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Identification of the Protein Import Components of the Rat Mitochondrial Inner Membrane, rTIM17, rTIM23, and rTIM44[1]

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We cloned rat liver mitochondrial 18.1, 21.9, and 51.0 kDa proteins with a significant structural homology to the components of the translocase of the yeast mitochondrial inner membrane, Tim17, Tim23, and Tim44. The 18.1 and 21.9 kDa proteins were synthesized as mature forms having four potential transmembrane segments and localized to the mitochondrial inner membrane. The 51.0 kDa protein is a precursor having a presequence of ~6 kDa which is cleaved during import into the mitochondria. The mature 45 kDa protein is located in the matrix, both in a soluble form and in a membrane-bound, alkali-extractable form. Immunofluorescence microscopy confirmed the location of all three proteins in the mitochondria. Antibodies against the 21.9 kDa protein, but not those against the 18.1 and 51.0 kDa proteins, inhibited the precursor import into the mitoplasts *in vitro*. Immunoprecipitation indicated that all three proteins interacted with the protein in transit to the matrix. Immunoprecipitation also revealed that the 18.1 kDa protein formed a complex with the 21.9 kDa protein and the 45 kDa protein with mHsp70; the latter complex was dissociated in an ATP- or ADP-dependent manner and the reaction was impeded by AMP-PNP or inorganic phosphate. These assays thus demonstrated the 18.1, 21.9, and 45 kDa proteins to be the translocator components of the rat mitochondrial inner membrane and, therefore, the functional homologues of Tim17, Tim23, and Tim44, respectively.

Key words: inner membrane, mitochondria, precursor, Tim proteins, protein import.

Mitochondria must import almost all of the proteins necessary for their function from the cytosol and this import is mediated by two distinct import machineries, namely the translocase of the outer membrane (Tom system) and the translocase of the inner membrane (Tim system) (*1, 2*). In yeast and fungi, Tom translocase is composed of at least 9 proteins: Tom5, Tom6, Tom7, and Tom40 form an import channel, whereas Tom72, Tom70, Tom37, Tom22, and Tom20 function as the import receptors (*3*). Seven proteins have been identified as the members of the Tim system in yeast: Tim11, Tim14, Tim17, Tim22, Tim23, Tim33, and Tim44 (*4-7*). Tim17 and Tim23 share sequence similarity and form a subcomplex which is believed to constitute the translocation channel of the inner mitochondrial membrane (*8-10, 5*). Tim23 and Tim22 also share sequence similarity, but they have a distinct function in the import reaction; Tim23 functions as the receptor for the matrix-targeted precursors in a $\Delta\Psi$-dependent manner (*11*), whereas Tim22 functions as an import factor for the ATP/ADP carrier family proteins of the inner membrane (*6*). Tim44, a peripheral protein bound to the matrix side of the inner membrane, recruits mHsp70 to the *trans* site of the import channel and the Tim44-mHsp70 complex drives, in collaboration with GrpE, translocation of the precursor proteins in an ATP-dependent manner (*12-17*). Tim11, which has been shown to be identical with the e-subunit of F_1F_o-ATPase (*18*), may function as a component of the protein sorting system for the mitochondrial intermembrane space (*7*). The function of other Tim components, however, remains to be clarified.

Although the components of the Tom system have so far been mainly identified in *Saccharomyces cerevisiae* and *Neurospora crassa* and those of the Tim system in *S. cerevisiae*, relatively little is known about the import machinery of other organisms; so far, Tom20 for humans and rats (*19, 20*), and Tim17 for humans, *Drosophila melanogaster*, and *Caenorhabditis elegans* and Tim23 for *Arabidopsis* have been reported (*21*).

Here we report the identification of rat homologues of Tim17, Tim23, and Tim44 proteins (we hereafter refer to them as rTim17, rTim23, and rTim44, respectively). The primary sequences predicted from cDNAs for rTim17, rTim23, and rTim44 showed an overall identity of 48, 28, and 30% with the yeast counterparts. Hydropathy plots

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; COX IV, cytochrome *c* oxidase subunit IV; DHFR, dihydrofolate reductase; MAO, monoamine oxidase; MDH, malate dehydrogenase; MSF, mitochondrial import stimulation factor; MTX, methotrexate; pAd, preadrenodoxin; PCR, polymerase chain raction; RACE, rapid amplification of the cDNA ends.

predicted that rTim17 and rTim23 contain four hydrophobic stretches which potentially span the membrane four times. rTim44 has no predicted transmembrane segments and is synthesized as a precursor with a presequence, which is proteolytically cleaved during import. rTim17 and rTim23 are localized exclusively to the inner mitochondrial membrane, whereas rTim44 is localized in the matrix both as a soluble form and as a form bound to the inner membrane. We also showed that the rat Tim proteins form complexes with the import-arrested protein. Furthermore, co-immunoprecipitation revealed the presence of subcomplexes of rat Tim proteins in the mitochondria.

These results suggest that the rat mitochondrial proteins identified in the present study function as components of the protein import machinery of the inner membrane, like their yeast counterparts and, therefore, that the essential parts of the import machinery are conserved among organisms.

EXPERIMENTAL PROCEDURES

Materials—The antibodies against MSF(L) and P450 (M1) were as previously described (*22, 23*). The antibodies against bovine malate dehydrogenase (MDH) and rat monoamine oxidase (MAO) were obtained from A. Ito, Kyushu University. The antibodies against subunit IV of rat cytochrome *c* oxidase (COX IV) were raised in rabbits by injecting a synthetic polypeptide corresponding to the N-terminal portion of COX IV, NH_2-AHGSVVKSEDYAL PSYVDRC-COOH. Monoclonal antibody against human Grp75 (SPA-825) which recognizes rat mHsp70 was purchased from StressGen. The rat liver cDNA library in λgt10 was as described (*24*). The rat liver cDNA library for RACE-PCR was a gift from G. Warren, the Imperial Cancer Research Fund. The cDNA encoding pAd-DHFR in which the entire preadrenodoxin (pAd) molecule was fused at the C-terminus with dihydrofolate reductase (DHFR) at the DNA level was a generous gift from T. Komiya, Kyushu University. Yeast mitochondrial processing peptidase was a generous gift from A. Ito.

cDNA Cloning of Rat Tim Proteins—Rat Tim17: The following oligonucleotides were synthesized based on a cDNA sequence encoding the complete human Tim17 [THC146733 from the human cDNA database (HCD)]: h18K1, 5′-ATGGAGGAGTACGCGCGAGA-3′ (coding strand) and h18K4, 5′-CTACTGATATTGTCGATAGTCTCC-3′ (anticoding strand). A 516-bp fragment of rat Tim17 was amplified from rat liver poly(A)$^+$-RNA by RT-PCR using the above oligonucleotides as the primers, then subcloned into pUC119 (pUC-Tim17) and used for the preparation of recombinant rTim17. To obtain full-size rTim17 cDNA, both the 5′ and 3′ regions of rat cDNA (~700 and ~500 bp, respectively) were amplified by 5′ and 3′ RACE-PCR using a rat liver RACE cDNA library as the template and h18K4 and AP1, and h18K1 and AP1 as the primers, respectively (Marathon cDNA Amplification Kit, Clontech), and both cDNA fragments were cloned into pGEM-T (Promega).

Rat Tim23: A ~360 bp cDNA sequence encoding a part of mouse Tim23 was assembled from three mouse EST clones: dbEST Ids 524452, 687546, and 792946. Based on this sequence, the following oligonucleotides were synthesized, m23-1: 5′-CTAGCTTTCTTTACCATTGG-3′ (coding strand) and m23-2: 5′-AGTGCATAGAGACTGGTGAG-3′ (anticoding strand). A 360 bp cDNA fragment was amplified from the rat liver poly(A)$^+$-RNA by RT-PCR using m23-1 and m23-2 as the primers and then used as a probe to screen a λgt10 rat cDNA library for rat Tim23. The ~1,100 bp cDNA encoding the entire rTim23 was thus obtained.

