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Identification and Characterization of a New Tom40 Isoform, a Central Component of Mitochondrial Outer Membrane Translocase

Jun-ya Kinoshita, Katsuyoshi Mihara* and Toshihiko Oka

Department of Molecular Biology, Graduate School of Medical Science, Kyushu University, Fukuoka 812-8582, Japan

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Newly synthesized precursors are transported into mitochondria through an outer membrane translocase, TOM. Tom40, a central pore-forming component, interacts directly with precursors to help them translocate across the outer membrane. We identified a new isoform of rat Tom40, Tom40B, which is conserved among mammals and exhibits significant similarities to Tom40 in other eukaryotes. Tom40B is an integral protein localized on the mitochondrial outer membrane, and expressed widely in all tissues examined except testis. Deletion mutant analysis revealed that the 28 amino acid residues at the carboxyl terminus were crucial for the mitochondrial targeting of Tom40B. Tom40B co-precipitated with other Tom components and formed a large protein complex. Furthermore, Tom40B directly bound to precursors of the matrix-targeted proteins with high affinities, comparable to those of Tom40A, a previously identified isoform. These findings indicate that Tom40B is a functional component of mitochondrial outer membrane translocase.

Key words: mitochondria, mitochondrial targeting sequence, presequence binding, TOM complex, Tom40 isoform.

Abbreviations: BN, blue native; ER, endoplasmic reticulum; HA, hemmagglutinin; MPP, mitochondrial processing peptidase; PBS, phosphate buffered saline; RT-PCR, reverse transcriptase-polymerase chain reaction; SCC, side chain cleavage; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; SPR, Surface Plasmon Resonance; TOM, translocase of the outer membrane.

Most mitochondrial proteins are synthesized on the free ribosomes in the cytoplasm and then targeted to the mitochondria. Precursor proteins, except a few outer membrane proteins, are translocated across the outer membrane through the translocase of the outer membrane (TOM) and destined for the matrix, inner membrane and outer membrane. The yeast TOM complex is composed of at least seven proteins (Tom70, Tom40, Tom22, Tom20, Tom7, Tom6 and Tom5). Tom70 and Tom20 contain large hydrophilic domains that extend into the cytoplasm and act as receptors for precursor proteins (*1–4*), whereas Tom40, Tom22 and three small subunits (Tom7, Tom6 and Tom5) form a core complex with a molecular size of ~400 kDa, which facilitates the translocation of preproteins across the outer membrane (*5*, *6*). Tom22 has a role in translocation not only as a central receptor of precursor proteins, but also as a multifunctional organizer of the TOM machinery (*7*). The small Tom subunits maintain the stability of the TOM complex and influence the translocation of precursor proteins into the organelle (*5*, *8–10*). Tom40 is a pore-forming component of the TOM complex that is essential for cell viability (*11*, *12*).

The enriched β-sheet structure of Tom40 contributes to forming a cation-selective high conductance channel that specifically binds to and translocates precursor proteins (*13–17*). Negative electron microscopy show that the TOM complex is organized as one to three rings with a diameter of 20 ~ 30Å (*15*, *18*). In addition to its structural role as the pore, Tom40 also binds precursors at both the *cis* (cytoplasm) and *trans* (intermembrane space) sites of the outer membrane (*19*, *20*). Refolded recombinant rat Tom40 protein binds to precursor proteins, initially through ionic interaction, followed by salt-resistant interaction with high affinity, depending on the unfolding state of the preproteins (*16*, *21*).

To date, several isoforms of TOM complex components have been reported. In yeast, Tom71, an isoform of Tom70, shares 53% amino acid sequence identity with Tom70 and has similar biochemical properties as a receptor for precursor proteins (*22*). In fly, Tom20boy and Tom40boy, homologues of Tom20 and Tom40, respectively, are expressed specifically in the male germ line (*23*). Tom20 isoforms, called Tom20 type I and type II, were discovered by a search using the hidden Markov model. Three dimensional models of both types of Tom20 revealed important differences in the amino acid residues lining the ligand-binding groove. Murine Tom20 type I is detected preferentially in testis, whereas the expression of Tom20 type II is ubiquitous (*24*).

In the present study, we identified Tom40B, a new isoform of rat Tom40 that was widely expressed in the tissue, except for the testis, localized on the mitochondrial outer membrane, and associates with other

*To whom correspondence should be addressed. Tel: +81 92 642 6176, Fax: +81 92 642 6183,
E-mail: mihara@cell.med.kyushu-u.ac.jp

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subunits of the TOM complex. Furthermore, Tom40B directly bound to the proteins with the presequences of both cytochrome P-450 side chain cleavage (SCC) and ATP synthase subunit 9 (Su9) with affinities as high as those of rat Tom40A, a previously identified isoform of Tom40. Our findings suggest that Tom40B functions as an alternative component of the pore-forming subunit in the TOM complex.

MATERIALS AND METHODS

Cloning of Rat Tom40B cDNA and Plasmid Construction—Using the first strand cDNA synthesized from rat brain total RNA with random hexamer as a primer, a 927-bp cDNA encoding rat Tom40B was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) with the following primers; for PCR, 5′CTGAAAGCTTCCACCATGGGGAACACCCTGG GCCTG3′ and 5′CGCAGGATCCGCCCACAGTGATGCTG AA3′, and subsequently cloned into a pGEM-T Easy Vector (Promega, Fitchburg, WI). The nucleotide sequence of the clone was determined with a Dye Terminator DNA Sequencing Kit (Applied Biosystems, Foster City, CA). The nucleotide sequence data reported in this article will appear in the DDBJ databases with the accession number AB274732. For the mammalian expression plasmid, the rat Tom40B coding region amplified by PCR was cloned between the HindIII and BamHI sites of pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA). Deletion mutants of Tom40B were generated using the QuikChange™ kit (Stratagene, La Jolla, CA). For construction of the *Escherichia coli* expression plasmid, the DNA fragment containing the entire coding region of rat Tom40B was inserted between the NdeI and BamHI sites of pET28a (Novagen, Madison, WI).

