

Characterization of rat TOM70 as a receptor of the preprotein translocase of the mitochondrial outer membrane

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Summary

We cloned a ~70 kDa rat mitochondrial outer membrane protein (OM70) with a sequence identity of 28.1% and 20.1% with *N. crassa* and *S. cerevisiae* Tom70, respectively. Even with this low sequence identity, however, the proteins share a remarkable structural similarity: they have 7-10 tetratricopeptide repeat motifs and are anchored to the outer membrane through the N-terminal transmembrane domain with the bulk portion located in the cytosol. Antibodies against OM70 inhibited import of preproteins, such as the ADP/ATP carrier and rTOM40, that use internal targeting signals but not the import of cleavable presequence-containing preproteins. Blue native gel electrophoresis and immunoprecipitation of digitonin-solubilized mitochondrial outer membranes revealed that OM70 was loosely associated with the ~400 kDa translocase complex of the mitochondrial outer membrane, which

contains rTOM22 and rTOM40. A yeast two-hybrid system demonstrated that OM70 interacted with rTOM20 and rTOM22 through the cytoplasmic domains. Thus, OM70 is a functional homologue of fungal Tom70 and functions as a receptor of the preprotein import machinery of the rat mitochondrial outer membrane. Furthermore, the N-terminal 66 residue region of OM70, which comprises a hydrophilic 41 residue N-terminal domain, a 22 residue transmembrane domain and three arginine residues, is sufficient to act as a mitochondria-targeting signal, and the arginine cluster is crucial for this function.

Key words: Mitochondrial protein import, Import receptor, Preprotein translocase, Mitochondrial outer membrane, Precursor proteins

Introduction

Most mitochondrial proteins are synthesized in the cytosol as preproteins, targeted to mitochondria by cytosolic factors such as hsp70 and mitochondrial import stimulation factor (MSF) and transported to the intramitochondrial compartments by the preprotein import machineries of the outer and inner membranes (TOM and TIM complexes, respectively) (Schatz and Dobberstein, 1996; Mihara and Omura, 1996; Neupert, 1997; Pfanner et al., 1997). The TOM complex of *S. cerevisiae* is composed of at least nine proteins (Tom72, 70, 40, 37, 22, 20, 7, 6 and 5) (Dekker et al., 1998; Ryan et al., 2000). Tom40, Tom22 and small Tom proteins form a ~400 kDa core complex in the outer membrane (Dekker et al., 1998). Tom70, Tom37, Tom22 and Tom20 function as import receptors. Tom72 has strong similarity to Tom70 and is weakly associated with the TOM complex (Bömer et al., 1996; Schlossmann et al., 1996). Its function, however, is not known. Using a heterologous system with rat liver MSF and yeast mitochondria, we demonstrated that the preprotein-MSF complex first docks onto the Tom70-Tom37 complex, then ATP releases MSF from the docking complex and the preprotein is transported into the mitochondria via the Tom20-Tom22 complex (Hachiya et al., 1995; Iwahashi et al., 1997). Preproteins with an unfolded conformation, however, bypass the Tom70-Tom37 complex, are targeted to the Tom20-Tom22 complex and are then translocated across the outer membrane (Hachiya et al., 1995).

Although the fundamental mechanisms of mitochondrial protein import seem to be conserved from lower eukaryotes to mammals, only limited information is available for higher eukaryotic systems. Recently, several mammalian counterparts were identified, and their roles are being studied. These mammalian proteins are TOM40 (Suzuki et al., 2000), TOM22 (Saeki et al., 2000), TOM20 (Hanson et al., 1996; Goping et al., 1995; Seki et al., 1995), TIM17 (Ishihara and Mihara, 1998), TIM23 (Ishihara and Mihara, 1998), TIM44 (Ishihara and Mihara, 1998) and DDP1, a homologue of yeast Tim8 (Koehler et al., 1999). A rat gene homologous to fungal Tom70 has been identified by differential display PCR as a thyroid-hormone-regulated gene that is located in specific brain regions, although its function remained to be determined (Alvarez-Dolado et al., 1999). In addition, several novel components that are thought to function as import receptors are also found in mammalian mitochondria. Human TOM34 contains a TPR sequence with a sequence similarity to those of fungal Tom70 and Tom20 (Nuttall et al., 1997). It is loosely associated with the outer membrane via its N-terminal hydrophobic segment, and antibodies against TOM34 inhibit mitochondrial import of matrix-targeted preproteins (Nuttall et al., 1997). Metaxin is a 35 kDa C-terminal membrane-anchor protein that has 25% sequence identity with the N-terminal region of yeast Tom37, and antibodies against metaxin also inhibit mitochondrial import of a matrix-targeted precursor

preadrenodoxin (pAd) (Armstrong et al., 1997). Whether metaxin is the mammalian counterpart of yeast Tom37 is not known. The antibodies against OM37, a 37 kDa outer membrane protein of rat liver mitochondria, inhibited mitochondrial docking of the pAd-MSF complex and the subsequent transport of pAd into mitochondria (Komiya et al., 1996; Komiya and Mihara, 1996). The homologue of OM37 is not found in yeast. These findings might reflect features of the preprotein import system unique to mammalian mitochondria and require further characterization.

In the present study, a 70 kDa outer membrane protein of rat liver mitochondria (OM70) was identified as the rat homologue of Tom70 and was characterized as the receptor of the rat TOM complex for a subset of preproteins that carry internal mitochondria-targeting signals. Furthermore, we demonstrated that the N-terminal segment, consisting of the N-terminal hydrophilic segment, the transmembrane domain (TMD), and the following three basic amino acid residues, is sufficient to function as a mitochondria-targeting signal. This structural feature of the mitochondria-targeting signal is also observed in the N-terminal membrane-anchor protein rTOM20 (Kanaji et al., 2000). An *in vitro* assay revealed that rTOM70 was targeted and inserted into the mitochondrial outer membrane independent of the import receptors, and this insertion strictly depended upon the basic amino-acid residues in the C-terminal flanking region of the TMD.

Materials and Methods

Materials

Antibodies against rTOM20 (Iwahashi et al., 1997), rTOM40 (Suzuki et al., 2000), rTIM23 (Ishihara and Mihara, 1998), cytochrome P450(M1) (Matsumoto et al., 1986) and cytochrome H450 (Ishihara et al., 1990) were prepared as previously described. Antibodies against rat OM37 were raised in rabbits against a recombinant cytoplasmic fragment of OM37 and antigen-selected. Monoclonal antibodies against hsp60 and cytochrome c were purchased from StressGen (SPA807) and PharMingen (65981A), respectively. The rat liver cDNA library in λ gt10 was prepared as previously described (Mihara, 1990). Rat liver mitochondria and the mitochondrial outer membrane were prepared as previously described (Hachiya et al., 1994; Iwahashi et al., 1994). 125 I-labeled pAd was prepared using Iodo-Beads (Pierce Chemical) (Komiya et al., 1996). 35 S-labeled recombinant pAd and recombinant MSFS were prepared as previously described (Alam et al., 1994).

cDNA cloning of rTOM70

A 927 bp cDNA sequence encoding part of a putative mouse homologue of fungal Tom70 was assembled from five partial mouse EST nucleotide sequences (dbEST IDs: 756557, 794014, 851708, 1188043, 851269). On the basis of this assembled sequence, the following oligonucleotides were synthesized: TOM70-1: 5'-GATGAATTTCGATGCTCAAGGCAAATACATG-3'; TOM70-2: 5'-CAGGGATCCTAGCCTTGCTGATAAGCTCC-3'.

Underlining in the TOM70-1 and TOM70-2 sequences denotes the restriction sites of *Eco*RI and *Bam*HI, respectively. A 650 bp cDNA fragment was amplified from the rat liver poly(A)⁺-RNA by RT-PCR using TOM70-1 and TOM70-2 as the primers. This cDNA fragment was used as a probe to screen the λ gt10 rat cDNA library for rat OM70, and an ~2.5 kbp cDNA encoding the entire OM70 was obtained.