Rat Tim44: A search of the mouse EST database revealed that three EST clones (EST Ids: 519324, 666290, and 793650) exhibited a significant homology to yeast Tim44. The clone 793650 coded for the N-terminal portion of Tim44, whereas the clones 519324 and 666290 partially overlapped to encode the C-terminal portion of Tim44. Based on this finding, the following oligonucleotides were synthesized, m44-1: 5′-AGGTGCCTAGGCAGCGGAAT-3′ (coding strand) and m44-2: 5′-TGTGCTGGAGGCTGAGATGT-3′ (anticoding strand). A 1,260 bp-fragment was amplified by RT-PCR from rat liver poly(A)$^+$-RNA using m44-1 and m44-2 as the primers and then was cloned into pGEM-T (pGEM-rTim44L). Since this fragment lacked the region coding for the N-terminal portion of Tim44, 5′ RACE was performed using 44GSP1 5′-TTCAACTCCTTCCTTGATCTGGC-3′ and AP-1 as the primers. The 5′ region of the 5′ RACE product was used as a probe to screen the rat liver cDNA library in λgt10 and afforded a 1.9 kbp cDNA encoding the entire rTim44.

Preparation of Antibodies against Rat Tim Proteins—Antibodies against rTim17: A 530 bp-cDNA fragment in pUC-Tim17 was cloned into pQE30 (Quiagen) to obtain pQE30-rTim17. XLI-Blue cells transformed with pQE30-rTim17 were grown in 1 mM IPTG for 2 h. rTim17, histidine-tagged at the N-terminus, was expressed as inclusion bodies, which were washed with Triton X-100 (*25*) and subjected to SDS-PAGE. A 19-kDa band was eluted from the gel and used for raising antibodies in rabbits. Monospecific IgGs were isolated from the sera using the antigen-conjugated Formylcellulofine beads (Seikagaku Kogyo).

Antibodies against rat Tim23: A 647-bp cDNA fragment was amplified by PCR using rTim23 cDNA as the template and the following oligonucleotides as the primers, Tim23-5′: 5′-ACGGGATCCATGGAGGGTGGCGGAGG-3′ (coding strand) and Tim23-3′: 5′-GTCGAATTCTCAGAGTGACTGTTGGAGC-3′ (anticoding strand), and then cloned into pET28a (pET28a-rTim23). BL21 (DE3) cells harboring pET28a-rTim23 were grown in the presence of 1 mM IPTG at 37°C for 70 min. The rTim23 (MW ~25kDa), histidine-tagged at the N-terminus and expressed as inclusion bodies, was eluted from SDS-PAGE gel and used for raising antibodies in rabbits.

Antibodies against rat Tim44: An 1,260 bp *Eco*RI-fragment coding for rTim44 lacking both the N-terminal 15 and the C-terminal 4 amino acid residues was excised from pGEM-rTim44L and cloned into pET28b to obtain pET28b-rTim44. BL21 (DE3) cells harboring pET28b-rTim44 were grown at 37°C in 1 mM IPTG for 2h. rTim44 (MW ~50 kDa), histidine-tagged at the N-terminus and expressed as inclusion bodies, was used to raise antibodies.

Subcellular and Submitochondrial Fractionations—Subfractionation of rat liver cells was performed as described (*20*). Submitochondrial fractionation was performed as follows. Mitochondria were diluted into 10 mM HEPES-KOH buffer (pH 7.4) ("hypotonic buffer") containing the

"protease inhibitor mix" (10 μg/ml each of leupeptin, antipain, chymostatin, and pepstatin) at a protein concentration of 1 mg/ml and incubated at 0°C for 30 min. The reaction mixture was centrifuged in a microfuge at 6,000 rpm for 5 min to isolate the mitoplasts. The mitoplasts were suspended in hypotonic buffer containing the protease inhibitor mix and the mixture was then sonicated with a Branson sonifier equipped with a microtip for 15 to 20 s at 0°C, followed by centrifugation at 10,000$\times g$ for 5 min to obtain the supernatant, which was centrifuged at 100,000$\times g$ for 20 min to separate the supernatant and membrane fractions. Submitochondrial fractionation by sucrose density gradient centrifugation was performed as follows. Mitochondria were suspended in hypotonic buffer containing 5 mM EDTA and the protease inhibitor mix and incubated at 0°C for 10 min. The mixture was briefly sonicated, layered over a linear gradient of sucrose from 0.6 to 1.6 M and centrifuged in a Hitachi RPS40T rotor at 100,000$\times g$ for 15 h at 4°C.

Protein Import into the Mitochondria and Mitoplasts—Rat liver mitochondria were prepared as described (*26*) except that Trasylol, DTT, and EDTA were omitted from buffer A ("homogenization buffer"). The mitoplasts were prepared as described above. If necessary, 1 mg/ml of mitochondria was pretreated with 10 μg/ml trypsin at 0°C for 30 min to inactivate the import activity of the mitochondria. After termination of the reaction by the addition of 250 μg/ml trypsin inhibitor and incubation at 0°C for 30 min, the mitochondria were subjected to hypotonic treatment as described. Protein import into the mitochondria or mitoplasts was performed as follows. The cDNA encoding pAd-DHFR was cloned into pSP65 and the protein (~43 kDa) was expressed *in vitro* using the transcription-translation system as described (*27*). Then 20 μg of mitochondria or mitoplasts was suspended in 50 μl of the homogenization buffer containing 1 mM ATP, 20 mM sodium succinate, 5 mM NADH, the protease inhibitor mix, 600 μg/ml cytochrome *c*, and 1 mg/ml fatty acid-free BSA ("import buffer"), and incubated with ^{35}S-labeled pAd-DHFR synthesized in reticulocyte lysate at 30°C for 15 to 30 min. After import, the mitochondria or mitoplasts were subjected to SDS-PAGE and the gels were analyzed with a Fuji Bioimage Analyzer (BAS2000).

Immunoprecipitation of the Import-Arrested Protein with the Antibodies against Rat Tim Proteins—^{35}S-Labeled pAd-DHFR (40 μl) synthesized in reticulocyte lysate was incubated at 0°C for 10 min in 1 ml of the import buffer containing 10 μM methotrexate and 1 mM NADPH and then 400 μg of mitoplasts was added. The mixture was incubated at 30°C for 15 min. The mitoplasts were isolated by centrifugation, washed once with the homogenization buffer and treated with 2 ml of 10 mM HEPES-KOH buffer (pH 7.4) containing 0.25% digitonin, 50 mM NaCl, and the protease inhibitor mix ("solubilization buffer") at 0°C for 30 min. The solubilized supernatant was divided into 4 aliquots. Each aliquot was pretreated with Protein A-Sepharose and the supernatant fractions were then mixed with the antibody-bound Protein A-Sepharose and incubated at 4°C for 90 min. As a negative control, reticulocyte lysate-synthesized pAd-DHFR was incubated with purified yeast mitochondrial processing peptidase at 30°C for 30 min, mixed with digitonin-solubilized mitoplasts and then subjected to immunoprecipitation as described above. The proteins adsorbed on Protein A-Sepharose were analyzed by SDS-PAGE and autoradiography.