Real-time PCR—Total RNA was extracted using TRIZOL (Invitrogen). First strand DNA was synthesized using the SuperScript III First-Strand Synthesis System for RT-PCR according to the manufacturer's instructions (Invitrogen). Primers for real-time quantitative PCR were designed using Primer Express software (Applied Biosystems). The primers used were as follows; for rat ß-actin, 5′CCAACCGTGAAAAGATGACC3′ and 5′GTACGACCAGAGCATACAGG3′; for rat Tom20, 5′GT GTGGACAGCCTCAGCAGTT3′ and 5′TTCTCTGACTAA TGGTTGGAAGCTT3′; for rat Tom22, 5′GATTTTCCAGG GCAGCTTTG3′ and 5′CTGTTGTTGCTCCATTTGCAA3′; for rat Tom40A, 5′CGGAAGTGCAAGGAGCTGTT3′ and 5′TGCCGAGGGCTACTGTATGG3′; for rat Tom40B, 5′GCCAGGGTACCACCTCCATA3′ and 5′CTGCCACTGC TGTCCATATCC3′; for rat truncated Tom40B, 5′ACCGG TTGTGCAAAGACACAGCA3′ and 5′CCAATCAGATCCG GATTTCCCAGGG3′. Real-time quantitative PCR was performed using QuantiTect SYBR Green PCR (Qiagen, Hilden, Germany) and analysed with an ABI PRISM 7000 Sequence Detection System (Applied Biosystems).

Cell Culture and Transfection—HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and high glucose under an atmosphere of 5% CO_2 at 37°C. DNA transfection was performed using FuGene6 Transfection Reagent, according to the manufacturer's instructions (Roche Applied Science, Indianapolis, IN).

Immunofluorescence Microscopy—HeLa cells were cultured on coverslips in 35 mm dishes. After transfection, the cells were further incubated for 24 h. The cells were washed twice with phosphate buffered saline (PBS), fixed with 2% paraformaldehyde at room temperature for 30 min and permeabilized with 0.1% Triton X-100. After several washes with PBS, the coverslips were incubated for 1 h with anti-haemagglutinin (HA) mouse monoclonal antibody (16B12; COVANCE) and anti-Tim17 rabbit anti-serum (*25*) in PBS containing 1% bovine serum albumin, followed by a 1 h incubation with the following secondary antibodies: Alexa Fluor® 488 goat anti-mouse IgG and Alexa Fluor® 568 goat anti- rabbit IgG (Molecular Probes, Eugene, OR). Fluorescent images were collected using a confocal laser microscopy (Radiance 2000; BIO-RAD, Hercules, CA).

Subcellular and Submitochondrial Fractionation—The cells were harvested and resuspended in ice-cold homogenization buffer [10 mM Hepes-KOH (pH 7.4), 0.2 M mannitol, 0.07 M sucrose, 1 mM EDTA, and protease inhibitor Complete EDTA Free (Roche Molecular Biochemicals, Mannheim Germany)]. The cells were disrupted by passing them through a 27-gauge needle 20 times. After centrifugation ($600 \times g$ for 5 min) to remove the nuclei and unbroken cells, the post-nuclear supernatant was further centrifuged at $6000 \times g$ for 10 min to obtain the mitochondria-enriched fraction (P6). The resulting supernatant was centrifuged at $10{,}000 \times g$ for 10 min to yield a pellet (P10), and then recentrifuged at $100{,}000 \times g$ for 20 min to separate the microsome-enriched fraction (P100) from the cytosol fraction (S100). The fractions were analysed by immunoblotting using the antibodies to HA (16B12; COVANCE), Tom22 (*26*), Sec61β (Upstate), and H450 (*27*). Sucrose density gradient centrifugation was performed as described previously (*28*).

Preparation of Anti-Tom40B Antibodies—His-tagged recombinant Tom40B was expressed in BL21(DE3) cells as inclusion bodies, and were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), followed by Coomassie brilliant blue staining. The Coomassie brilliant blue-stained band was excised from the gel and used to raise antibodies in rabbits using the RiBi Adjuvant systems (RIBI Immunochem Research Inc., Hamilton, MT).

Immunoprecipitation of TOM Complex—The mitochondria isolated from rat brain were solubilized with 10 mM HEPES-KOH (pH7.4) buffer containing 1% (w/v) digitonin, 100 mM NaCl, 1 mM phenylmethylsulphonyl fluoride and 10% (v/v) glycerol on ice for 30 min, followed by centrifugation at $100{,}000 \times g$ for 10 min. The supernatant was subjected to immunoprecipitation with either control IgG or anti-Tom40B IgG. The immunoprecipitates were washed with the same buffer three times, and analysed by SDS–PAGE and immunoblotting.

Purification and Refolding of Tom40B—Purified refolded Tom40B was prepared as described previously (*16*). In brief, His-tagged Tom40B was expressed in BL21(DE3) cells as inclusion bodies, and solubilized in 20 mM Tris–HCl (pH 8.0) buffer containing

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6 M guanidine HCl, 500 mM NaCl and 1 mM 2-mercaptoethanol (2ME). The ultracentrifuged supernatant was passed through a HiTrap chelating HP column equilibrated with the same buffer and washed with 20 mM Tris–HCl (pH 8.0) buffer containing 0.5 M NaCl, 6 M urea, 1 mM 2ME and 5 mM imidazole. For refolding of Tom40B, the washing buffer was slowly exchanged to the refolding buffer [20 mM Tris–HCl (pH 8.0), 0.5 M NaCl, 0.5% Brij35 and 1 mM 2ME]. After the refolding step, Tom40B was eluted by the refolding buffer containing 300 mM imidazole. The eluted proteins were dialysed with 20 mM Tris–HCl (pH 8.0) buffer containing 100 mM NaCl, 0.5% Brij35, 1 mM 2ME and 15% (w/v) glycerol, and used for surface plasmon resonance analysis.