Preparation of antibodies against OM70

An 1830 bp cDNA fragment was amplified by PCR using OM70 cDNA as the template and the following oligonucleotides as the primers: TOM70-3: 5'-GATCATATGGCCGCCTCTAAGCCC-3'; TOM70-4: 5'-GCGGGATCCTATAATGTTCGGTGGTT-3'.

Underlining in TOM70-3 and TOM70-4 denotes the restriction sites of *Nde*I and *Bam*HI, respectively. The obtained fragment was subcloned into the pET28b vector (Novagen) to create pET28b-NHIS70, which tags (His)₆ to the N-terminus of the expressed protein. His-tagged OM70 was expressed in BL21 (DE3) cells as inclusion bodies, which were separated by SDS-PAGE and the Coomassie-brilliant-blue-stained band was excised from the gel and used to raise antibodies in rabbits using the Ribi Adjuvant system (RIBI Immunochem Research Inc.).

Construction of expression plasmids for rTOM fusion proteins

The expression vector for rTOM70-HA was constructed as follows. The coding region of rTOM70 cDNA was amplified by PCR using pET28b-NHIS70 as the template and the following oligonucleotides as the primers: TOM70-5: 5'-TGCGGTACCACCATGGCCGCCTCTAAGCCCGTAGAG-3'; TOM70-6: 5'-CGCGGATCCTAATGTTCGGTGGTTTAAATCCGTA-3'.

Underlining in TOM70-5 and TOM70-6 denotes the restriction sites of *Kpn*I and *Bam*HI, respectively. The isolated fragment was subcloned into *Kpn*I-*Bam*HI-digested pcDNA3.1 in which HA-tag sequence had been inserted to create pcDNA-rTOM70HA. pcDNA-rTOM70S6HA was constructed by the Kunkel method using pcDNA-rTOM70HA as the template. rTOM70-GFP was constructed using pET28b-NHIS70 as the template and the following oligonucleotides as the primers: TOM70-7: 5'-TGCTCTAGAACCATGGCCGCCTCTAAGCCCGTAGAG-3'; TOM70-8: 5'-CGCTCTAGATAATGTTCGGTGGTTTAAATCCGTA-3'.

Underlining in TOM70-7 and TOM70-8 denotes the restriction sites of *Xba*I. The obtained PCR fragment was subcloned into the *Xba*I site of pRcG (Kanaji et al., 2000) to create pRc-rTOM70GFP. cDNAs coding rTOM70(1-69)GFP, rTOM70(42-69)GFP, rTOM70(1-66)GFP and rTOM70(1-65)GFP were all constructed by PCR using pRc-rTOM70GFP as the template and appropriate oligonucleotides as the primers: the 5'-upstream primers containing *Hind*III site and 3'-downstream primers containing *Xba*I site. Thus obtained fragments were subcloned into *Hind*III-*Xba*I digested pRcG.

Subcellular and submitochondrial fractionations

Subcellular fractionation of the rat liver was performed as described previously (Ishihara and Mihara, 1998). Submitochondrial fractionation by sucrose density gradient centrifugation was performed as follows. Mitochondria were diluted into 10 mM HEPES-KOH buffer (pH 7.4) containing 1 mM EDTA and the protease inhibitor cocktail (PIC: 5 μ g/ml each of leupeptin, antipain, chymostatin, and pepstatin) ('hypotonic buffer') and incubated at 0°C for 30 minutes. The mixture was sonicated on ice 5 times for 30 seconds each time and centrifuged at 5,000 *g* for 10 minutes to obtain the supernatant. This fraction was layered over a linear gradient of sucrose from 0.6 to 1.6 M in hypotonic buffer and centrifuged at 100,000 *g* for 15 hours at 4°C.

Protein import into mitochondria

The reaction mixtures containing 25 μ g mitochondria and rabbit reticulocyte-lysate-synthesized 35 S-Su9-DHFR, ADP/ATP carrier (AAC) or rTOM40 were incubated in 50 μ l of 10 mM HEPES-KOH buffer (pH 7.4) containing 1 mM ATP, 20 mM sodium succinate, 5 mM NADH, 1 mg/ml fatty acid-free bovine serum albumin and PIC ('import buffer') at 30°C for 30 minutes. When the import of 125 I- or 35 S-labeled recombinant pAd was assayed, 1/10 of the volume of

rabbit reticulocyte lysate or 20 µg/ml MSFS (Alam et al., 1994) was added to the reaction mixture, respectively. To examine the effect of antibodies against the import components on the preprotein import, mitochondria were pretreated with the antibodies in the homogenization buffer (Ishihara and Mihara, 1998) at 0°C for 30 minutes, washed once with the homogenization buffer, then subjected to the import reactions. The import of ³⁵S-rTOM70 into isolated rat liver mitochondria was performed as described above. After import, the reaction mixture was treated with 100 mM Na₂CO₃ (pH 11.5) as described previously (Kanaji et al., 2000) and the mitochondria and supernatant were subjected to SDS-PAGE and the gels analyzed for rTOM70 using a Bioimage Analyzer FLA2000 (Fuji). The membrane-insertion of ³⁵S-AAC and ³⁵S-rTOM40 was assessed as follows. After the import, the reaction mixtures were incubated with 100 µg/ml of proteinase K at 0°C for 30 minutes under hypotonic (for AAC) or isotonic (for rTOM40) conditions, followed by incubation with 1 mM PMSF at 0°C for 15 minutes. The reaction mixtures were centrifuged, the precipitates were resolved by SDS-PAGE, followed by fluorimaging analysis.

Immunoprecipitation of the TOM complex

The mitochondrial outer membranes were incubated with 10 mM HEPES-KOH buffer (pH 7.4) containing 2% (w/v) digitonin, 50 mM NaCl, 1 mM PMSF and 10% (v/v) glycerol ('solubilization buffer') at 0°C for 30 minutes, followed by centrifugation at 100,000 g for 15 minutes. The supernatant was incubated first with Protein A-Sepharose, centrifuged and the supernatant was incubated with anti-rTOM20, anti-rTOM40 or anti-rTOM70 IgG-bound Protein A-Sepharose at 4°C for 3 hours. The reaction mixtures were centrifuged at 5,000 rpm for 5 minutes, and the precipitates were washed three times with the solubilization buffer and suspended in the SDS-PAGE loading buffer. The eluted proteins were separated by SDS-PAGE and the gels were analyzed by immunoblotting.

Blue native PAGE

Blue native PAGE was performed as previously described (Schägger and von Jagow, 1991; Schägger et al., 1994). Mitochondrial outer membranes (50 µg) were solubilized in 50 µl solubilization buffer, and insoluble material was removed by centrifugation for 15 minutes at 100,000 g. The supernatant was mixed with 5 µl sample buffer (5% Coomassie brilliant blue G-250, 100 mM Bis-Tris, pH 7.0, 500 mM 6-aminocaproic acid) and electrophoresed through 5 to 16% polyacrylamide gradient gels. For the second dimensional gel analysis, individual lanes were excised from the first dimensional gel and subjected to Tricine SDS-PAGE.