Detection of the rTim44-mHsp70 and rTim17-rTim23 Complexes by Immunoprecipitation—One milligram of mitochondria was incubated at 30°C for 20 min either in 1 ml of the homogenization buffer containing 1 mM ATP and the protease inhibitor mix (for ATP-replenishment) or in 1 ml of the homogenization buffer containing 10 units of apyrase, 12.5 μg/ml oligomycin and the protease inhibitor mix (for ATP-depletion). The mitochondria were then treated with the solubilization buffer at 0°C for 30 min, followed by centrifugation at 15,000$\times g$ for 5 min. If necessary, the mitochondria were treated with the solubilization buffer in which 50 mM NaCl was replaced with 100 mM KCl and then subjected to immunoprecipitation in the presence of the indicated nucleotides, inorganic phosphate, or EDTA. Four micrograms of monospecific IgGs against rTim17, rTim23, or rTim44 was added to the supernatant together with 10 μl Protein A-Sepharose and the mixture was incubated at 4°C for 90 min. Protein A-Sepharose was washed 3 times with solubilization buffer and then treated with gel-loading buffer. The eluted proteins were separated by SDS-PAGE and the gel was analyzed by immunoblotting with monoclonal antibody against mHsp70 or specific IgGs against rTim17, rTim23, or rTim44.

Immunofluorescence Microscopy—Immunofluorescence microscopy was performed as described (*27*). Briefly, normal rat kidney (NRK) cells were grown on glass coverslips in culture dishes and the cells were fixed with 50% acetone and 50% methanol for 2 min at room temperature. The coverslips were incubated with the monoclonal antibody against mHsp70 and IgGs against rTim17, rTim23, or rTim44 at room temperature for 1 h. The coverslips were washed and then incubated with the secondary antibodies, FITC-conjugated goat antibodies against rabbit IgG and rhodamine-conjugated goat antibodies against mouse IgG. The images were obtained and analyzed by the use of a confocal laser-scanning microscope (Zeiss, Germany).

RESULTS

Isolation of Rat cDNAs for Tim17, Tim23, and Tim44—A search of the dbEST database revealed mouse cDNA fragments encoding partial sequences with a significant homology to either Tim23 (Ids 524452, 687546, and 792946) or Tim44 (Ids 519324, 666290, and 793650) of *S. cerevisiae*. In addition, a search of the human cDNA database (HCD) revealed a human cDNA encoding a homologue of Tim17 of *S. cerevisiae* (Accession number THC146733). Based on the nucleotide sequence of these clones, we isolated three cDNAs encoding the rat counterparts. The isolated cDNAs encode proteins of 171 amino acid residues (MW 18.1 kDa), 209 amino acid residues (MW 21.9 kDa), and 453 amino acid residues (MW 51.0 kDa), and the predicted sequences showed an overall identity of 48, 28, and 30% with Tim17, Tim23, and Tim44 of *S. cerevisiae*, respectively (Fig. 1A). Rat Tim23 also exhibited a low degree of identity of 20% with yeast Tim22 (*6*).

Hydropathy profiles of Tim17, Tim23, and Tim44 of rats showed a significant similarity to those of yeast (Fig. 1B). rTim17 has four hydrophobic stretches which potentially constitute transmembrane segments (amino acid residues

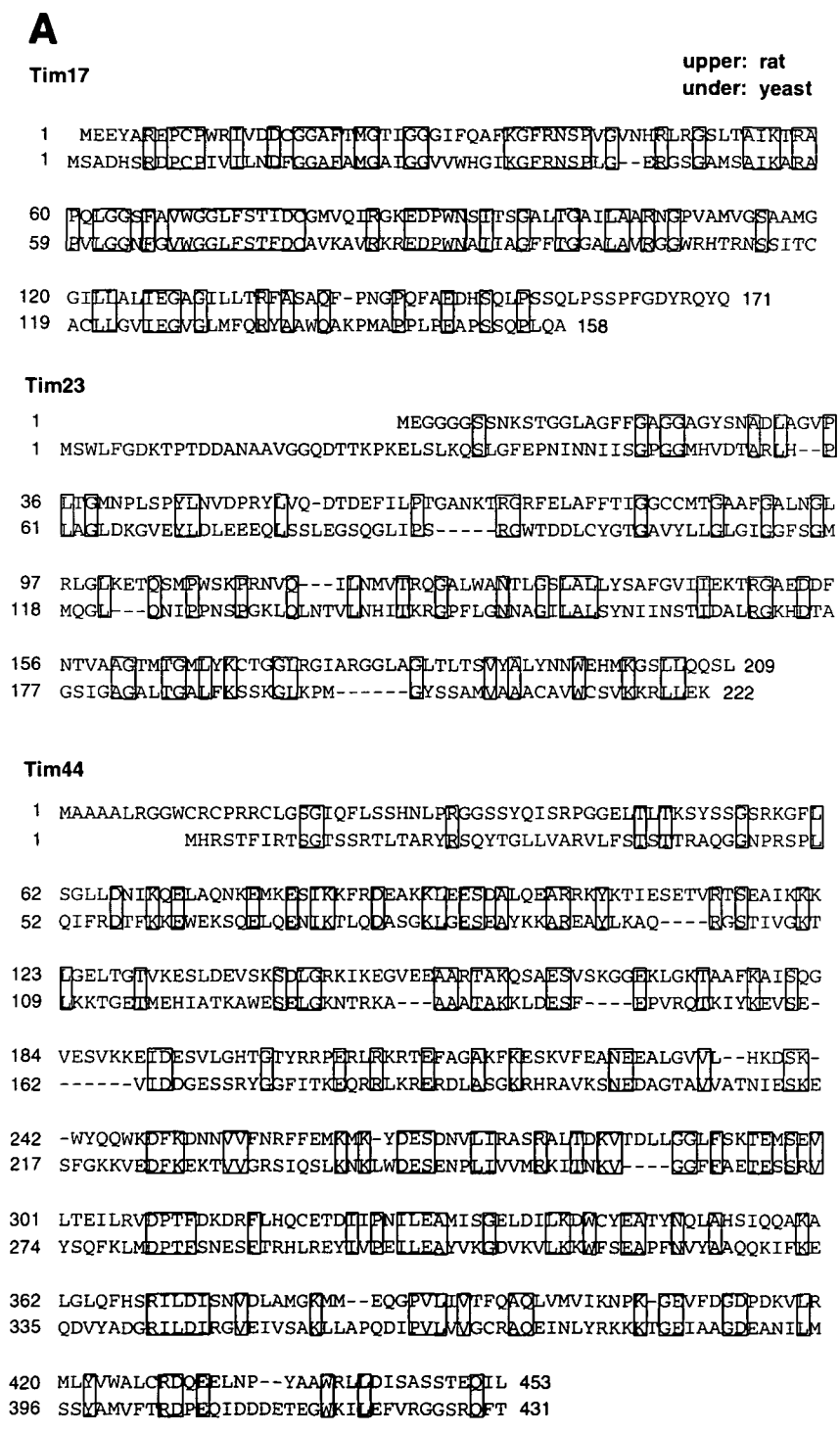


Fig. 1. **(A) Comparison of the deduced amino acid sequences of Tim17, Tim23, or Tim44 from rats and *S. cerevisiae*.** The sequences of Tim17, Tim23, or Tim44 were aligned using the FASTA program. Identical amino acid residues are boxed. **(B) Comparison of hydropathy profiles of Tim17, Tim23, or Tim44 from rats and *S. cerevisiae*.** Evaluation of hydropathy was performed according to the program of Kyte and Doolittle at a span setting of 10 amino acid residues. The putative transmembrane segments are underlined. The accession numbers for the DNA sequences reported here are AB006450, AB006451, and AB006452 in the DDBJ/EMBL/GenBank DNA databases for rTim17, rTim23, and rTim44, respectively.

