, MIM23, and MIM44 were cross-linked to the same preprotein in transit, strongly suggesting that all three MIM proteins function at the same preprotein import site of the mitochondrial inner membrane. Other major cross-linking products contained mitochondrial hsp70, suggesting the view that hsp70, in cooperation with the MIM proteins, plays an important role in preprotein translocation across the inner membrane [28–30].

Our results suggest the following conclusions about the mitochondrial inner membrane import machinery. (i) MIM17 and MIM23 share a homologous hydrophobic domain, each including four hydrophobic segments. The termini of these MIM proteins are located on the intermembrane space side. Most hydrophobic segments probably do not act as independent membrane anchor sequences, but may associate in a cooperative manner [19,20]. The similarities between the second and third hydrophobic segments of MIM17 and MIM23 are higher than that between the first and fourth segments. Conversely, the predicted hydrophobicity is higher for the first and fourth segments than that for the middle segments of both proteins. We speculate that the less conserved and more hydrophobic segments are important for anchoring the proteins in the inner membrane, while the more conserved and less hydrophobic middle segments are mainly in the interior and may be important for a transport function. MIM23 contains an amino-terminal hydrophilic domain protruding into the intermembrane space. Deletion of the amino-terminal domain or first hydrophobic segment or deletion of the fourth hydrophobic segment of MIM23 impair the growth of yeast, but are not lethal, while deletions in the region of the middle segments are lethal [11] (P.D. and M.M., unpublished results). This suggests that a core domain including the less hydrophobic segments II and III plus one of the more hydrophobic segments I or IV is sufficient for the basic function of MIM23. (ii) Despite the similarity of their hydrophobic domains, MIM17 and MIM23 are not functionally interchangeable, but both are required for a functional inner membrane import site. (iii) Cross-linking shows that MIM17 is in close proximity to a preprotein in transit across the inner membrane. Other cross-linking products of the same preprotein included MIM23, MIM44, and mitochondrial hsp70. Thus the same preprotein is in close proximity to the three MIM proteins and mt-hsp70, suggesting that these proteins function at the same inner membrane import site. Interestingly, our mislocalization-based genetic selection for mitochondrial import mutants led to the identification of exactly those four components [4,5,7]. We propose that MIM17 and MIM23 are integral parts of a putative protein-conducting channel of the mitochondrial inner membrane [22,31], and that MIM44 and mt-hsp70 function as binding proteins required for driving the import of preproteins.

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