Semi-quantitation of Tom40A and Tom40B in Rat Brain Mitochondria—Purified recombinant Tom40 proteins and isolated rat brain mitochondria were subjected to SDS–PAGE, followed by immunoblotting using Immun-Star™ AP kit (BIO-RAD). The intensity of each band was analysed by Image Gauge software (FUJIFILM). Amounts of both Tom40 isoforms in rat brain mitochondria were calculated using standard curves of the recombinant proteins.

Surface Plasmon Resonance (SPR) Measurements—Refolded Tom40B was prepared as described previously (*16*). The surface plasmon resonance (SPR) measurements were performed at 25°C with Biacore 3000 (Biacore AB, Tokyo, Japan). The purified His-tagged proteins were immobilized onto a CM5 sensor chip using the amine-coupling method according to the manufacturer's protocol. Binding analyses were performed in the running buffer [20 mM Hepes-KOH buffer (pH 7.4), 150 mM NaCl and 0.05% Brij35] at a flow rate of 20 μl/min. To avoid aggregation of pSU9-DHFR, the binding analysis was performed in the running buffer containing 15% glycerol and 0.1% Brij35. For kinetic analysis, purified SCC(1–19)DHFR of different concentrations (18.8–300 nM) was passed through the flow cells at a rate of 20 μl/min. SCC(1–19) peptides were flowed at a concentration ranging from 1.56 to 25 μM. Total 18.8 nM of pSU9-DHFR was used for kinetics analysis. Binding curves were analysed using BIA-Evaluation software (version 3.2). The kinetic data fitting was performed using a Langmuir 1:1 binding model.

RESULTS

Identification of a New Tom40 Isoform, Tom40B—While searching for additional genes coding for a subunit of the mitochondrial TOM complex in the genomic DNA databases, we found a DNA sequence highly similar to that of rat Tom40. According to a predicted open reading frame, we amplified a full-length cDNA using RT-PCR with rat brain total RNA. The cDNA clone contained a 927-bp coding region for a 308-amino acid polypeptide (Fig. 1A). As expected, the deduced amino acid sequence shared 59.4%, 25.0% and 21.4% identity with rat, *Saccharomyces cerevisiae*, and *Neurospora crassa* Tom40, respectively (Fig. 1B). We therefore named the rat protein Tom40B, and the previously identified Tom40 (*28*) was renamed Tom40A. To determine the Tom40B expression in various tissues, real time RT-PCR analysis was performed using total RNA prepared from liver, kidney, brain, heart and testis. As previously reported (*26*, *29*), the known subunits of the TOM complex (Tom22 and Tom20) were ubiquitously expressed in all the tissues tested. Tom40A expression was also detected in all the tissues, and Tom40B was expressed in all the tissues examined except testis (Fig. 1C). Similar results were obtained using another set of RT-PCR primers for Tom40B (data not shown), indicating that the expression pattern of Tom40B analysed by RT-PCR represents the tissues distribution of the Tom40B transcripts. The human transcripts (accession number; AK022832) coding for truncated Tom40B has been found in the NCBI database. Therefore, the expression of the rat transcript corresponding to the truncated form was also analysed by real time RT-PCR. The transcripts for truncated Tom40B were expectedly detectable in all tissues except testis, although its expression was 100-fold lower than the full-length transcripts (data not shown), indicating that rat Tom40B is expressed at least in liver, kidney, brain and heart.

Tom40B is Localized in the Mitochondrial Outer Membrane—To investigate the intracellular localization of Tom40B, HA-tagged Tom40B was exogenously expressed in HeLa cells. Immunofluorescence microscopy showed that Tom40B co-localized with Tim17, a mitochondrial protein (Fig. 2A). Cell fractionation analysis revealed that Tom40B co-fractionated with Tom22 in the mitochondria-enriched fraction (P6), but not with Sec61β, an endoplasmic reticulum (ER) protein, or cytochrome H450, a cytosolic protein (Fig. 2B). Furthermore, in the P6 fraction, Tom40B was resistant to alkali extraction (Fig. 2C). The results indicated that Tom40B is a mitochondrial membrane protein.

To generate an antibody specific to Tom40B, a His-tagged recombinant protein was expressed in *E. coli*, separated by SDS–PAGE, excised from the gel, and used as an antigen. The affinity-purified antibody to Tom40B recognized a 34-kDa band in rat brain cell extract (Fig. 2D). In contrast, Tom40A was detected as a 37-kDa band in the same cell extract, indicating that the anti-Tom40B antibody specifically recognized Tom40B, but not Tom40A. To examine the submitochondrial localization, mitochondria prepared from rat brain was sonicated under hypotonic conditions and subjected to sucrose density gradient centrifugation. Tom40B was recovered in the upper fraction of the tube, and co-migrated with monoamine oxidase, an outer membrane protein, but not with Tim17, an inner membrane protein (Fig. 2E). Furthermore, Tom40B possessed no presequences cleaved by mitochondrial processing peptidase (MPP) (Supplementary Fig. 1). These results indicate that Tom40B is localized on the mitochondrial outer membrane. We next determined the membrane topology of Tom40B. The anti-Tom40B antibody recognized the full-length protein, but not the N-terminal deletion mutant proteins, Tom40B(Δ2–40)-HA and Tom40B(Δ2–25)-HA (Fig. 2F), indicating that the antibody bound to at least the initial 25 amino acid residues of rat Tom40B. Protease protection assay showed that C-terminal HA-tagged Tom40B was resistant to proteinase K under isotonic conditions, as analyzed by