17-34, 59-76, 89-105, and 107-134), although the junction between the 3rd and 4th transmembrane segments was somewhat ambiguous. Since the overall hydrophobicity profile of rat Tim17 is similar to that of yeast Tim17, they probably share the same topology in the membrane.

Rat Tim23 has a hydrophilic N-terminal half (amino acids 1-74) rich in charged amino acid residues and hydrophobic stretches of residues 75-96, 124-145, 155-168, and 180-197. This feature is conserved for yeast Tim23, except that it contains a longer N-terminal hydrophilic segment of 100 amino acid residues, thus suggesting that the two molecules share a similar topology in the membrane. It has been shown that both the N- and C- terminal portions of yeast Tim23 are extruded into the intermembrane space, and that the N-terminal half is enriched in acidic amino acid residues and potentially forms a coiled-coil, leucine-zipper structure (*11*). However, the "Coils" program failed to predict the coiled-coil structure for the corresponding region of rTim23 (data not shown). Furthermore, yeast Tim17 is believed to assume a similar membrane topology

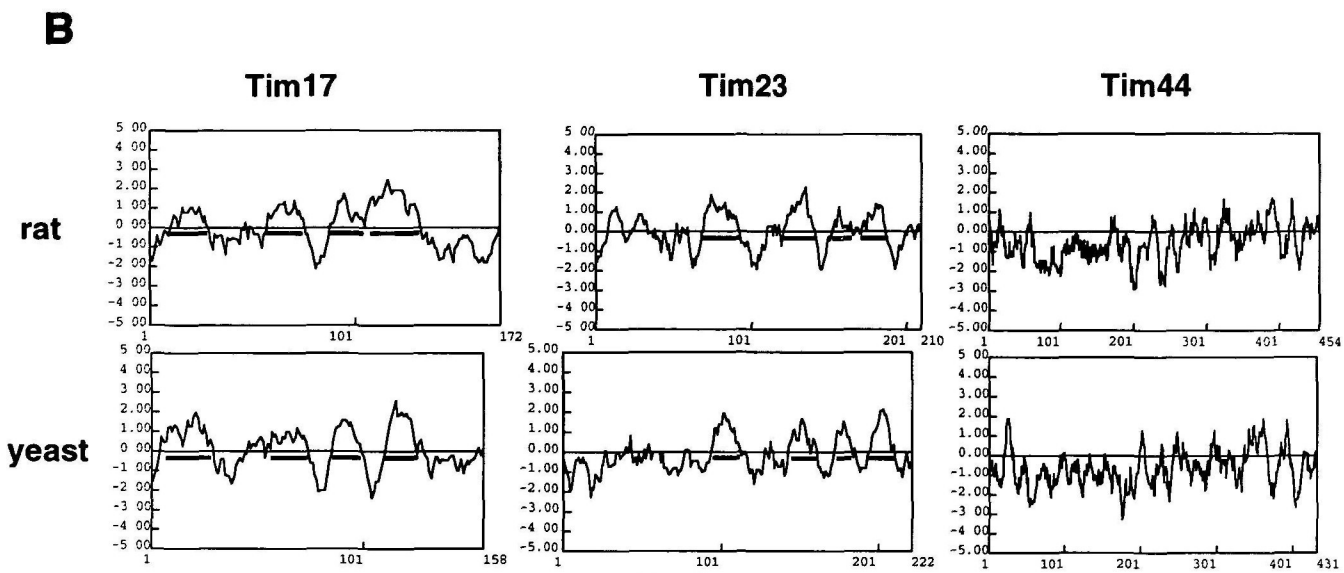


Fig. 1B

to that of yeast Tim23. The topology of rTim23 and rTim17 in the membranes remains to be determined experimentally.

When rTim17 or rTim23 synthesized in the reticulocyte lysate was incubated with mitochondria, it was imported into the mitochondria without a processing of the presequence and became resistant to proteinase K treatment (Fig. 2), indicating that rTim17 and rTim23 carry an internal mitochondrial targeting signal, as the yeast counterparts do.

The hydrophobicity profile of rTim44 indicates that it is rather hydrophilic and apparently does not contain any hydrophobic stretch that may function as a transmembrane segment (Fig. 1B). rTim44 was synthesized *in vitro* as a 51 kDa protein (Fig. 2, lane 1) and when incubated with mitochondria, it was imported into the mitochondria concomitantly with processing of the presequence. The mature rTim44 of ~45 kDa was resistant to externally added proteinase K (Fig. 2, lanes 2 through 4). Thus, rTim44 was synthesized as a precursor having a presequence of ~6 kDa. Although the precise processing point of the rTim44 precursor by the mitochondrial processing peptidase is unknown, a visual inspection revealed a potential cleavage site at Gly33-Gly34 or at Pro42-Gly43 (*28*), and the sequence of the N-terminal fragment produced thereby seems to possess features that are characteristic of mitochondrial targeting sequences (*29*). Indeed, a mutant precursor in which the N-terminal 15 amino acid residues have been deleted could not be imported into the mitochondria, indicating that the N-terminal portion of the precursor actually functions as the mitochondria targeting signal (data not shown). Furthermore, the *in vitro* import of the precursors for rTim17, rTim23, and rTim44 was inhibited by a protonophore, CCCP, indicating that the import requires $\Delta\Psi$ of the inner membrane (Fig. 2, lane 4). For unknown reasons, the import of the precursors for rTim17 and rTim23 was less sensitive to CCCP than that for the matrix-targeted precursors. Similar results were obtained in yeast mitochondria (*21*).

Based on this structural similarity and the experiments

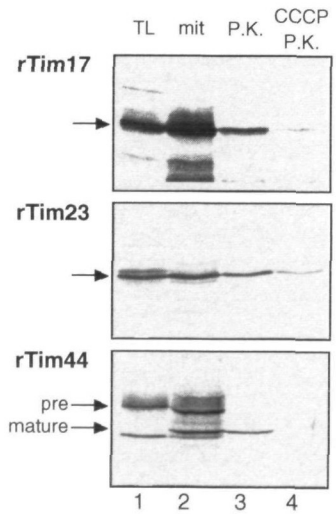


Fig. 2. **Import of reticulocyte lysate-synthesized rTim17, rTim23, and rTim44 into rat liver mitochondria *in vitro*.** *In vitro*-synthesized rTim17, rTim23, or rTim44 was incubated in the import buffer with mitochondria at 30°C for 40 min in the absence (lanes 2 and 3) or presence of 200 μM CCCP (lane 4). After import, the reaction mixtures were treated with 50 μg/ml proteinase K at 0°C for 20 min (lanes 3 and 4). The reaction mixtures were analyzed by SDS-PAGE and autoradiography. TL, 20% of input (lanes 1). The positions of rTim17, rTim23, and precursor and the mature form of rTim44 are indicated by arrows.

described below, we conclude that the proteins encoded by these clones function as components of the import machinery of the rat mitochondrial inner membrane and that they are the structural and functional homologues of yeast Tim17, Tim23, and Tim44.