Yeast two-hybrid assay

Maintenance and transformation of yeast cells, using the

A

Tom70						
Rats	MAASKPVEAA	MAAAAAPASG	NGVGSSTGTA	APGSGAGTLP	RW-----HVA	45
N. crassa	MAPTIPPPSV	PIPAATPVT-	-----VP	ADSSIWDRVS	NWVSEHKAVV	41
Rats	LAIGAP--LL	LGAGAMYLWS	RRRRRREAGG	R-GDASGLKR	NSEKKTPEGR	92
N. crassa	YTIAGVSVVI	TTAGVVVYLR	KGSEQKESGP	KLSKKERRKR	KQAEKASTSK	91
Rats	ASPALGSGPD	GSG--DSLEM	SSLD-----	-----RAQA	AKNK--GNKY	126
N. crassa	TEEAAPTQPK	AAAVESADEL	PEIDEESVVR	LSEDERKAYA	AKLKELGKKA	141
Rats	FKAGKYEQAI	QCYTEAISLC	PTEKNADLST	FYQNRAAAFE	QLQKWKEVAQ	176
N. crassa	YGSKDFNKAI	DLYSKAI-IC	KPD-----PV	YYSNRAACHN	ALAQWEQVVA	185
Rats	DCTKAVELNP	KYVKALPFRRA	KAHEKLDNKK	ECLLEDVTAVC	IILEGFQNEQS	226
N. crassa	DTTAALKLDP	HYVKALNRRA	NAYDQLSRYR	HALLDFTASC	IIDGFRNEQS	235
Rats	MLLADKVLKL	LGKENAKEKY	KNREPLMPSF	QFIKSYFSSF	TDDIISQPM-	275
N. crassa	AQAVERLLKK	FAENKAKEIL	ETKPKPLPSS	TFVGNLYQSF	RSKPRPEGLE	285
Rats	----LKGEKS	DEDKDKEGEA	LEVKENSGLY	KAKQYMEEEN	YDKIISECSK	321
N. crassa	DSVELSEETG	LGQLQLGLKH	LESKTGTGYE	EGSA-----A	FKKAL-----	325
Rats	EIDAQKGYMA	EALLLRATFY	LLIGSANAAK	PDLDKVISLK	EANVKLRANA	371
N. crassa	DLGELGPHEA	LAYNLRGTFH	CLMGKHEEAL	ADLSKSTELD	PA----MTQS	371
Rats	LIKRGTMCMQ	QQQPMLSTQD	FNMAAEIDPM	NSDVYHHRGQ	LKILLDLVEE	421
N. crassa	YTKRASMNLE	LGHDPKAEED	FNKATEQNAE	DPDIYYHRAQ	LHFTKGEPAE	421
Rats	AVADFDACIR	LRPKFALAQA	QKCFALYRQA	YTANNSSQVQ	AAMKGFEETVI	471
N. crassa	AAKDYQKSID	LDSDFIFPSHI	QLGVTQYKM-	-----GSTA	SSMATFRRCM	464
Rats	KKFPRCAEGY	ALYAQALTDQ	QQFGKADEMY	DKCIDLEPD-	-----NATTY	515
N. crassa	KNFDQTPDVI	NYYGELLLDQ	NKFQEAIEKF	DTAIALEKET	KPMCMMNVLP	514
Rats	VHKGLLQLQW	KQDLDKGLEL	ISKAIEIDNK	CDPAYETMGT	IEVQRGNMEK	565
N. crassa	INKALALFQW	KQDYAEAEQL	CEKALIIDPE	CDIAVATMAQ	LLLQOQGVVE	564
Rats	AIDMFKAIN	LAKSEMEMAH	LYSLCDAAHA	QTEVAKYKYL	KPPTL-----	610
N. crassa	ALKFFERAAE	LARTEGELVN	ALSYAEATRT	QIQVQENYPE	LASKLQGMMSG	614
Rats	-----	-----	-----	-----	-----	619
N. crassa	GPGMR-----	-----	-----	-----	-----	619

B

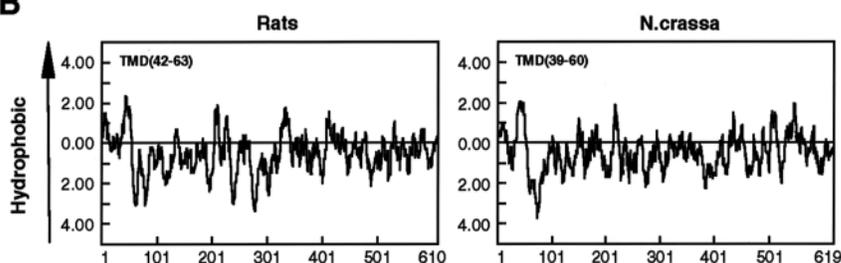


Fig. 1. Comparison of the deduced amino-acid sequences of Tom70 from rats and *N. crassa*. (A) The sequences of rats and *N. crassa* Tom70 were aligned using the Multiple Sequence program (DNASIS). The amino-acid residues conserved among species were shaded, TMDs were boxed and TPR motifs were underlined and numbered. TPR motifs were searched for using the Protein Families database alignment program (Pfam). (B) Comparison of hydropathy profiles of Tom70 from rats and *N. crassa* using the Kyte and Doolittle algorithm at a span setting of 10 amino-acid residues.

MATCHMAKER GAL4 Two-Hybrid System 2 (Clontech), were performed according to the manufacturer's protocol. The cytosolic domain of rTOM20 (D25-E145), rTOM22 (M1-R82), rTOM70 (R64-L610) and OM37 (R41-D338) were amplified by PCR, and the PCR fragments were inserted separately downstream of the GAL4 DNA-binding domain (BD) in the pAS2-1 plasmid (*TRP1*) or into the

GAL4-activating domain (AD) in the pACT2 plasmid (*LEU2*). Two-hybrid interactions were assayed using either the *HIS3* reporter or the *lacZ* reporter systems. Cotransformation of two hybrid vectors into *S. cerevisiae* Y190 (*MATa*, *ura3-52*, *his3-200*, *ade2-101*, *lys2-801*, *trp1-901*, *leu2-3*, *112*, *gal4Δ*, *gal80Δ*, *cyh2*, *LYS2::GALIUS-HIS3TATA-HIS3*, *MEL1URA3::GALUAS-GALITATA-lacZ*) was performed according to the manufacturer's instruction. The transformants were screened for their potential to grow on synthetic complete medium lacking tryptophan, leucine and histidine. The transformants were also assayed for β -galactosidase activity of the *lacZ* reporter gene according to Adams et al. (Adams et al., 1997).

Transfection of COS-7 cells and immunofluorescence microscopy

COS-7 cells were cultured on coverslips in 35-mm dishes in 2 ml of DMEM supplemented with 10% fetal calf serum at 37°C overnight under an atmosphere of 10% CO₂ in air. Transfection was performed using FuGene 6 reagent (Roche Molecular Biochemicals). The cells were incubated for 24 hours. When mitochondria were to be stained, 100 nM MitoTracker Red CMX Ros (Molecular Probes, Eugene, OR) was added to the medium and incubated for 20 minutes before fixation. The cells were fixed with 50% methanol/50% acetone for 2 minutes at room temperature. The coverslips were incubated with rabbit polyclonal (BABCO) or mouse monoclonal (BABCO) anti-HA epitope tag antibodies at room temperature for 1 hour, washed and then incubated with FITC-conjugated goat antibodies against rabbit IgGs. To monitor the effect of brefeldin A (BFA) on intracellular localization, cells were treated with 5 μ g/ml BFA for 1 hour. To stain the endoplasmic reticulum (ER), the fixed cells on coverslips were incubated with rabbit anti-rat Calnexin (StressGen) antibodies for 1 hour and then with FITC-conjugated goat antibodies against rabbit IgGs. The images were obtained and analyzed using a confocal microscope, Radiance 2000 (BioRad).