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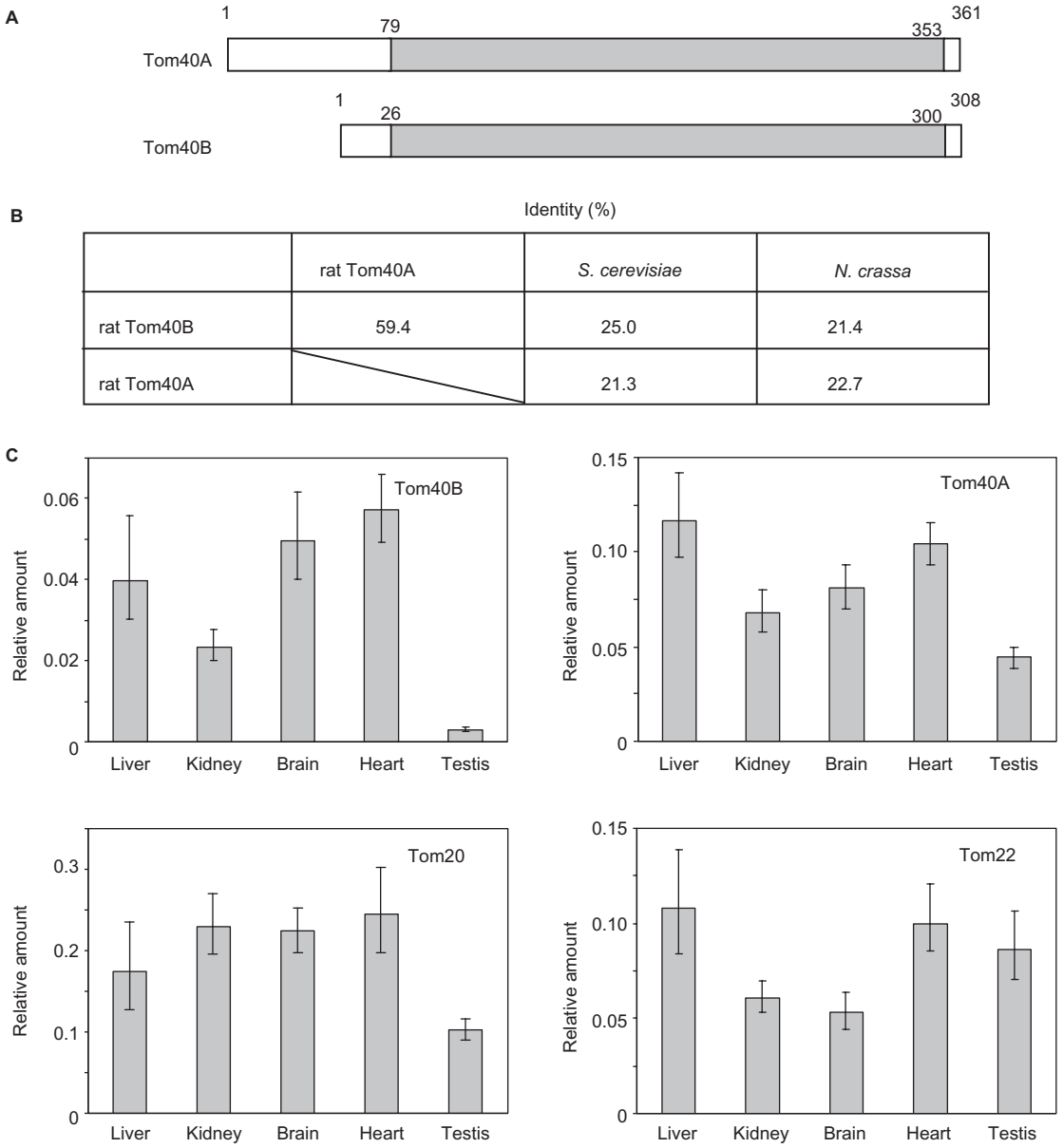

Fig. 1. **Domain structure and tissue distribution of Tom40B.** (A), Schematic representation of rat Tom40A and Tom40B. Porin conserved domains are indicated by the grey bar. (B), Identity of the amino acid sequence of Tom40B or Tom40A with *S. cerevisiae* and *N. crassa* Tom40. (C), Quantitative real-time RT-PCR analysis of Tom40B expression. Normalization was performed using actin mRNA levels as controls in the same reaction as described under 'MATERIALS AND METHODS'. *Error bars* represent SE from three independent experiments.

Fig. 2. **Tom40B is a mitochondrial outer membrane protein.** (A), HeLa cells expressing Tom40B-HA were co-stained with anti-HA and anti-Tim17 and viewed by confocal microscopy. A merged green and red fluorescence image is shown in the right panel. Scale bar, 15 μm. (B), HeLa cells expressing Tom40B-HA were fractionated into P6 (mitochondria-enriched fraction), P10, P100, and S100 fractions (see 'MATERIALS AND METHODS'). The isolated fractions were subjected to SDS–PAGE followed by immunoblotting using antibodies against HA for Tom40B or against the indicated proteins. Tom22, Sec61β and H450 were used as markers for the mitochondrial, microsomal and supernatant fractions, respectively. (C), Mitochondria were extracted with 100 mM sodium carbonate (pH 11.5). After centrifugation at 100,000 × *g* for 15 min, the resultant pellet (P) and the supernatant (S) fractions were analysed by immunoblotting with specific antibodies as indicated. (D), Mitochondria prepared from rat brain were subjected to SDS–PAGE and immunoblotting with antibodies as indicated. (E), Mitochondria prepared from rat brain were sonicated and then layered over a linear sucrose gradient (0.6–1.6 M). After centrifugation, the samples were fractionated from the top to the bottom of the tubes. The fractions were analysed by immunoblotting with antibodies against the indicated proteins. (F), HeLa cells were transfected with vector alone or expressing Tom40B-HA(Δ2–40) or Tom40B-HA(Δ2–25). Whole cell lysates were analysed by immunoblotting with antibodies against Tom40B or HA. (G), Mitochondria from HeLa cells expressing Tom40B-HA were treated with 50 μg/ml proteinase K (ProK) for 30 min on ice in the presence or absence of 1% Triton X-100 (T × 100) under isotonic or hypotonic conditions. The reaction mixtures were analysed by SDS–PAGE and immunoblotting with the indicated antibodies.