Subcellular Localization of rTim17, rTim23, and rTim44—The subfractionation of the rat liver and the subsequent immuno-blotting showed that rTim17 (~18 kDa), rTim23 (~22 kDa), and rTim44 (~45 kDa) were cofractionated with a mitochondrial marker, COX IV, but not with cytochrome P450(M1) or with the large subunit of

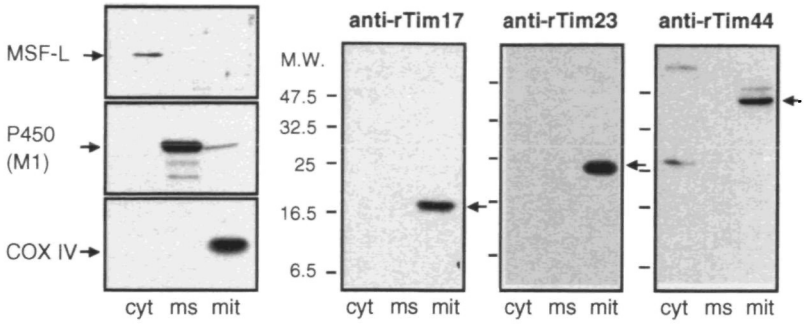


Fig. 3. **Analysis of the subcellular localization of rTim17, rTim23, and rTim44 in rat liver cells.** Twenty-five micrograms each of mitochondrial (mit), microsomal (ms), and cytosolic (cyt) proteins from rat liver were subjected to SDS-PAGE and immunoblotting using IgGs against rat MSF (large subunit), cytochrome P450(M1), COX IV, rTim17, rTim23, or rTim44. The immunoreacted bands were visualized by ECL (Pharmacia).

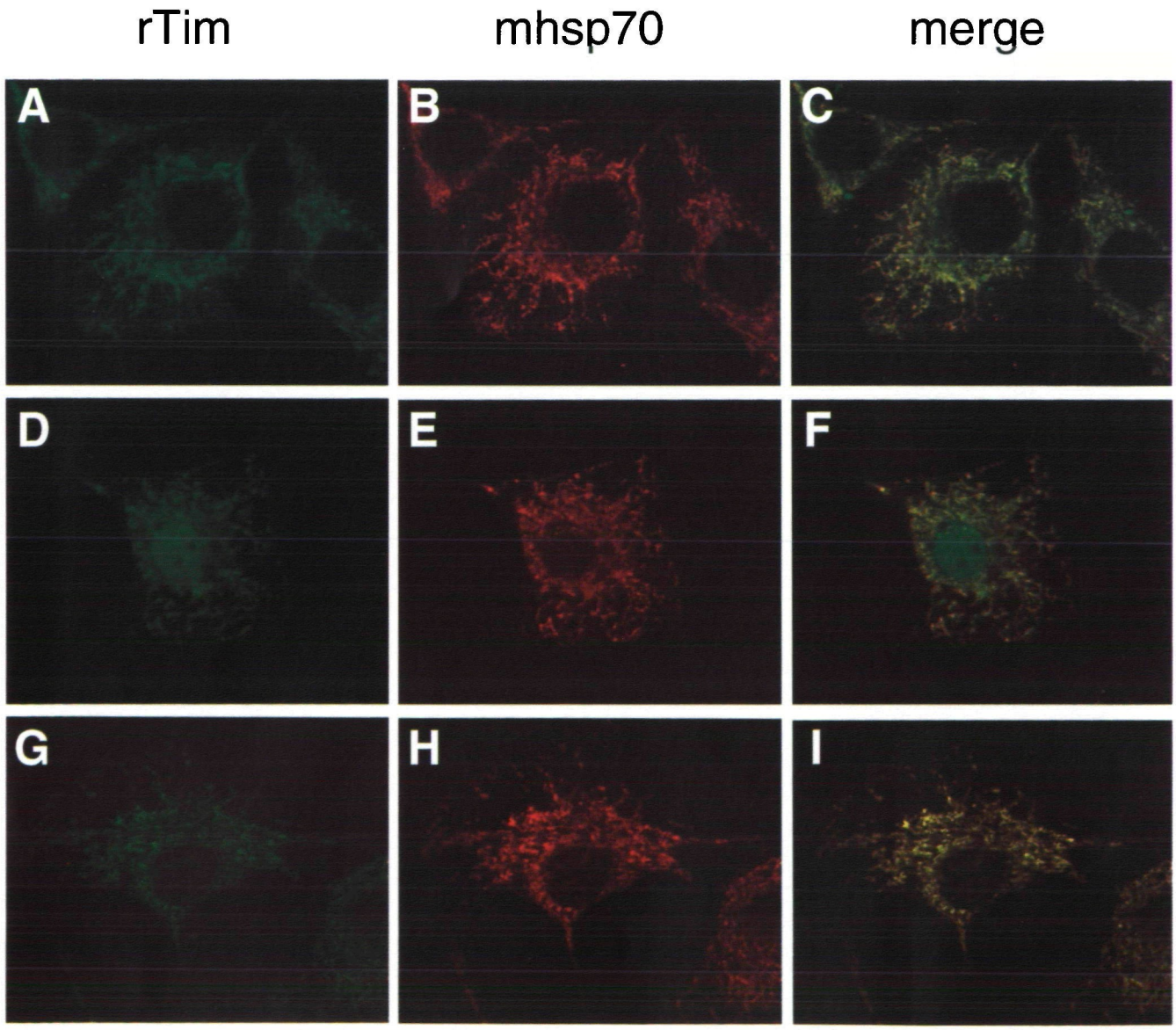


Fig. 4. **Immunofluorescence detection of rTim17, rTim23, and rTim44 in NRK cells.** NRK cells were fixed and stained with rabbit IgGs against rTim17 (A, B, and C), rTim23 (D, E, and F), or rTim44 (G, H, and I) and then with FITC-conjugated goat antibodies against rabbit IgG. As a reference, the cells were stained simultaneously with mouse monoclonal antibody against mHsp70 and with rhodamine-conjugated goat antibodies against mouse IgG. Images were obtained and analyzed by confocal laser-scanning microscope. rTim, staining for Tim proteins (green); mHsp70, staining for mHsp70 (red); merge, merged images of staining for rTim proteins and mHsp70 (yellow). Other conditions are described in the "EXPERIMENTAL PROCEDURES."

MSF, marker proteins of rat liver microsomes (*22*) and cytosol (*23*), respectively (Fig. 3A).

The expression levels of rTim17, rTim23, and rTim44 in the rat liver mitochondria were roughly estimated from immuno-blots, using the recombinant rat Tim proteins as standards, to be in the molar ratio of 0.3:1:1, respectively, and rTim44 represented ~0.04% of mitochondrial protein. In contrast, the expression levels of Tim17 and Tim23 in the yeast mitochondria are comparable, with a 1:1 stoichiometry, whereas the amount Tim44 is 3-10 times less; yeast Tim44 represented 0.1-0.25% of the mitochondrial proteins (*13, 15*).

The subcellular location of rTim17, rTim23, and rTim44 was also determined in NRK cells by immunofluorescence microscopy (Fig. 4). All three proteins exhibited a filamentous distribution throughout the cells (stained green; panels A, D, and G) with a distribution similar to that of mHsp70 (stained red; panels B, E, and H), the 70 kDa heat shock protein localized in the mitochondrial matrix (*30*). In addition, the merged images of the stainings for rTim17, rTim23, and rTim44 with mHsp70 also revealed obvious co-localization (yellow color; panels C, F, and I), confirming the results of subcellular fractionation. For unknown reasons the nuclear matrix was also stained by anti-rTim23 IgGs (Fig. 4, D and F), although a potential nuclear localization signal was not apparent in the primary sequence of rTim23.