Results

Isolation of rat TOM70 cDNA

In the EST database, there were five partially overlapping sequences predicted to encode the partial amino acid sequence of the mouse counterpart of fungal Tom70. We screened the rat λ gt10 cDNA library for the rat counterpart using the assembled nucleotide sequence as the probe and obtained a cDNA clone of ~2.5 kbp that carried a putative open reading frame of 1830 bp encoding a 67,441 Da protein. We concluded that this clone encoded the entire region of the authentic OM70 protein because (1) the nucleotide sequence around the putative initiator methionine fits well with the Kozak motif (GTCATGG for A/GxxATGG), and (2) the in vitro-translated protein as well as the authentic protein, as detected by immunoblotting or autoradiography, exhibited the same mobility on SDS-PAGE (Fig. 2A), and the molecular size estimated from the mobility coincided well with that calculated from the predicted sequence (Fig. 1).

The sequence identity of OM70 with *N. crassa* and *S. cerevisiae* Tom70 was 28.1% and 20.1%, respectively (Fig. 1A). The predicted sequence of OM70 exhibited a similar hydrophathy profile to *N. crassa* and *S. cerevisiae* Tom70 and also contained a TMD in the N-terminus (Fig. 1B). The cytoplasmic domain of OM70 contained 10 TPR motifs, and 7 and 9 TPR motifs in *S. cerevisiae* and *N. crassa*, respectively. OM70, as demonstrated by the experiments described below, constitutes a structural and functional homologue of fungal

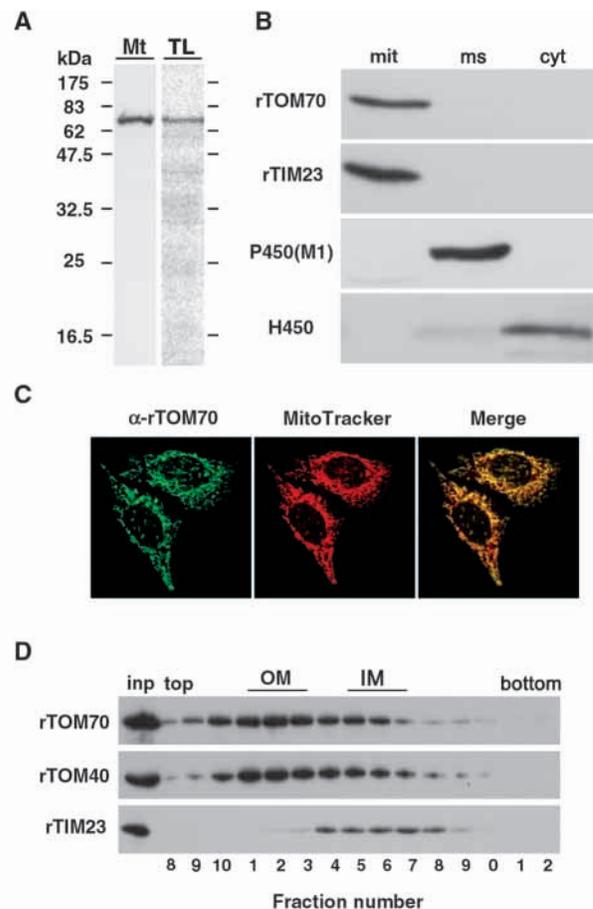


Fig. 2. Subcellular localization of rTOM70 in rat liver cells. (A) Comparison of SDS-PAGE profiles of mitochondrial authentic OM70 (Mt) and the in vitro synthesized OM70 (TL) as detected by autoradiography and immunoblotting, respectively. (B) 50 μ g each of mitochondria (mit), microsomes (ms) and cytosol (cyt) proteins from the rat liver were subjected to SDS-PAGE and immunoblotting using antibodies against the indicated proteins. The immunoreacted bands were visualized by ECL (Amersham). (C) Immunofluorescence detection of TOM70 in HeLa cells. HeLa cells were incubated with MitoTracker at 37°C for 20 minutes, fixed, stained with rabbit IgGs against rTOM70 and then stained with FITC-conjugated goat antibodies against rabbit. Images were obtained and analyzed using confocal fluorescence microscopy. (D) Submitochondrial localization of rTOM70. Submitochondrial fractionation by sucrose density gradient centrifugation was performed as described in the Materials and Methods. After centrifugation, the samples were fractionated from the top of the tubes. The fractions were TCA-precipitated and subjected to SDS-PAGE and then the proteins were detected by immunoblotting with antibodies against the indicated proteins. Inp represents 20 μ g of the unfractionated sample.

Tom70; thus, we refer to this protein as rTOM70 hereafter. While this study was in progress, the amino-acid sequences of the predicted human and *Drosophila* homologues of Tom70 with 92% and 40% identity to the rat sequence, respectively, were deposited in the database (GenBank accession numbers NM_014820 and AE003634, respectively). Partial DNA sequence of a rat homologue of the fungal Tom70 had also been deposited in the database (EMBL accession number: AJ243368).

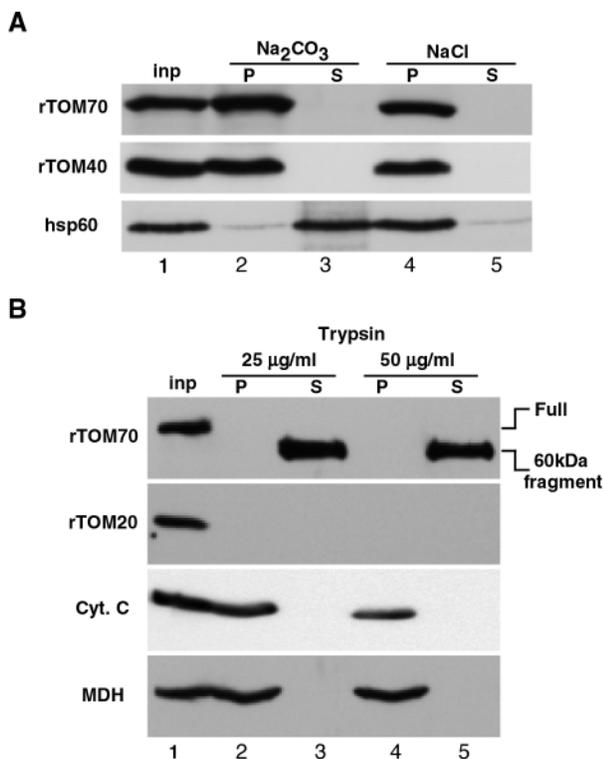


Fig. 3. Characterization of rTOM70 as a mitochondrial integral membrane protein. (A) Mitochondria were treated with either 100 mM Na₂CO₃ (pH 11.5) or 1 M KCl at 0°C for 30 minutes. The reaction mixtures were centrifuged to separate the supernatant (S) and precipitate (P) fractions, and each fraction was subjected to SDS-PAGE followed by immunoblot analysis using antibodies against the indicated proteins. (B) Rat liver mitochondria were treated with the indicated concentrations of trypsin at 0°C for 20 minutes and then soybean trypsin inhibitor was added to the reaction mixtures and incubated at 0°C for 20 minutes. The reaction mixtures were centrifuged to separate the supernatant and precipitate fractions, and each fraction was resolved by SDS-PAGE and analyzed by immunoblotting with the antibodies against the indicated proteins.

Subcellular and submitochondrial localization of rTOM70
 Subfractionation of the rat liver and subsequent immunoblotting indicated that rTOM70 was cofractionated with the mitochondrial marker protein, rTIM23, but not with either cytochrome P450 (M1) or cytochrome H450, the marker proteins of rat liver microsomes (Matsumoto et al., 1986) and cytosol (Ishihara et al., 1990), respectively (Fig. 2B). Immunofluorescence microscopy also revealed that the endogenous TOM70 colocalized with MitoTracker as a filamentous structure in HeLa cells (Fig. 2C).