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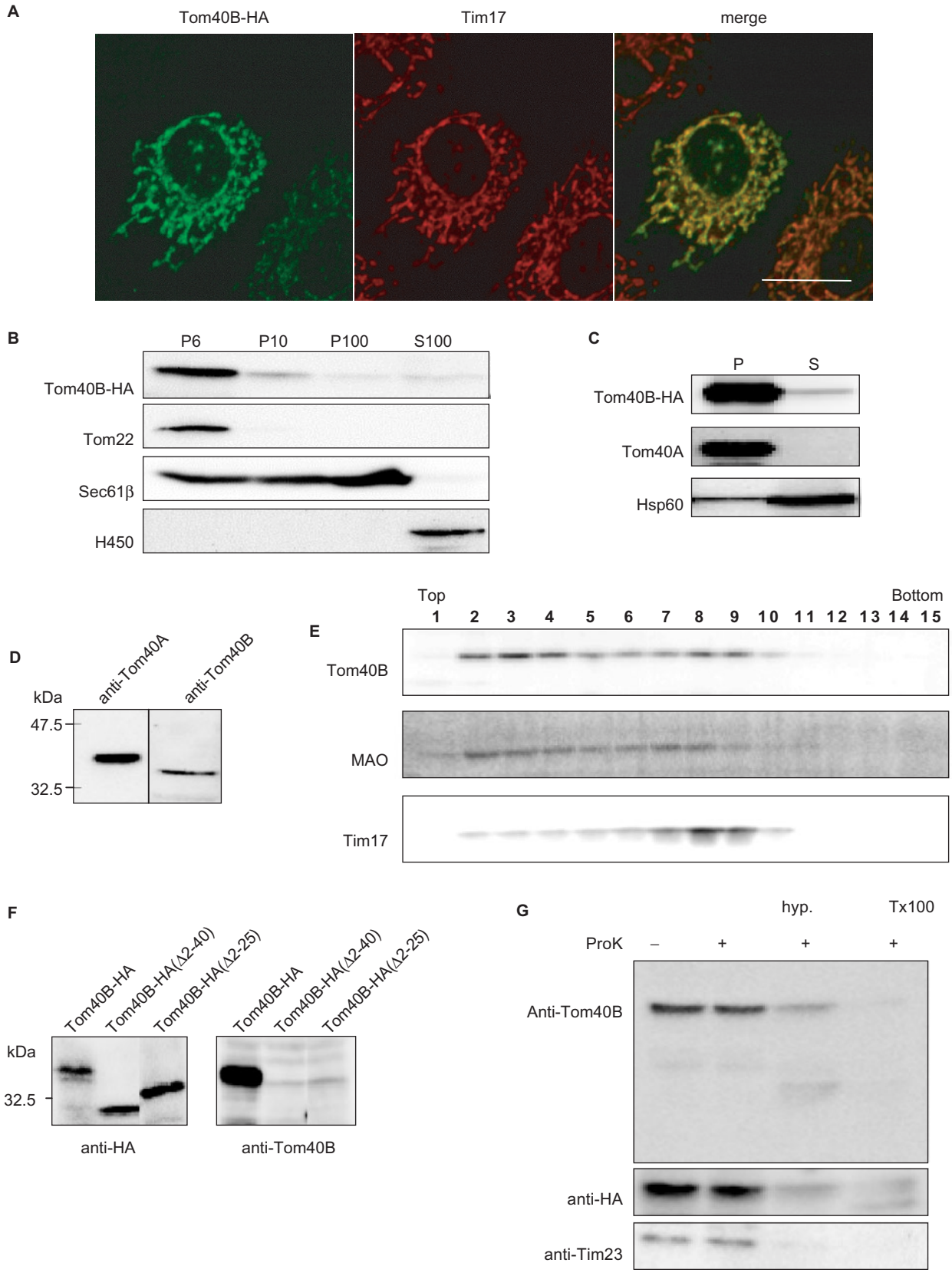

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immunoblotting with both anti-Tom40B and anti-HA antibodies. Under hypotonic conditions, however, the protein lost reactivity to both antibodies to a significant extent, suggesting that both termini of Tom40B are exposed to the intermembrane space (Fig. 2G). As a control, the inner membrane protein Tim23 that extrudes its C-terminal domain into the intermembrane space was digested by proteinase K under the same hypotonic conditions.

Tom40B is a Component of the TOM Complex—The yeast TOM complex consists of at least five subunits; Tom40, Tom22, Tom7, Tom6 and Tom5 (*5*, 15, 28, 30) forming a protein complex with a molecular size of ~400 kDa (*6*). To examine whether Tom40B acts as a component of the TOM complex, mitochondria from rat brain were solubilized with digitonin and subjected to blue native (BN) PAGE. Endogenous rat Tom40B migrated as a single band with a molecular size of ~470 kDa in brain, similar to the molecular sizes of Tom40A and Tom22 on the BN-PAGE (Fig. 3A). These results suggest that Tom40B formed a complex with other Tom components. To further test the interaction of Tom40B with other Tom components, the solubilized TOM complex was immunoprecipitated with the anti-Tom40B antibody. Tom40A, Tom22 and Tom70 co-precipitated with Tom40B, but monoamine oxidase, an outer membrane protein, did not (Fig. 3B), strongly suggesting that Tom40B was incorporated into the TOM complex. For stoichiometric analysis, Tom40B or Tom40A in the rat brain mitochondria was semi-quantified by immunoblotting (Fig. 3C). The mitochondrial fraction from rat brain contained Tom40B (48 pmol/mg of protein) and Tom40A (30 pmol/mg), indicating that in the rat brain the Tom40B existed in equal or slightly higher amounts than Tom40A.

The C-terminal Segment of Tom40B is Required for Targeting to Mitochondria—The signals of β-barrel proteins for targeting to the mitochondrial outer membranes are poorly understood. To search for the domain required for mitochondrial targeting of Tom40B, a series of deletion mutations were prepared (Fig. 4A). The deletion mutants were expressed in HeLa cells and analysed with immunofluorescence microscopy. While the six mutants localized on mitochondria as well as wild-type Tom40B (data not shown), only the Δ281–308 mutant was detected in both the mitochondria and cytoplasm (Fig. 4B, middle panel). Cell fractionation results supported that the Δ281–308 mutant was detected in both supernatant and pellet fractions, but the other mutants were recovered only in the pellet fraction (Fig. 4C). These results indicated that the 28 amino acid residues at the C-terminus are necessary for the targeting of Tom40B to mitochondria. Interestingly, replacement of the C-terminus (281–308) of Tom40B with that of Tom40A did not influence mitochondrial targeting activity (Fig. 4B, lower panel), suggesting that 19 amino acid residues at the C-terminus conserved between Tom40A and Tom40B would be crucial for mitochondrial targeting of Tom40B.