Submitochondrial Localization of Rat Tim17, Tim23, and Tim44—We then examined the submitochondrial localization of rat Tim proteins. As shown in Fig. 5A, the mitochondrial matrix enzyme, malate dehydrogenase (MDH), was found in the mitoplasts (mp) (lane 2) and was located mainly in the supernatant fraction after the sonication of the mitoplasts (lane 4). COX IV was exclusively found in the membrane (lane 3) and was resistant to alkali-extraction (lane 6). Likewise, rTim17 and rTim23 were retained in the mitoplasts (lane 2), and all of them were found in the membrane fractions after alkali-extraction (lane 6), indicating rTim17 and rTim23 to be intrinsic inner membrane proteins. On the other hand, rTim44 was detected in the mitoplasts (Fig. 5A, lane 2; Fig. 5B, lane 3) and it was resistant to externally added proteinase K (Fig. 5B, lane 4), which indicates that rTim44 is located on the matrix side of the mitochondria. When the mitochondria were subjected to brief sonication under hypotonic conditions, 40% of total rTim44 was found in the soluble fraction and 60% in the membrane fraction (Fig. 5A rTim44, lanes 3 and 4; see also Fig. 8C, rTim44). Furthermore, the fraction which was found in the membrane was alkali-extractable (Fig. 5A, lanes 3, 5, and 6), indicating it to be a peripheral membrane protein bound to the matrix side of the inner membrane.

To confirm further the above results, the mitochondria were briefly sonicated under a hypotonic condition and the proteins were separated by sucrose density gradient centrifugation (Fig. 5C). rTim17 and rTim23 were cofractionated with COX IV, whereas rTim44 exhibited an apparent double-distribution in the sucrose gradient; one peak cosedimented with the outer mitochondrial membrane (lanes 6 through 9) and the other sedimented slightly faster than the inner mitochondrial membrane (lanes 2 through 4). We found that rTim44 which cosedimented with the outer membrane was exclusively detected in the supernatant after ultracentrifugation to remove the membrane fragments (data not shown), suggesting that Tim44 in the matrix is in an oligomeric state and is sedimented by chance at around the outer membrane fractions. On the other hand, the highly oligomerized form, as well as the inner membrane-bound form, of rTim44 seemed to be recovered in the faster-sedimenting fractions. Taken together, the results indicate that rTim44 is present in the mitochondrial matrix in a soluble form as well as in the form bound to the matrix side of the inner membrane. In marked contrast, yeast Tim44 is a peripheral inner membrane protein and is located exclusively on the matrix side of the inner membrane with the C-terminus anchoring

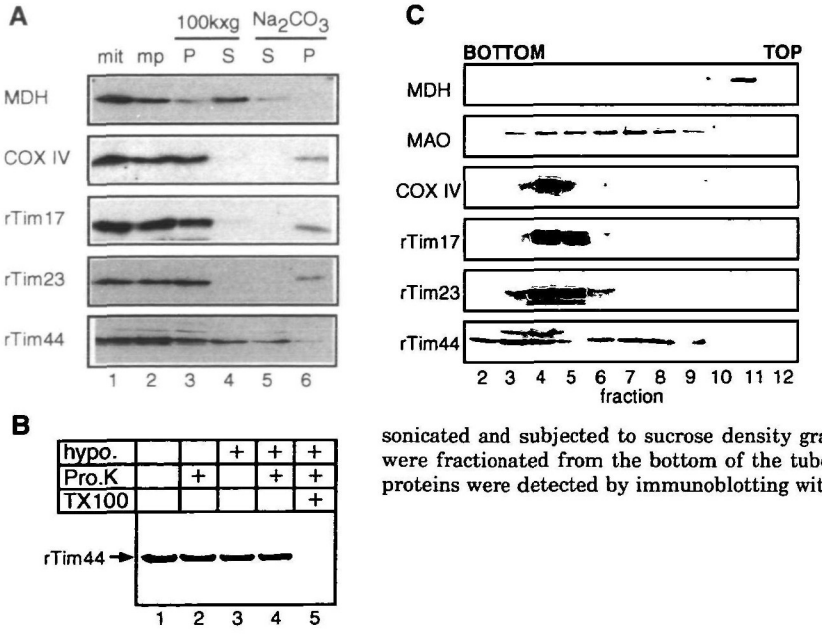
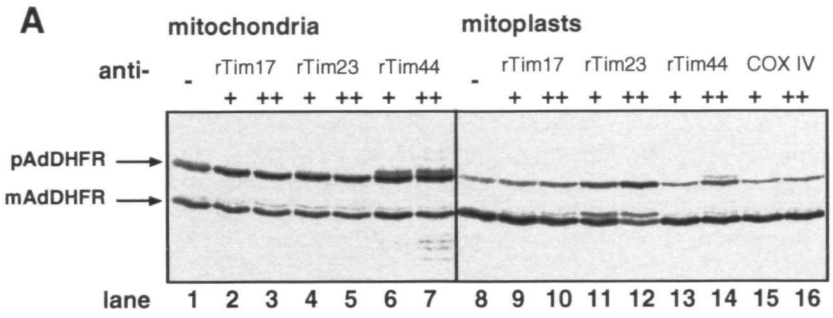


Fig. 5. **Submitochondrial localization of rTim17, rTim23, and rTim44.** (A) Mitoplasts (mp) were sonicated and centrifuged at 100,000×*g* to separate the supernatant (S) and the membrane (P) fractions. The membrane fraction was treated with 0.1 M Na_2CO_3 and ultracentrifuged to separate the supernatant (S) and the precipitate (P). Each fraction was subjected to SDS-PAGE, followed by immunoblotting with IgGs against MDH, COX IV, rTim17, rTim23, or rTim44. (B) Rat liver mitochondria were treated with or without 100 μg/ml proteinase K at 0°C for 20 min under isotonic or hypotonic condition or in the presence or absence of 1% Triton X-100 in the indicated combinations. After the reaction, proteins were recovered by TCA-precipitation and analyzed by SDS-PAGE followed by immunoblotting with IgGs against rTim44. (C) Mitochondria were sonicated and subjected to sucrose density gradient centrifugation. After centrifugation, the samples were fractionated from the bottom of the tubes. Each fraction was separated by SDS-PAGE and the proteins were detected by immunoblotting with IgGs against the indicated proteins.

to the membrane, probably through integral membrane proteins (*12, 13*). The mode of the membrane binding of rTim44 and the relation between the free and membrane-bound forms still remain to be elucidated.

Antibodies against rTim23 Inhibit Precursor Import into the Mitoplasts—We next examined the effect of antibodies against rTim17, rTim23, and rTim44 on the precursor import into the mitochondria or mitoplasts. As shown in Fig. 6, IgGs against rTim17 or rTim44 inhibited neither the import of pAd-DHFR into the mitochondria nor the import into the mitoplasts (Fig. 6A, lanes 1 through 7, 9, 10, 13, and 14 and Fig. 6B). In contrast, monospecific IgGs against



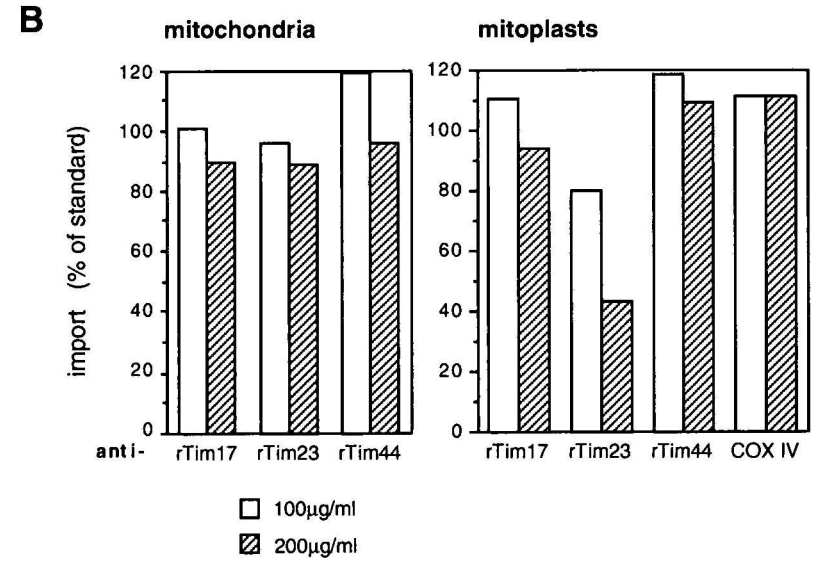


Fig. 6. **The effect of antibodies against rTim17, rTim23, and rTim44 on the precursor import into mitoplasts.** (A) The mitochondria or mitoplasts prepared from trypsin-pretreated mitochondria were incubated at 0°C for 30 min with 100 μg/ml (+) or at 200 μg/ml (++) IgGs against the indicated proteins, and then subjected to the import of reticulocyte lysate-synthesized ^{35}S-pAd-DHFR at 30°C for either 30 min (for mitochondria) or 15 min (for mitoplasts). The reaction mixtures were analyzed by SDS-PAGE and subsequent autoradiography. (B) The import efficiencies were quantified. Import is expressed relative to control import without IgG added (100%).