To examine the submitochondrial localization of rTOM70, mitochondria were sonicated under hypotonic conditions and subjected to sucrose density gradient centrifugation. As shown in Fig. 2D, rTOM70 co-fractionated with the marker protein of the outer membrane, rTOM40, but not with rTIM23, an intrinsic inner membrane protein (Ishihara and Mihara, 1998). rTOM70 was resistant to alkaline carbonate (pH 11.5) extraction or high salt-treatment of the mitochondria (Fig. 3A). The intrinsic inner membrane protein, rTOM40, behaved similarly to rTOM70 in these treatments, whereas hsp60, the

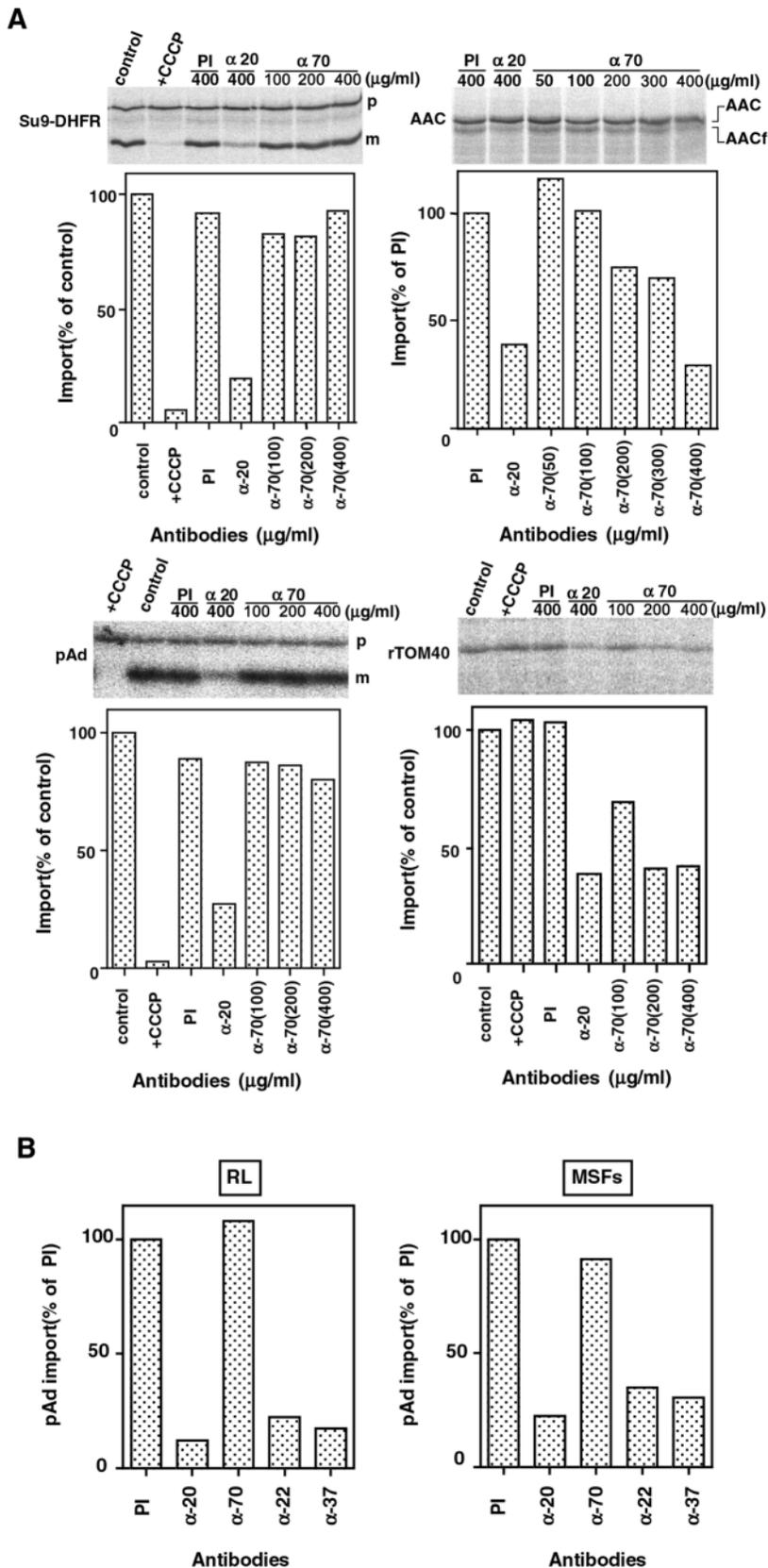
soluble protein in the matrix, was easily solubilized by alkaline-carbonate treatment. The topology of rTOM70 in the outer membrane was then probed by trypsin treatment of the mitochondria. As shown in Fig. 3B, 25 µg/ml trypsin cleaved rTOM70, and a cytoplasmic fragment of ~60 kDa was released into the supernatant fraction, whereas rTOM20, which is the major import receptor, with the N-terminal membrane anchor extruding the bulk portion to the cytosol (Iwahashi et al., 1997), was completely digested by this treatment. The matrix-localizing malate dehydrogenase (MDH) as well as the intermembrane space-localizing cytochrome c was also resistant to this treatment, indicating that both the outer and inner mitochondrial membranes remained intact during these treatments.

These results and the predicted primary amino-acid sequence indicate that rTOM70 is an integral membrane protein that is anchored to the outer membrane through the N-terminal TMD, extruding the bulk TPR-containing portion to the cytosol with a membrane topology similar to that of fungal Tom70 (Söllner et al., 1990).

Antibodies against rTOM70 inhibit mitochondrial import of preproteins with the internal targeting signal

We examined the effect of antibodies against rTOM70 on the preprotein import into mitochondria. As shown in Fig. 4A, specific IgGs against rTOM70, at concentrations as high as 400 µg/ml, did not inhibit mitochondrial import of pre-Su9-DHFR and pAd, the preproteins with the matrix-targeted and cleavable presequence, whereas anti-rTOM20 IgGs at the same concentration inhibited their import (Fig. 4A, left panels). We then examined the effect of the antibodies on the mitochondrial import of the AAC, the inner membrane protein with the internal targeting signal (Pfanner et al., 1987; Wiedemann et al., 2001). The import of AAC was assessed by measuring the formation of the fragment AACf, which is produced from correctly inserted AAC by proteinase K digestion of the mitoplasts (Wiedemann et al., 2001; Kübrich et al., 1998; Ishihara and Mihara, 1998). IgGs against rTOM70 inhibited AAC import in a dose-dependent manner (Fig. 4A, right panel). Similarly, they inhibited insertion of rTOM40 into mitochondria (Fig. 4A, right panel). Anti-rTOM20 IgGs also inhibited both insertion reactions. These results were consistent with the reported specificity of fungal Tom70, which functions as the receptor of preproteins with an internal targeting signal, such as the phosphate carrier, AAC, Tom40 (Keil et al., 1993) and Tim 54 (Kurz et al., 1999).

In rat liver mitochondria, antibodies against OM37 inhibit both mitochondria binding of the pAd-MSF complex and ATP-induced subsequent transport of pAd across the outer membrane via rTOM20, thus inhibiting the overall reaction of pAd import into the mitochondria (Komiya et al., 1996; Komiya and Mihara, 1996). To probe the function of rTOM70 in the preprotein transport pathways of the rat mitochondrial outer membrane, we examined the effects of anti-rTOM70 and anti-OM37 IgGs on the MSF-assisted mitochondrial import of pAd. As shown in Fig. 4B (right panel), anti-OM37 IgGs and anti-TOM20 IgGs efficiently inhibited the MSF-supported import of pAd, consistent with our previous report (Komiya et al., 1996). Anti-rTOM70 IgGs did not inhibit the same import. The same results were obtained with rabbit reticulocyte lysate



to provide cytoplasmic chaperones (Fig. 4B, left panel). Taken together, these findings indicate that rTOM70 functioned as the import receptor for preproteins with an internal targeting signal

Fig. 4. Effect of antibodies against rTOM70 on mitochondrial preprotein import.

