Identification of Mammalian TOM22 as a Subunit of the Preprotein Translocase of the Mitochondrial Outer Membrane*

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A mitochondrial outer membrane protein of ~22 kDa (1C9-2) was purified from Vero cells assessing immuno-reactivity with a monoclonal antibody, and the cDNA was cloned based on the partial amino acid sequence of the trypsin-digested fragments. 1C9-2 had 19–20% sequence identity to fungal Tom22, a component of the preprotein translocase of the outer membrane (the TOM complex) with receptor and organizer functions. Despite such a low sequence identity, both shared a remarkable structural similarity in the hydrophobic profile, membrane topology in the Ncyt-Cin orientation through a transmembrane domain in the middle of the molecule, and the abundant acidic amino acid residues in the N-terminal domain. The antibodies against 1C9-2 inhibited the import of a matrix-targeted preprotein into isolated mitochondria. Blue native polyacrylamide gel electrophoresis of digitonin-solubilized outer membranes revealed that 1C9-2 is firmly associated with TOM40 in the ~400-kDa complex, with a size and composition similar to those of the fungal TOM core complex. Furthermore, 1C9-2 complemented the defects of growth and mitochondrial protein import in Δtom22 yeast cells. Taken together, these results demonstrate that 1C9-2 is a functional homologue of fungal Tom22 and functions as a component of the TOM complex.

Most mitochondrial proteins are encoded by the nuclear genome and are synthesized in the cytosol as preproteins. They are guided to the mitochondrial surface by cytoplasmic chaperones (1) and are then transported to the intramitochondrial compartments by the preprotein import machinery of the outer and inner membranes: the TOM1 and TIM complexes, respectively (2–8). Extensive genetic and biochemical studies in Saccharomyces cerevisiae and Neurospora crassa have identified components of these complexes. The S. cerevisiae TOM complex is composed of at least nine proteins, Tom71, Tom70, Tom40, Tom37, Tom22, Tom20, Tom7, Tom6, and Tom5. Tom70, Tom37, Tom22, and Tom20 function as import receptors. Tom71 has strong similarity to Tom70 and is weakly associated with the TOM complex (9), although its function is unclear. Tom40 is deeply embedded in the outer membrane in a predicted β-barrel structure and functions as the central component of the translocation channel (10–14). Tom6 and Tom7 modulate the dynamics of the TOM channel (15, 16). Tom5 is tightly associated with Tom40 and represents the connecting link between import receptors and the translocation channel (17). Thus, Tom40, Tom22, and three smaller Tom proteins form the general preprotein import pore (the TOM core complex) of ~400 kDa (14, 18). A recent study revealed that Tom22 not only functions as the import receptor, but also regulates the TOM complex organization (19). The mitochondrial inner membrane has at least two separate import machineries. The Tim23-Tim17 system mediates, in conjunction with Tim44 and mHsp70 in the matrix, mitochondrial transport of matrix-targeted preproteins (8, 20). The Tim54-Tim22-Tim18 system acts with the small Tim proteins in the intermembrane space to mediate insertion of the metabolite carrier proteins, such as the ADP/ATP carrier, as well as several TIM components, such as Tim23, Tim22, and Tim17, into the inner membrane (21–27).

Despite extensive knowledge of fungal systems, relatively little is known about the import machinery of mammalian mitochondria. Several mammalian counterparts have been identified (for review, see Ref. 28): TOM20 (29–32); TIM17, TIM23, and TIM44 (33); and DDP1, a homologue of Tim3 (34). In addition, TOM34 and metaxin have been identified as unique components of the mammalian mitochondrial import system. Human TOM34 was cloned using a degenerate tetrapeptide repeat sequence present in Tom70 and Tom20 (35). Metaxin has a 25% sequence identity to yeast Tom37 in the N-terminal region, although whether it is a mammalian counterpart of Tom37 is not yet known (36). TOM34 and metaxin might function as the import receptor. Furthermore, we have recently identified and characterized the rat homologue of Tom40 2 and rat TOM70.3

This study reports the identification and characterization of a mammalian homologue of Tom22. This protein was found serendipitously during the study of the diphtheria toxin recep-

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2 H. Suzuki, Y. Okazawa, T. Komiyi, S. Kitada, A. Ito, and K. Mihara, manuscript in preparation.
3 H. Suzuki and K. Mihara, unpublished observations.
tor complex using monoclonal antibodies prepared against the membrane fraction of Vero cells as antigens (37, 38). We found that one of the monoclonal antibodies reacted with the ~22-kDa mitochondrial outer membrane protein 1C9-2. The cDNA was cloned based on the partial amino acid sequence of the purified protein. Its primary sequence was 19–20% identical to that of fungal Tom22, the multifunctional regulator of the protein translocase complex of the mitochondrial outer membrane (the TOM complex). Despite this low sequence identity, 1C9-2 and fungal Tom22 shared characteristic structural similarities in the hydrophathy profile, the membrane topology, and the distribution of acidic amino acid clusters in the extramembrane domains. Furthermore, 1C9-2 is a component of the mammalian TOM complex containing TOM40 and several components of smaller molecular size, and the anti-1C9-2 antibody inhibited mitochondrial protein import. Most notably, it complemented the functional defect in Δtom22 yeast cells. We conclude that 1C9-2 is the functional homologue of Tom22.