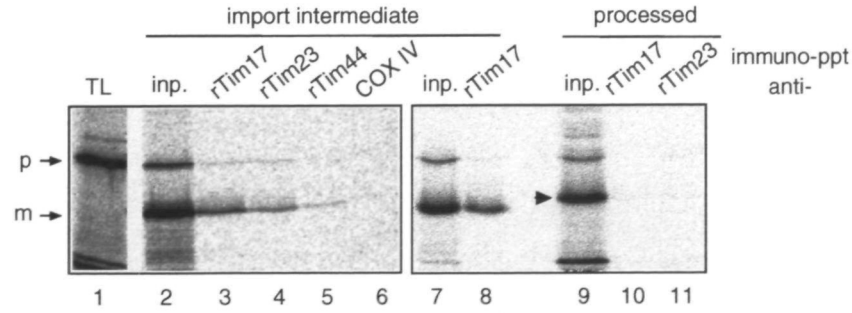


Fig. 7. **Interaction of the import-arrested precursor with rTim17, rTim23, or rTim44.** Reticulocyte lysate-synthesized ^{35}S-pAd-DHFR was incubated with 10 μM methotrexate and 1 mM NADPH, then subjected to import into the mitoplasts. The mitoplasts were then isolated and treated with 0.25% digitonin-50 mM NaCl. The solubilized supernatant was divided into 4 aliquots and subjected to immunoprecipitation with IgGs against rTim17, rTim23, rTim44, or COX IV. The immunoprecipitates were analyzed by SDS-PAGE followed by autoradiography. As a control, reticulocyte lysate-synthesized ^{35}S-pAd-DHFR was incubated with mitochondrial processing peptidase, mixed with digitonin-solubilized mitochondria in the presence of methotrexate and NADPH, and subjected to immunoprecipitation with IgGs against rTim17 or rTim23. The positions of the precursor (p) and mature (m) proteins are indicated. TL: 20% of input ^{35}S-pAd-DHFR. inp: 20% of the immunoprecipitation reaction mixture. Note that purified mitochondrial processing peptidase cleaved the presequence of pAd-DHFR at a position upstream of the cleavage site observed with mitochondria to produce a higher molecular weight mature peptide (indicated by an arrowhead).

rTim23 inhibited the precursor import into the mitoplasts dose-dependently, whereas they had no effect on the import into the mitochondria (Fig. 6A, lanes 4, 5, 11, and 12; and Fig. 6B). Similar results were reported for yeast Tim23, which exposes the N-terminal ~100 amino acid domain and a small C-terminal domain to the intermembrane space, suggesting that rTim23 assumes a topology in the inner membrane similar to that of yeast Tim23 and that the intermembrane space domain of rTim23 is important for the precursor import. On the other hand, IgGs against rTim23 failed to inhibit the insertion of AAC into the inner membrane (N. Ishihara and K. Mihara, in preparation), confirming the finding in yeast that import of the AAC precursor is mediated by Tim22, whereas import of matrix-targeted precursors is mediated by the Tim23/Tim17 complex (*6*). The failure of anti-rTim44 antibodies to inhibit protein import into the mitoplasts is accounted for by the fact that Tim44 is localized exclusively on the matrix side of the mitochondria.

rTim17, rTim23, and rTim44 Interact with the Import-Arrested Protein—We then addressed whether rat Tim proteins physically interact with the precursor in transit to the matrix. To accumulate the import-arrested protein, pAd-DHFR was synthesized in the reticulocyte lysate, incubated with methotrexate (MTX) and NADPH (*31*) at 0°C for 10 min and then subjected to import into mitoplasts. The mitoplasts were then solubilized by addition of digitonin and subjected to immunoprecipitation with anti-rTim17, rTim23, or rTim44 IgGs. As shown in Fig. 7, anti-COX IV IgGs did not precipitate the import-arrested protein at all (lane 6), whereas monospecific IgGs against rTim17, rTim23, or rTim44 precipatated the processed form of pAd-DHFR (lanes 3-5 and 8), although the efficiencies varied with the antibodies. In the absence of MTX and NADPH, the amount of the import-arrested protein coprecipitated decreased to less than one-fourth of the original amount (data not shown), confirming that the immunoprecipitated signals actually depended upon the import-arrest of the precursor. Furthermore, when the mitochondrial lysate was mixed with pAd-DHFR that had been partially processed by mitochondrial processing peptidase and subjected to immunoprecipitation with IgGs against rTim17 or Tim23, the processsed form of pAd-DHFR was not precipitated (Fig. 7, lanes 10 and 11), ruling out the possibility that the processed form of pAd-DHFR interacted nonspecifically with the Tim proteins. Thus, rTim17, rTim23, and rTim44 physically interacted with the precursor in transit to the matrix.

The rTim17-rTim23 and the rTim44-mHsp70 Complexes in Rat Liver Mitochondria—It has been reported that Tim17 forms a stoichiometric complex with Tim23, and constitutes a membrane potential-driven proteinaceous import channel for a subset of precursor proteins in yeast mitochondria (*5*). We therefore examined whether rTim17 and rTim23 interact in rat liver mitochondria. Rat liver mitochondria were solubilized with digitonin, and first subjected to immunoprecipitation with IgGs against rTim17, rTim23, or rTim44. The immunoprecipitates were separated by SDS-PAGE and the co-precipitated proteins were then probed by immunoblotting with IgGs against mHsp70, rTim44, rTim23, or rTim17 (Fig. 8). Clearly, IgGs against rTim23 precipitated rTim17 with a high efficiency, irrespective of the presence or absence of ATP (Fig. 8A, lanes 4 and 5). On the other hand, IgGs against rTim17 precipitated only a small amount of rTim23 (lanes 2 and 3). This may partly reflect the lower abundance of rTim17 relative to rTim23, assuming that they also form a 1:1 stoichiometric complex. In yeast mitochondria, the interactions of the Tim17-Tim23 complex with mHsp70 and the Tim44-mHsp70 complex with Tim23 have been reported for the *in vitro* imported components (*32*), but no such interactions have been observed for the authentic components of rat liver mitochondria (Fig. 8A, lanes 2 through 7).