(A) Mitochondria were preincubated with the indicated concentrations of preimmune IgG (PI), anti-rTOM70 IgG ($\alpha 70$) or anti-rTOM20 IgG ($\alpha 20$) at 0°C for 30 minutes, reisolated and subjected to the import of pre-Su9-DHFR, pAd, AAC and rTOM40. When import of AAC or rTOM40 was assayed, the reaction mixtures were treated with proteinase K at 0°C for 30 minutes under hypotonic (for AAC) or isotonic (for rTOM40) conditions after import. After the reaction, 1 mM PMSF was added to the reaction mixtures, the mixture was centrifuged, the precipitates were resolved by SDS-PAGE and the gels were analyzed by FLA2000. Other conditions were as described in the Materials and Methods. The import efficiencies were expressed relative to the control import in the absence of IgG or in the presence of preimmune IgG. Where indicated, the import reactions were performed in the presence of 10 μM CCCP. (B) Effect of anti-rTOM70 IgGs on the MSF- or reticulocyte-lysate-supported mitochondrial import of pAd. ^{35}S -pAd or ^{125}I -pAd was subjected to the import reaction in the presence of MSFs (right) or rabbit reticulocyte lysate (left), respectively, using the mitochondria that had been treated with the indicated IgGs (200 $\mu\text{g}/\text{ml}$ each) and reisolated. After the reaction, the reaction mixtures were resolved by SDS-PAGE and the gels were analyzed by autoradiography. Other conditions were as described in Materials and Methods.

and did so probably upstream of OM37 or OM37-independently, upstream of rTOM20.

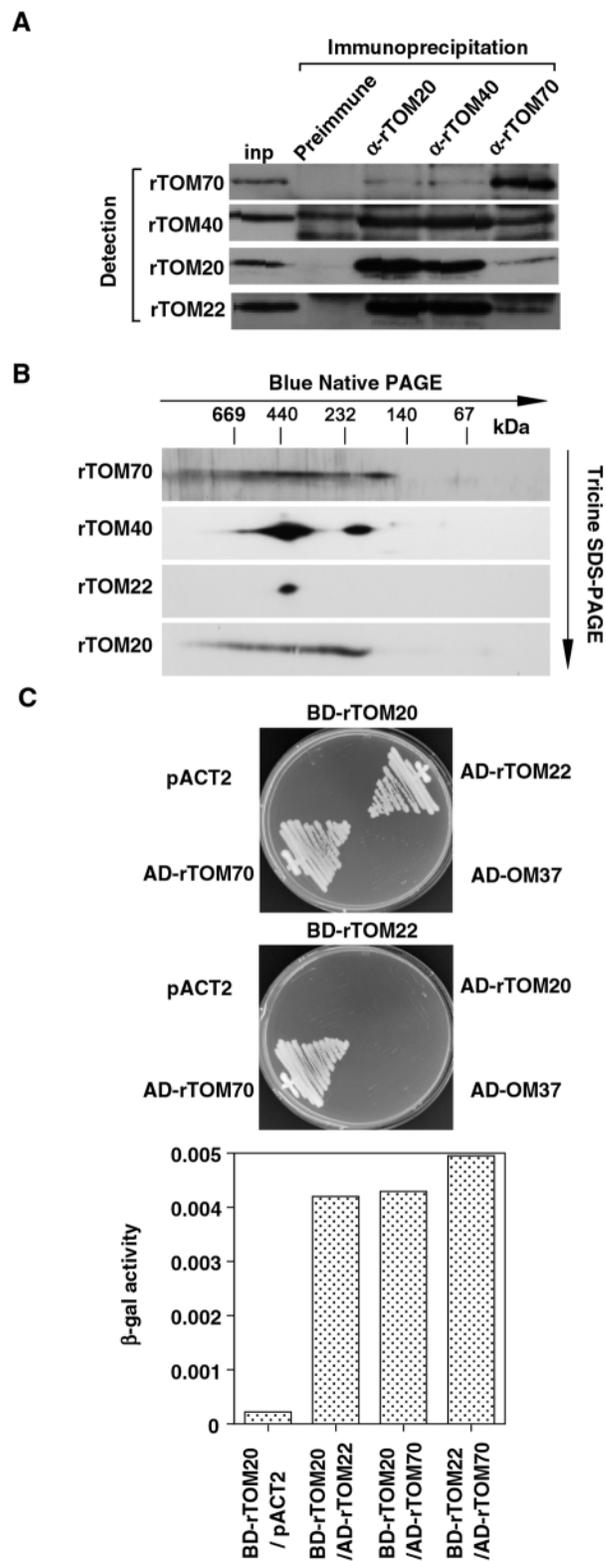
rTOM70 is loosely associated with the ~ 400 kDa TOM complex containing rTOM40 and rTOM22

S. cerevisiae Tom40 forms an ~ 400 kDa complex with Tom22, Tom7, Tom6 and Tom5 in digitonin-solubilized membranes (Dekker et al., 1998), whereas most of the import receptors were not observed in the complex. A similar complex was detected in *N. crassa* mitochondria, although Tom5 was not identified (Künkele et al., 1998; Ahting et al., 1999). We therefore examined the interaction of rTOM70 with the other import components of rat mitochondrial outer membranes using immunoprecipitation. Rat liver mitochondrial outer membranes were solubilized using 2% digitonin-50 mM NaCl and subjected to immunoprecipitation using antibodies against rTOM20, rTOM40 or rTOM70. The import components of the outer membrane recovered in the precipitates were then analyzed by immunoblotting. As shown in Fig. 5A, antibodies against rTOM20 and rTOM40 efficiently precipitated the other import components, whereas the antibodies against rTOM70 only inefficiently precipitated

Fig. 5. rTOM70 is loosely associated with the ~400 kDa complex containing rTOM22 and rTOM40. (A) Interaction of rTOM70 with the import components of the outer membrane as probed by coimmunoprecipitation. The outer membranes were solubilized with 2% digitonin-50 mM NaCl, and aliquots of the supernatant were subjected to immunoprecipitation at 4°C for 3 hours using preimmune, anti-rTOM20, anti-rTOM40 or anti-rTOM70 IgGs. The immunoprecipitates were resolved by SDS-PAGE and the proteins were visualized by immunoblotting with antibodies against the indicated proteins. Inp represents 20% of an aliquot of the supernatant fraction. Other conditions were as described in the Materials and Methods. (B) Analysis of rat TOM proteins with blue native PAGE. Rat liver mitochondrial outer membranes were solubilized with 2% digitonin-containing buffer, and solubilized supernatant was subjected to blue native PAGE as described in the Materials and Methods. Gel slots were excised and subjected to the second dimensional Tricine SDS-PAGE. The gels were analyzed by immunoblotting with the indicated IgGs. Marker proteins used were serum albumin, 66 kDa; lactate dehydrogenase, 140 kDa; catalase, 232 kDa; apoferritin, 440 kDa; and thyroglobulin, 669 kDa. (C) Interaction of rTOM70 with rTOM22 and rTOM20 as revealed by the yeast two-hybrid assay. Host strain Y190 was transformed with two plasmids, one encoding the GAL4 DNA-binding domain (BD) fused to the cytosolic domain of either rTOM20 (BD-rTOM20) or rTOM22 (BD-rTOM22) and the other encoding the GAL4-activating domain (AD) fused to the cytosolic domains of the import components (AD-rTOM20, AD-rTOM22, AD-rTOM70, or AD-OM37). Positive interactions were verified by growth of the transformants on synthetic complete medium without histidine (upper panel) or β -galactosidase activity (lower panel). As a control, the host strain was transformed with an empty vector encoding AD (pACT2).

rTOM20, rTOM22 and rTOM40, indicating that rTOM70 loosely interacted with the rat TOM complex. Solubilization by 2% digitonin-200 mM NaCl completely disrupted this weak interaction (data not shown).