Physical Interaction of Tom40B with Mitochondrial Targeting Sequence—We previously demonstrated that purified Tom40A directly binds loosely folded matrix-targeted preprotein with high affinity (*16*). The interaction of Tom40B with precursors of the matrix-targeted proteins was examined using SPR. Recombinant Tom40A and Tom40B proteins for SPR were expressed in *E. coli*,

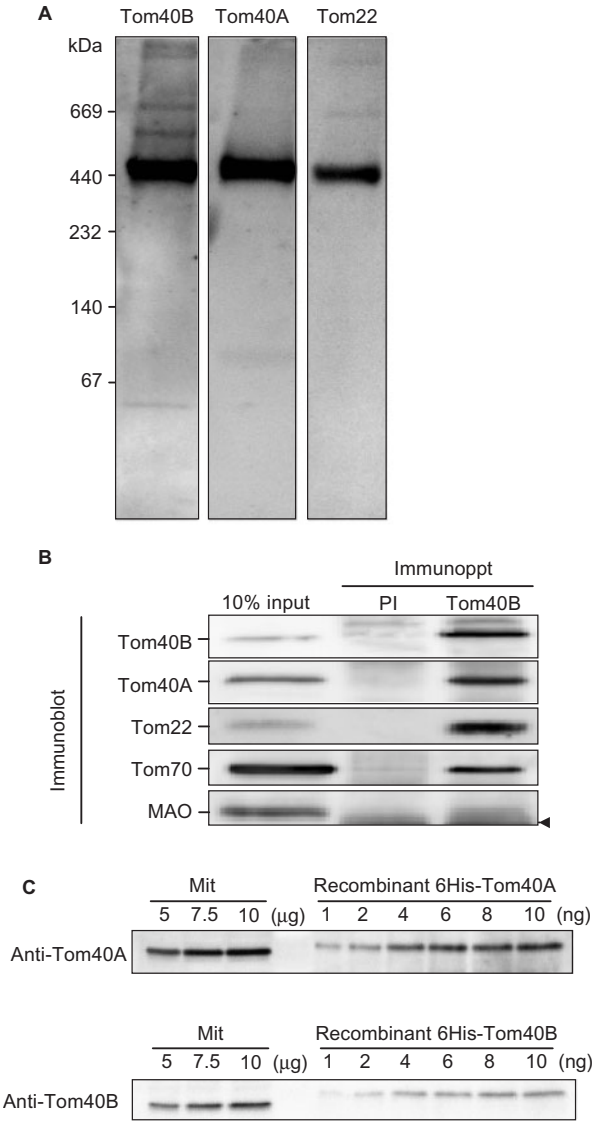

Fig. 3. **Assembly of Tom40B into the TOM complex.** (A), Isolated mitochondria from rat brain were solubilized in 1% digitonin buffer and subjected to BN-PAGE followed by immunoblotting with antibodies against the indicated proteins. (B), Mitochondria from rat brain were solubilized in digitonin and aliquots of the supernatant were subjected to immunoprecipitation with preimmune (PI) or anti-Tom40B IgGs. The immunoprecipitated materials were subjected to SDS–PAGE followed by immunoblotting with antibodies against the indicated proteins. The arrowhead on the right side of the blots indicates the position of the immunoglobulin heavy chain. (C), Isolated mitochondria from rat brain and purified recombinant 6His-Tom40A or 6His-Tom40B were subjected to SDS–PAGE and followed by immunoblotting with the indicated antibodies. The immunoblots were performed using CDP-star as a substrate, and the images were analysed by Image Gauge Software (FUJIFILM). R^2 values of approximation lines were greater than 0.933.

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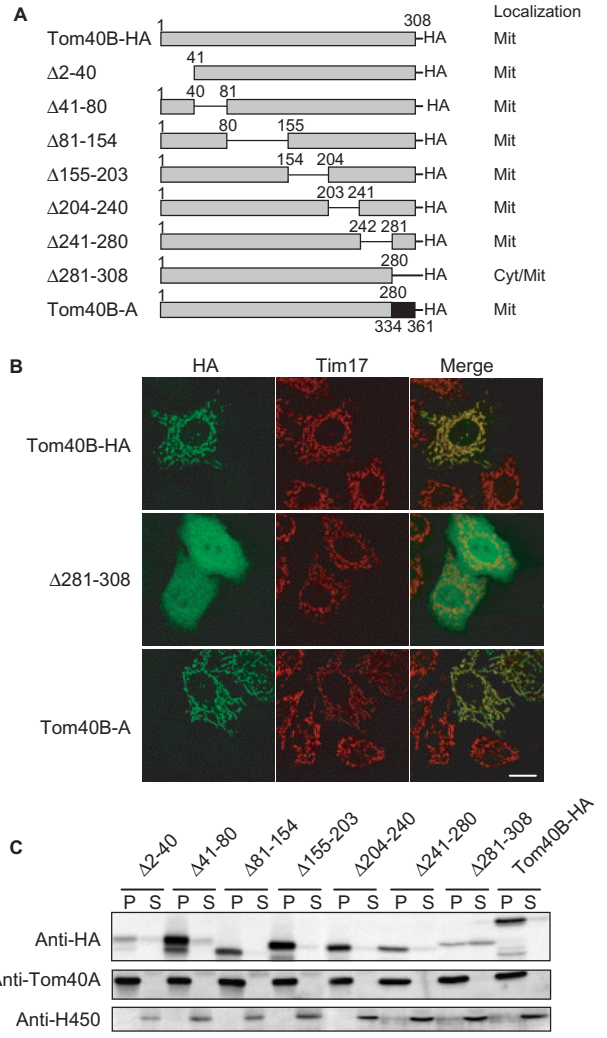