It is well established in yeast mitochondria that mHsp70 is recruited to the import machinery of the inner membrane by Tim44 and functions as the translocation motor for the precursor chain. We therefore examined whether rTim44 is present as a complex with mHsp70 in rat mitochondria

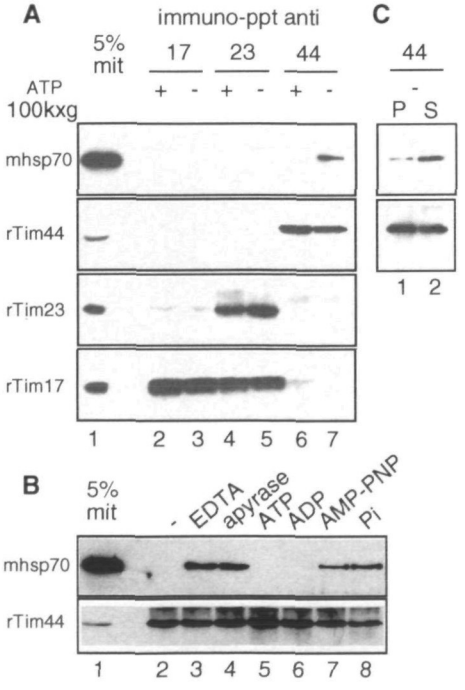


Fig. 8. **The rTim 17-Tim23 and the rTim44-mHsp70 complexes in rat liver mitochondria as detected by immunoprecipitation.** (A) Rat liver mitochondria that had been incubated with 1 mM ATP (ATP+) or with apyrase plus oligomycin (ATP−) were solubilized in 0.25% digitonin-50 mM NaCl. The supernatant fractions were each divided into 3 aliquots and each aliquot was subjected to immunoprecipitation with IgGs against rTim17, rTim23, rTim44. The immunoprecipitates were resolved by SDS-PAGE and analyzed by immunoblotting with IgGs against mHsp70, rTim44, rTim23, or rTim17. The immunoreacted bands were visualized by ECL. As a reference standard, 5% of the original amount of mitochondria was applied to lane 1. (B) Rat liver mitochondria that had been incubated with 5 mM EDTA, 10 units/ml apyrase plus 20 mM oligomycin ("apyrase"), 1 mM ATP, 5 mM ADP, 3 mM AMP-PNP, or 4 mM NaH_2PO_4 ("P_i") at 30°C for 20 min were solubilized in 0.25% digitonin-100 mM KCl and subjected to immunoprecipitation with IgGs against rTim44 as in (A). (C) The mitochondria were sonicated and then ultracentrifuged to separate the supernatant (S) and the membrane (P) fractions. The membrane fraction was solubilized in 0.25% digitonin and both fractions were subjected to immunoprecipitation with anti-rTim44 IgGs. The immunoprecipitates were resolved by SDS-PAGE and the proteins were detected by immunoblotting with the indicated IgGs.

and found that mHsp70 was precipitated with IgGs against rTim44 in the absence of ATP, whereas, in its presence, the complex was dissociated and mHsp70 was no longer precipitated (Fig. 8A, lanes 6 and 7). Since the mitochondrial matrix contains a high concentration of K^+ ion and a low concentration of Na^+ ion, we examined the interaction of mHsp70 and rTim44 in the presence of 100 mM KCl by immunoprecipitation with IgGs against rTim44 (Fig. 8B). It should be noted here that a significant amount of Mge1p, the mitochondrial homologue of bacterial GrpE, was present in the mitochondrial lysate used (*33*). In the presence of ATP or ADP, rTim44 did not bind to mHsp70 (lanes 5 and 6). The same result was obtained in the absence of added nucleotides (lane 2). On the other hand, formation of the mHsp70-rTim44 complex was observed when the lysate prepared from apyrase/oligomycin-treated mitochondria was used (lane 4), indicating that the lysate contained a low concentration of ATP or ADP, which induced dissociation of the mHsp70-rTim44 complex. Furthermore, a nonhydrolyzable ATP analogue, AMP-PNP, inorganic phosphate or EDTA stabilized the mHsp70-rTim44 complex (lanes 3, 7, and 8). These results were essentially the same as those obtained for yeast mitochondria; Mge1p stabilized the mHsp70-Tim44 complex in the presence of nonhydrolyzable ATP analogues, whereas it destabilized the complex in the presence of ADP, whose action was counteracted by inorganic phosphate or EDTA (*33*). We calculated that roughly 2-3% of the total mHsp70 formed a complex with rTim44. Since we have shown that rTim44 is present in the mitochondrial matrix both in soluble and in inner membrane-bound forms, we estimated the amount of mHsp70 which complexed with the soluble rTim44 and that which complexed with the membrane-bound rTim44. The immunoprecipitation with IgGs against rTim44 revealed that less than 20% of mHsp70 that was complexed with rTim44 was in the membrane-bound form, whereas most of the remaining mHsp70 was in the soluble form (Fig. 8C). In this connection, yeast Tim44 was reported to have a motif of 18 amino acid residues with similarity to the J-domain of Sec63p, the domain by which ER luminal hsp70, Kar2p, is recruited to the import machinery of the ER (*15*). However, no such sequence was apparent in the structure of rTim44 and the mode of interaction between rTim44 and mHsp70, as well as the way in which the matrix mHsp70 is recruited to the import machinery, still remains to be analyzed.

DISCUSSION

The protein import machinery of the inner mitochondrial membrane has been analyzed precisely in *S. cerevisiae*. Recently human Tim17 was described as the first example of a component of mamalian Tim import machinery (*21*). We found, by a databank search, human or mouse partial amino acid sequences with a significant similarity to Tim17, Tim23, or Tim44 of *S. cerevisiae*. Based on such information, we cloned the complete cDNAs for the rat counterparts, expressed them in *E. coli* and raised antibodies. All three proteins were located in mitochondria and exhibited an overall structural similarity to either yeast Tim17, Tim23, or Tim44. Furthermore, they also exhibited a functional similarity to the yeast counterparts in several respects: (i) they all interacted with the transport-arrested mitochondrial protein, (ii) some fractions of rTim23 are present as a form complexed with rTim17 in the mitochondria, (iii) matrix rTim44 formed an ATP-dissociable complex with a portion of mHsp70, and (iv) IgGs against rTim23 inhibited precursor import into the mitoplasts.

A significant dissimilarity was also noted as follows: (i) the relative content of Tim17, Tim23, and Tim44 in molar ratio was 0.3:1:1 in rats, but 1:1:0.1-0.3 in yeast, (ii) interactions between the rTim17-rTim23 complex and mHsp70 and between the rTim44-mHsp70 complex and rTim23 were reported in yeast, whereas no such interaction was apparent in the rat mitochondria, and, most of all, (iii) yeast Tim44 is reported to be present on the matrix side of the inner membrane as a peripheral membrane protein (*13*), whereas rTim44 exhibited a double distribution in the matrix; 40% was soluble in the matrix while the remaining portion was bound to the inner membrane. Most of the mHsp70 that had been complexed with rTim44 was present in the matrix in a soluble form. These findings may reflect a possible difference in the mechanism of protein import between mammals and yeast. Are the membrane-bound and the soluble forms of rTim44 thus playing, in collaboration with mHsp70 or independently, distinct roles in protein translocation across the inner membrane? Or, in contrast, is the matrix-soluble rTim44-mHsp70 complex recruited *en bloc* to the *trans*-site of Tim machinery in response to the emerging precursor chain to function as the translocation motor? Clearly, experiments involving functional complementation of yeast *tim* strains by rat cDNAs, as well as more biochemical work, are required to clarify these questions.

In summary, we isolated and characterized the rat homologues of Tim17, Tim23, and Tim44. To our knowledge, this is the first time a functional characterization of Tim17, Tim23, and Tim44 has been achieved in an organism other than *S. cerevisiae*. These results, in conjunction with the fact that a mammalian homologue of Tom20 has been found, indicate that the mitochondrial import machineries of the outer and inner membranes are essentially conserved among species, even though subtle variations do exist among different species.

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