We then examined the interaction of rTOM70 with the TOM complex using blue native PAGE, which allows separation of the protein complex under native conditions. As shown in Fig. 5B, the major fraction of rTOM40 migrated as an ~400 kDa complex, and the small fraction of rTOM40 was detected in the ~190 kDa complex. rTOM22 was contained in the ~400 kDa complex. In contrast to the results obtained by immunoprecipitation, rTOM70 and rTOM20 were mostly dissociated from the ~400 kDa complex, probably because of destabilization by the negative charge of the Coomassie brilliant blue (Ahting et al., 1999). We then examined the interaction of rTOM70 with other import receptors using a yeast two-hybrid assay. When assayed using BD-rTOM20 as bait, yeast cells expressing either AD-rTOM22 or AD-rTOM70 grew on synthetic medium without histidine (Fig. 5C, upper panel), and the cell lysates exhibited β -galactosidase activity (Fig. 5C, lower panel). Neither AD-OM37 nor the empty vector pACT2 gave positive interaction signals. When BD-rTOM22 was used as bait, only the cells expressing AD-rTOM70 gave positive interaction signals (Fig. 5C) and neither rTOM20 nor OM37 exhibited positive signals. Thus, rTOM70 interacts with rTOM20 and rTOM22 through the cytosolic domains in vivo. In yeast, Tom20 directly interacts through its TPR motif with Tom70 (Haucke et al., 1996). The involvement of the TPR sequences in rTOM70 in the interactions described herein remains to be determined.



Characterization of the mitochondria-targeting signal of rTOM70

In yeast, amino acids at positions 1 through 10 of Tom70 comprise a hydrophilic, positively charged segment, which functions as the matrix-targeting signal and a segment with nonpolar amino-acid residues at positions 11 through 29

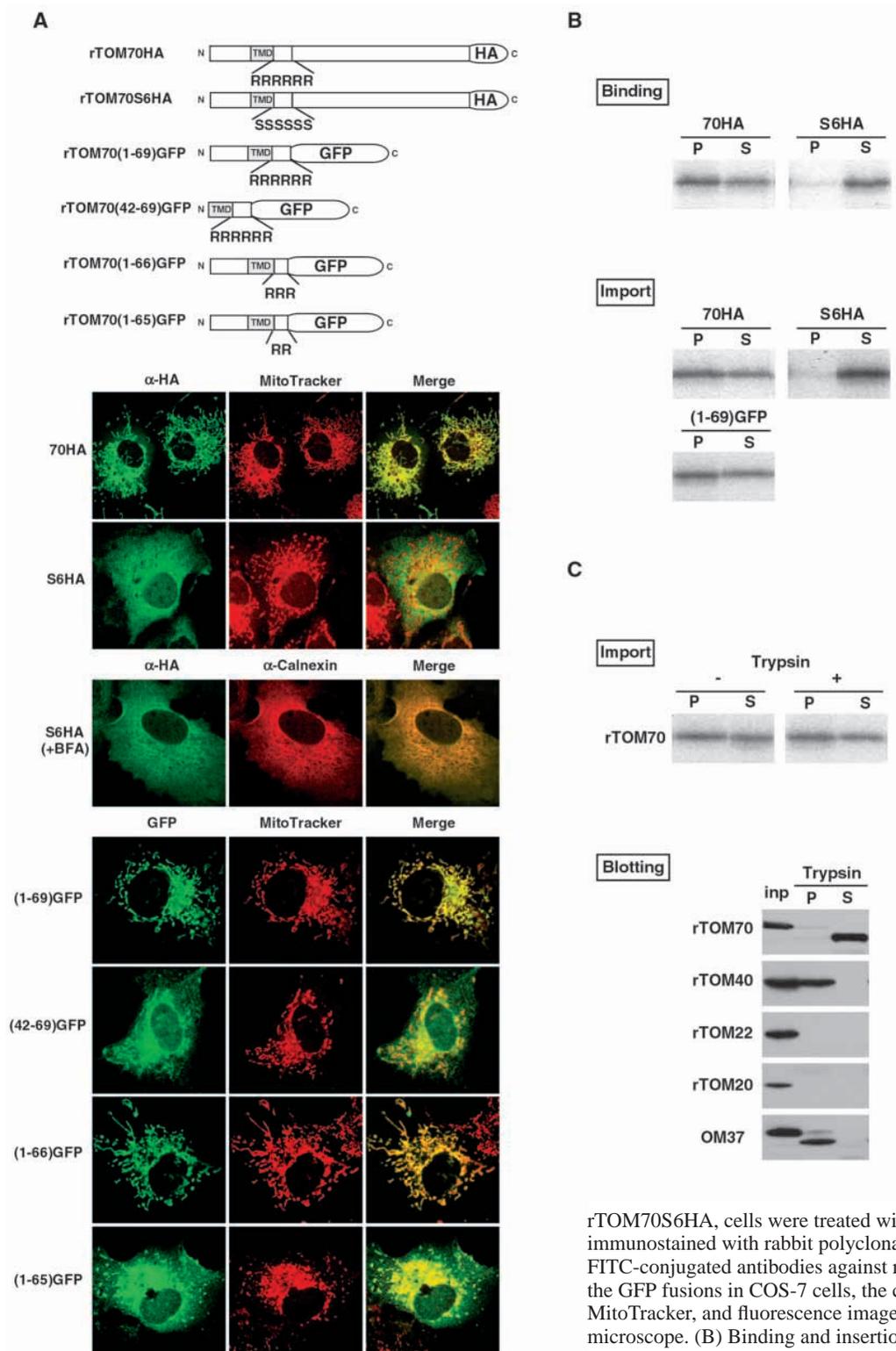


Fig. 6. Characterization of the mitochondria-targeting signal of rTOM70. (A) Basic amino acids at the C-terminal flanking region of the TMD are critical for targeting rTOM70. COS-7 cells were transfected with the indicated constructs in the expression vectors. After 24 hours, the cells were incubated with MitoTracker, then the cells were fixed and immunostained with IgG against rabbit polyclonal anti-HA epitope tag antibodies and FITC-conjugated antibodies against rabbit IgG. To monitor the localization of

rTOM70S6HA, cells were treated with 5 μ g/ml BFA for 1 hour, fixed and immunostained with rabbit polyclonal IgGs against rat Calnexin and FITC-conjugated antibodies against rabbit IgGs. To detect localization of the GFP fusions in COS-7 cells, the cells were co-stained with MitoTracker, and fluorescence images were obtained using a confocal microscope. (B) Binding and insertion of rTOM70HA, rTOM70S6HA or rTOM70(1-69)GFP into mitochondria in vitro. Reticulocyte-lysate-synthesized 35 S-labeled proteins were subjected to mitochondrial import. (C) Membrane integration of rTOM70 into trypsin-treated mitochondria. Mitochondrial import assay of rTOM70 was performed using 20 μ g/ml trypsin-treated or untreated mitochondria as described in (B). An aliquot of sodium-carbonate-treated mitochondria was centrifuged to separate the membrane (P) and supernatant (S) fractions, and both fractions were subjected to immunoblot analysis using the antibodies against the indicated proteins.