EXPERIMENTAL PROCEDURES

Monoclonal Antibody—BALB/c mice were immunized by subcutaneous injection of gelatin- and albumin-extracted formalin-fixed yeast cells (monkey kidney-derived cells) (37). Splenocytes from the immunized mice were fused with X63-Ag8-653 mouse myeloma cells as described previously (38), and the hybridoma producing an antibody reacting with an ~22-kDa mitochondrial protein was selected. We refer to this antigen as 1C9-2.

Immunofluorescence Microscopy—Vero cells were plated at a density of 1 × 10⁴ cells/well the day before staining. The cells were incubated with 300 nm MitoTracker Green FM (Molecular Probes, Inc.) for 40 min, fixed in 3.7% formaldehyde in phosphate-buffered saline, and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline. The cells were incubated with anti-1C9-2 monoclonal antibody for 30 min at room temperature and then with Cy3-conjugated donkey anti-mouse IgG (Amersham Pharmacia Biotech) and 4′,6-diamidino-2-phenylindole (DAPI). The cells were then rinsed and mounted in Mountant (Invitrogen). After staining with DAPI, the 1C9-2 band was excised and subjected to in-gel digestion with trypsin (39). The digested protein-1C9-2 was eluted with 60% acetonitrile containing 0.1% trifluoroacetic acid. The eluate was diluted with 0.5 volume of deionized water containing 0.1% trifluoroacetic acid and applied to a Waters Sep-Pak tC2 cartridge. The cartridge was washed with the same buffer; incubated with 20 mM 2-mercaptoethanol for 5 min, the reaction mixtures were analyzed by SDS-PAGE and immunoblotting using mouse anti-FLAG monoclonal antibody (Sigma) or anti-1C9-2 monoclonal antibody.

Blue Native Gel Analysis—Blue native PAGE was performed essentially as described previously (41, 42). The mitochondrial outer membranes (50 µg) were solubilized with 50 µl of 10 mM HEPES-KOH (pH 7.4) containing 0.22 M mannitol, 0.07 M sucrose, and phenylmethylsulfonyl fluoride, and 10 µg of pUC19/MV/CMV/h1C9-2/FLAG was used for raising polyclonal antibodies. The proteins were electroeluted from the gel and subjected to second dimension Tricine/SDS-PAGE.

Preparation of Polyclonal Antibodies against Human 1C9-2—The proteins glutathione S-transferase-1C9-2 and maltose-binding protein-1C9-2 were expressed in Escherichia coli using the pGEX-2T vector (Amersham Pharmacia Biotech) and the pMAL-c2 vector (New England Biolabs Inc.), respectively, and purified. The glutathione S-transferase-1C9-2 fusion protein was used for raising the polyclonal antibodies in rabbits. Monospecific IgGs for 1C9-2 were isolated from the serum using maltose-binding protein-1C9-2-conjugated Affi-Gel 15 (Bio-Rad) and following protein A-Sepharose (Amersham Pharmacia Biotech).

Determination of Topology of 1C9-2 in Mitochondria—1C9-2 carrying the C-terminal FLAG epitope tag was used to determine the membrane orientation of 1C9-2. A linker encoding the 8-amino acid FLAG tag sequence (DYKDDDK) was cloned into the XbaI/ApaI site of pPR/CMV (Invitrogen) to obtain pRC/CMV/FLAG. cDNA encoding human 1C9-2 was inserted into the HindIII-XbaI site of pRC/CMV/FLAG to generate pRC/CMV/h1C9-2/FLAG. L cells (mouse fibroblast-derived cells) were plated at a density of 5 × 10⁵ cells/cm² the day before staining. The cells were incubated with 100,000 x g for 30 min and sonicated. The solution was layered over a 12-mL linear gradient of 0–1.6 M sucrose in 10 mM HEPES-KOH (pH 7.3) containing the protease inhibitor mixture. After centrifugation at 100,000 x g for 15 min, the fractions were collected from the top of the gradient.