Fig. 4. **A C-terminal segment is crucial for mitochondrial targeting of Tom40B.** (A), Schematic representation of Tom40B deletion mutants and Tom40B-A chimeric protein. Grey and solid boxes represented portion of Tom40B and Tom40A, respectively. Mit and Cyt represented miochondria and cytoplasm, respectively. (B), The indicated Tom40B deletion mutants and chimeric protein were expressed in HeLa cells, and their intracellular localizaton was examined as in Fig. 2A. Scale bar, 15 μm. (C), The indicated Tom40B deletion mutants were expressed in HeLa cells and the cells were fractionated by centrifugation at $10000 \times g$ for 10 min to the pellet (P) and supernatant (S) fractions, followed by immunoblotting with the indicated antibodies.

purified under denaturing conditions (Fig. 5A) and refolded as previously described (*16*). The purified recombinant proteins were immobilized on a BIAcore sensor chip, followed by the injection of either SCC(1–19)DHFR or pSU9-DHFR. Tom40A strongly interacted with SCC(1–19)DHFR and pSU9-DHFR, and Tom40B also bound both of the precursor proteins with affinities comparable to those of Tom40A (Fig. 5B). Methotrexate strongly inhibited the binding of SCC(1–19)DHFR to Tom40B (Fig. 5C, +MTX), suggesting that the binding to Tom40B was dependent on not only the presequence but also on the unfolded mature region of the preprotein. In addition to the preproteins, the SCC(1–19) peptide was used to determine the kinetic parameters. The K_D value for the binding of SCC(1–19) peptide to Tom40B was similar to that for binding to Tom40A (Table 1). Similar results were obtained with the binding of SCC(1–19)DHFR, although the binding affinity of the synthetic peptide was lower than that of the precursor protein (Table 1).

DISCUSSION

By searching the rat genomic DNA database, we identified a new gene coding for an isoform of Tom40, a central pore-forming component of Tom. Tom40B was widely expressed in all tissues examined except testis, located on the mitochondrial outer membrane, and formed a protein complex with other components of the TOM complex as well as Tom40A. The peptide corresponding to a presequence of the matrix-targeted protein SCC directly bound to recombinant Tom40B protein with a high affinity comparable to that of Tom40A. Furthermore, the genes for Tom40B were in the human, monkey, mouse and dog DNA databases (human, NM_032174; monkey, XM_525231; mouse, NM_001037170; dog, XM_847162). Tomm40L deposited in the NCBI database appears to be human and mouse orthologues of rat Tom40B. The results indicate that Tom40B is presumably conserved among mammals and acts as a functional subunit of the mitochondrial translocon.

The TOM core complex is composed of at least five subunits; Tom40, Tom22 and the three small Tom proteins (*5*, *6*). Stoichiometric analysis of the yeast TOM complex revealed that a single complex includes four to six copies of Tom40 (*6*). Electron micrographs of the purified TOM complex suggested that at least two pores exist in a single complex (*31*, *32*). Tom40B co-immunoprecipitated with Tom40A as well as Tom22, and the molecular size of the complex containing Tom40B was similar to that of Tom40A on BN-PAGE. The findings suggest the following possibilities; (i) Tom40B and Tom40A function as pores in the same complex and (ii) a Tom40B-containing complex is tightly associated with a TOM complex including Tom40A. Further study on the structure of the complex is necessary.

Tom40B is composed of a short N-terminal region and a β-barrel domain homologous to both Tom40A and porin. The β-barrel domain of Tom40A strongly contributes to the binding of preproteins and has the characteristics of a protein channel (*16*). Purified recombinant Tom40B also bound to both preprotein and presequence peptides with the same affinity as Tom40A, suggesting that Tom40B functions as a translocation channel for preproteins. The length of the N-terminal region of Tom40 is dependent on the species. Mammalian Tom40A proteins have an extended N-terminal region compared to Tom40 in other eukaryotes. The extended N-terminus is exposed to the cytoplasm (*16*). In contrast, the N-terminal end of rat Tom40B and *N. crassa* Tom40 face the intermembrane

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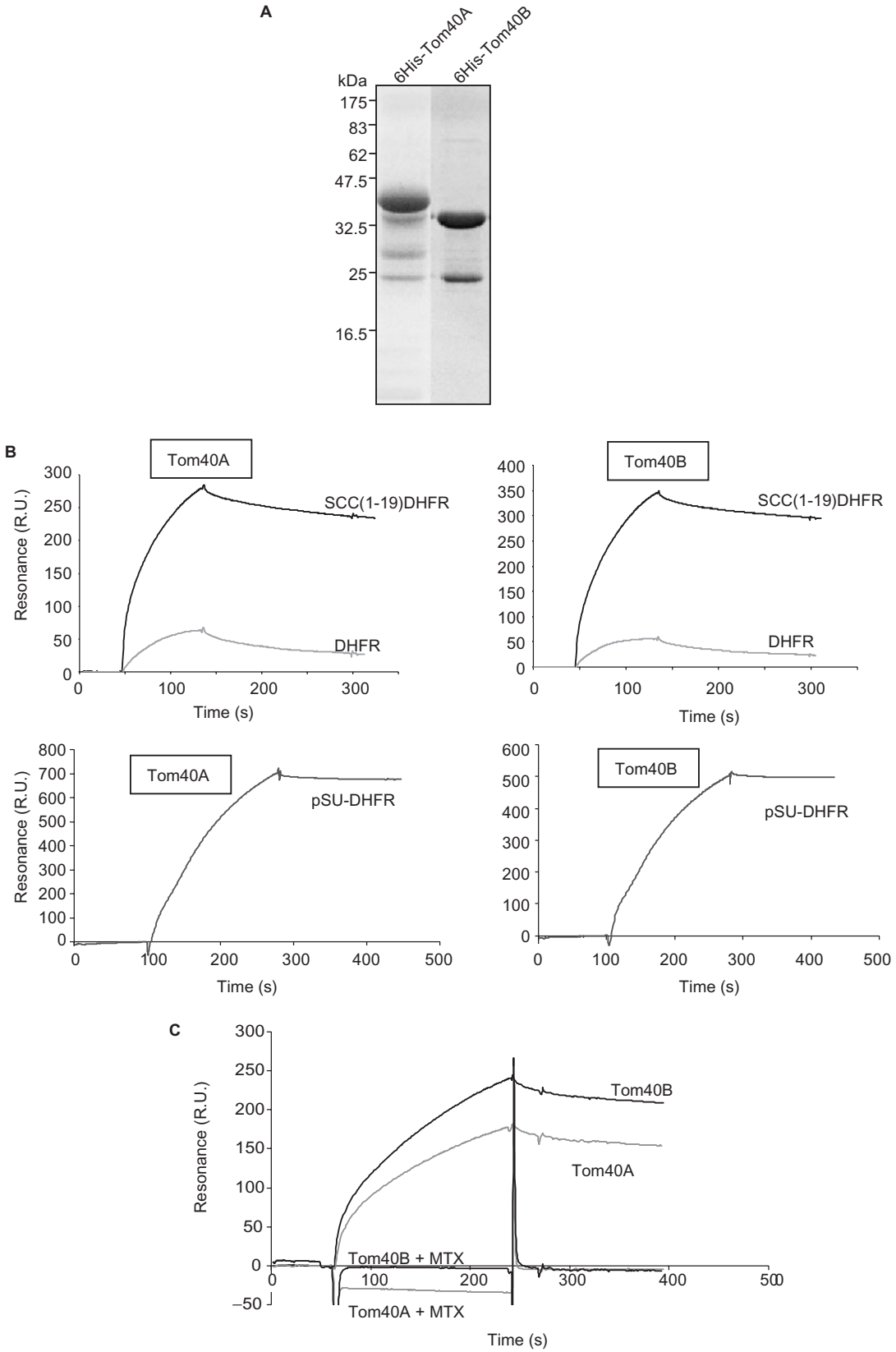