functions as the stop-transfer sequence (Hase et al., 1984). McBride et al. (McBride et al., 1992) later demonstrated that the nonpolar segment (amino acids 11-29) functions as the signal-anchor sequence, and the hydrophilic N-terminal segment increases the efficiency of binding and insertion of Tom70. In contrast, however, the hydrophilic N-terminal segments of rats or *N. crassa* Tom70 do not resemble the matrix-targeting signal; the segment of rTOM70 comprises an N-terminal hydrophilic stretch at amino-acid positions 1 through 41, the hydrophobic TMD at positions 42 through 63 and six arginine residues. We recently demonstrated for the N-terminal membrane-anchor protein rTOM20 that the TMD hydrophobicity and at least one net positive charge at the C-terminal flanking segment are critical for mitochondria targeting (Kanaji et al., 2000). We examined whether the targeting signal of rTOM70 follows these criteria. For this purpose, we constructed rTOM70HA in which the hemagglutinin A (HA)-tag was fused to the C-terminus of rTOM70. When expressed in COS-7 cells, rTOM70HA colocalized with MitoTracker in a dispersed filamentous structure (Fig. 6A upper panel). In contrast, a mutant in which six arginine residues in the C-terminal flanking region of the TMD were changed to serine residues (rTOM70S6HA) localized to the reticular structure throughout the cells as well as to the plasma membranes; a structure that is distinct from mitochondria identified by MitoTracker. In the presence of BFA, which blocks anterograde vesicular transport from the ER to the Golgi compartment, rTOM70S6HA colocalized with the ER marker Calnexin (Fig. 6A lower panel), indicating that the fusion protein was localized in the secretory pathway and transported back to the ER in the presence of BFA. The GFP-fusion construct carrying the N-terminal segment at amino-acid positions 1 to 69 of rTOM70 [rTOM70(1-69)GFP] colocalized well with MitoTracker to the mitochondria (Fig. 6A). rTOM70(42-69)GFP, the construct in which the N-terminal hydrophilic segment (residues 1-41) of TOM70(1-69)GFP was deleted also localized to mitochondria, although the targeting fidelity was slightly lower and a small fraction localized to the organelles of the secretory pathway (Fig. 6A). This effect remains to be investigated.

The above results were also confirmed in an in vitro import system. As shown in Fig. 6B, reticulocyte-lysate-synthesized rTOM70HA was targeted to and imported into the mitochondrial membrane. The same result was obtained for rTOM70(1-69)GFP. In contrast, rTOM70S6HA, which failed to be targeted to mitochondria in vivo, also failed to bind and insert into the mitochondrial membrane in vitro. We further found that rTOM70(1-66)GFP carrying three arginine residues in the C-terminal vicinity of the TMD localized correctly to mitochondria, whereas rTOM70(1-65)GFP carrying two arginine residues in the same flanking region did not (Fig. 6A). Therefore, the segment of amino-acid residues 1 through 66 were sufficient to function as a mitochondria-targeting signal. These results also suggested that at least three basic residues in the C-terminal flanking region of the TMD were necessary. We then examined the requirements of the import receptors for the insertion of rTOM70 into a mitochondrial outer membrane using an in vitro import system and found that the membrane insertion of rTOM70 was not affected by trypsin-treatment of mitochondria, which degraded rTOM20, rTOM22, OM37 and rTOM70 but not rTOM40 (Fig. 6C). Thus, rTOM70 was

targeted to and inserted independently of the import receptors into the outer membrane, probably directly into rTOM40, although involvement of the other integral components of the rat TOM core complex (OM5, OM7.5, and OM10) (Suzuki et al., 2000) or the OM37 fragment is not yet known.

Discussion

We identified OM70 as a receptor of the preprotein translocase system of the mammalian mitochondrial outer membrane, TOM70. Although the sequence identity was low (28.1% and 20.1% identity with *N. crassa* and *S. cerevisiae* Tom70, respectively), OM70 exhibited structural and functional similarity to fungal Tom70. Therefore, mammalian mitochondria contain a similar set of the import receptors to yeast: TOM70, TOM22 and TOM20. In addition, they contain several novel components that seem to function as the import receptor: TOM34 (Nuttall et al., 1997), metaxin (Armstrong et al., 1997), and OM37 (Komiya et al., 1996). The precise function of most of these components in preprotein import is not known at present; that is, it is not known whether they function as independent import receptors with distinct recognition specificity or whether they function sequentially in the import pathway etc. We previously demonstrated that OM37, the 37 kDa outer membrane protein of rat liver mitochondria, functions as the receptor of the preprotein-MSF complex upstream of rTOM20 in the preprotein transport pathway; the antibodies against OM37 inhibited both docking of the pAd-MSF complex and translocation of the pAd to TOM20 (Komiya et al., 1996; Komiya and Mihara, 1996). The antibodies against OM37 also inhibited mitochondrial import of AAC (M.M. and K.M., unpublished). In the present study, anti-rTOM70 antibodies inhibited mitochondrial import of AAC but not the import of the cleavable presequence-containing preproteins. Taken together, these results suggest either that rTOM70 functions upstream of OM37 and rTOM20 as the receptor of preproteins with an internal mitochondria-targeting signal such as AAC and rTOM40 or that rTOM70 functions in the import pathway upstream of rTOM20 but independently of OM37. The functional characterization of these components remains to be analyzed in detail.

We previously demonstrated that TMD hydrophobicity and a net positive charge at the C-terminal flanking segment of TMD are critical for mitochondria targeting in rTOM20 (Kanaji et al., 2000). These structural features are conserved among several other mitochondrial outer membrane proteins, including Tom20 from various species, Tom70 from *N. crassa* and rats and OM37. Here we demonstrated that the amino acids at positions 1 through 66 of rTOM70 are sufficient to function as the mitochondria-targeting signal, and the basic amino-acid cluster in the C-terminal flanking region of TMD is critical for this function. These features are in marked contrast to *S. cerevisiae* Tom70. It has a composite topogenic signal at the N-terminus: a hydrophilic, positively charged 11 residue segment that functions as the matrix-targeting signal and a 19 residue TMD that functions as the stop-transfer sequence (Hase et al., 1984). The in vitro studies demonstrated that the TMD functions as the signal-anchor sequence and is both necessary and sufficient for targeting, and residues 1 through 10 enhanced the rate of import (McBride et al., 1992). Therefore, targeting and insertion mechanisms of rTOM70 are quite different from

S. cerevisiae Tom70. Rather, the structural feature of the TMD and the flanking regions of rTOM70 resemble those of *N. crassa* Tom70. The rTOM70 mutant, in which the basic amino-acid residues at the C-terminal flanking region of the TMD were replaced by serine, was unable to be targeted to mitochondria in vivo and, instead, was mistargeted to the secretory pathway. This was clearly demonstrated using an in vitro import assay; rTOM70S6HA failed to bind to and insert into the mitochondria. Therefore, the region covered by the N-terminal hydrophilic segment (residues 1-41) and the following TMD does not function by itself as the matrix-targeting signal. Targeting and insertion strictly required the downstream basic amino-acid residues. We speculate that the TMD of rTOM70 is recognized by SRP as soon as it emerges from the ribosome, but the basic amino-acid residues at the C-terminal flanking segment interfere with its function and eventually target the nascent rTOM70 to mitochondria, as is the case for rTOM20 (Kanaji et al., 2000).

rTOM70 was efficiently inserted in vitro into the trypsin-treated mitochondria in which the import receptors rTOM70, rTOM20, rTOM22 and OM37 were removed. Thus, as is the case for fungal Tom70 (Schlossmann and Neupert, 1995), rTOM70 bypasses the import receptors and inserts into the TOM core complex, probably directly into rTOM40. This is in marked contrast to the results demonstrated by McBride et al. (McBride et al., 1992). Insertion into rat heart mitochondria of a hybrid protein pOMD29 in which the N-terminal mitochondria-targeting signal (residues 1-29) of yeast Tom70 was fused to dihydrofolate reductase is significantly reduced by pretreatment of the mitochondria with trypsin (McBride et al., 1992). In contrast, import of *N. crassa* Tom70 is not affected by trypsin pretreatment of the *N. crassa* mitochondria (Schlossmann and Neupert, 1995), again suggesting a similarity in the insertion mechanism of Tom70 between rats and *N. crassa*.

We speculate that a cytoplasmic factor recognizes these structural features of the mitochondria-targeting signal of N-terminal anchored outer membrane proteins and directs them to the TOM complex. These possibilities as well as the mechanism of membrane integration require further analysis.

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