Fig. 5. **Analysis of preprotein binding to Tom40B using SPR**. (A), Coomassie brilliant blue-stained SDS–PAGE profiles of purified Tom40A and Tom40B. (B), Tom40A or Tom40B was immobilized on a sensor chip and then 300 nM of DHFR (grey line), SCC(1–19)DHFR (black line), or 150 nM pSU9-DHFR were injected. (C), SCC(1–19)DHFR in the running buffer in the presence or absence of 50 μM methotrexate (MTX) was injected into a Tom40A (grey line) or Tom40B (black line)-immobilized sensor chip. The large peaks at 250 s were due to replacement of the MTX-containing buffer with the MTX-free buffer.

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Table 1. **Kinetic parameters by surface plasmon resonance.**

Ligand	Analyte	k_a (1/Ms)	k_d (1/s)	K_D (M)
Tom40B	SCC(1–19)DHFR	6.96×10^4	4.98×10^{-4}	7.16×10^{-9}
	pSU9-DHFR	2.92×10^4	5.26×10^{-5}	1.80×10^{-9}
	SCC(1–19)peptide	1.58×10^3	3.66×10^{-3}	2.32×10^{-6}
Tom40A	SCC(1–19)DHFR	5.48×10^4	5.62×10^{-4}	1.02×10^{-8}
	pSU9-DHFR	2.72×10^4	7.62×10^{-5}	2.81×10^{-9}
	SCC(1–19)peptide	1.69×10^3	3.27×10^{-3}	1.93×10^{-6}

space [Fig. 2G and (*31*)]. How the extended N-terminal domain of Tom40A contributes to translocase function is still unknown.

Newly synthesized Tom40 is translocated through the TOM complex to the intermembrane space. Following the small Tim proteins, Tom40 is integrated into the membrane via the sorting assembly machinery complex (*33–36*). Additional components, including Mim1/Tom13, are required for assembly of the complex (*37*, *38*). The signals for mitochondrial targeting of Tom40 remain unclear. Analysis using a series of Tom40B deletion mutants indicated that the 28 amino acid residues at the C-terminal end were necessary for the targeting to mitochondria. Replacement of the C-terminal region of Tom40B with that of Tom40A still retained mitochondrial targeting activity. Nonetheless, deletion of the same region of Tom40A did not influence the mitochondrial targeting (Supplementary Fig. 2), suggesting that Tom40B may have a unique targeting pathway of which Tom40A is independent.

The discovery of the Tom40 isoform leads to the question of the physiologic function of the two different isoforms. Recently, some TOM complex subunit homologues were reported. In *Drosophila melanogaster*, Tomboy20 and Tomboy40, homologues of Tom20 and Tom40, are expressed specifically in the male germ line (*23*). In addition to fly, Tom20 isoforms, called Tom20 Type I and Type II, are conserved between animals, and mouse Tom20 Type I is also expressed specifically in testis whereas the expression of another isoform is ubiquitous (*24*), suggesting that certain isoforms of the TOM complex have a role in spermatogenesis. Unexpectedly, the Tom40B transcripts were widely detected in all tissues examined except testis, implying that there may be a third Tom40B isoform expressed only in testis.

In yeast, a pore-forming component of the ER translocon also has two isoforms; Sec61p and Ssh1p. Sec61p forms heterotrimeric complexes required for co- and post-translational translocation of nascent polypeptides, while Ssh1p is a component of the complex that functions exclusively in the co-translational pathway (*39*). Moreover, Δ*ssh1* mutant cells have strong defects in signal recognition particle dependent/independent translocation and ER dislocation of unfolded proteins (*40*). Ssh1p might function as a reserve for translocons providing extra capacity and physiologic flexibility in the ER. As mentioned earlier, Tom40B had a high affinity to the preprotein and formed a complex with other Tom components, indicating that it is a functional subunit of the TOM complex. Therefore, it is possible that Tom40B has a role in an alternative pathway of either targeting and/or translocation of preproteins to increase the capacity of mitochondrial import. Further investigation, however, is necessary to elucidate the physiologic significance of the isoforms of the TOM complex in mitochondrial translocases.

Supplementary data are available at *JB* online.

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