Identification of Human TOM22—cDNA Cloning of Human 1C9-2—For constitutive expression of 1C9-2 in yeast cells, cDNA encoding human 1C9-2 was inserted downstream of the alcohol dehydrogenase promoter of the yeast expression vector pMD288 (2µ, TRP1) to create pMD288/h1C9-2. The yeast strain MNMS-1C, which has a chromosomal TOM22 gene disrupted by HIS3 that is rescued by pYE-Ura3/TOM22, in which TOM22 is placed under a GAL1 promoter (43), was transformed with either pMD288 or pMD288/h1C9-2 and selected using synthetic medium plates (lacking His, Ura, and Trp) containing 2% galactose. Similarly, the cells grown on the first 2% glucose-containing synthetic medium plate and incubated at 30 °C for 3 more days were streaked onto the synthetic medium plates (lacking His, Ura, and Trp) containing 2% galactose. The transformants were streaked onto the synthetic medium plates (lacking His, Ura, and Trp) containing the C-terminal FLAG epitope tag. The cells were subjected to SDS-PAGE and immunoblotting using mouse anti-FLAG monoclonal antibody (Sigma) or anti-1C9-2 monoclonal antibody.
RESULTS

Isolation of cDNA Clone Encoding 1C9-2—We obtained a monoclonal antibody reacting with an ~22-kDa protein (1C9-2) of mammalian cell mitochondria during the course of screening monoclonal antibodies raised against the membrane fraction of Vero cells (monkey kidney-derived cells) for those recognizing the diphtheria toxin receptor complex. Immunofluorescence microscopy revealed that 1C9-2 colocalized with MitoTracker (Fig. 1A). Western blot analysis with this antibody revealed that 1C9-2 was expressed ubiquitously in various human tissues as well as in several cultured cell lines (Fig. 1B). 1C9-2 was purified from cultured Vero cells, and amino acid sequences of several trypsin-digested fragments were determined. A database search revealed that the sequences (boxed in Fig. 2A) are all found in human expressed sequence tag clones (GenBank/EBI Data Bank accession number AA316462 for the longest clone). Because the assembled sequences of these clones did not contain an in-frame initiator methionine, 5'-RACE and 3'-RACE were performed using a cDNA library of CMK cells (human myeloid leukemia-derived cells). Human 1C9-2 cDNA thus obtained coded for a 15521-Da protein containing 142 amino acid residues (Fig. 2A). The N-terminal region, which was missing in the database, was characteristic in that it contains highly negative charges. The entire sequence of 1C9-2 was not found in the cDNA data banks, but the gene encoding the entire sequence was deposited in the GenBank/EBI Data Bank as clone 508I15, which lies within human chromosome 22q12–13 (accession number AL021707). Comparison of both nucleotide sequences revealed that the 1C9-2 gene consists of four exons (Fig. 2B). The exon junctions are shown in Fig. 2A (arrowheads). The data base search for homologous proteins revealed a Caenorhabditis elegans protein with 27% sequence identity (accession number AB71053), which was annotated to have sequence similarity to Tom22 from S. cerevisiae. The predicted sequence of human 1C9-2 showed overall identities of 19 and 20% to Tom22 from S. cerevisiae and N. crassa, respectively. Although the sequence identity was low, these proteins shared a significant structural similarity in the distribution of clusters of acidic amino acid residues along the extramembrane domains (Fig. 3A) and in the hydropathy profile (Fig. 3B). The hydropathy plot shows
the presence of two hydrophilic segments (residues 1–82 and 102–142), which are separated by a hydrophobic segment of 19 amino acid residues. Of note, the N-terminal segment is extremely rich in acidic amino acid residues (21 negative charges in 82 total residues). The C-terminal tails of *N. crassa* and *S. cerevisiae* Tom22, however, have an overall negative charge with a net charge of 2. In marked contrast, 1C9-2 has a C-terminal tail with a neutral net charge.

**Intracellular Localization of 1C9-2**—We examined the intracellular localization of 1C9-2 in rat liver cells by Western blotting using anti-human 1C9-2 polyclonal antibody. As shown in Fig. 4A, this antibody recognized 1C9-2 in rat liver cells, although with a lower reactivity. Using cDNA cloning, we confirmed the presence of the rat counterpart with a sequence

**FIG. 3.** A, comparison of the deduced amino acid sequences of Tom22 from *N. crassa* (N.c.), *S. cerevisiae* (S.c.), and *C. elegans* (C.e.), and human 1C9-2 (H.s.). The positions of conserved acidic amino acids and basic amino acids are indicated by filled and empty boxes, respectively. The putative transmembrane domain is underlined. **B**, comparison of hydropathy profiles of human 1C9-2 and *N. crassa* Tom22 using the algorithm of Kyte and Doolittle at a span setting of 20 amino acid residues.

**FIG. 4.** Subcellular and submitochondrial localization and membrane topology of 1C9-2. A, subcellular localization 1C9-2 in rat liver cells. 10 µg each of the mitochondrial, microsomal, and cytosolic fractions were resolved by SDS-PAGE and subjected to Western blot analysis with the indicated antibodies. Because only antibodies against rat liver marker proteins were available to us, we used rat liver for the fractionation experiments. Several nonspecific bands were observed with polyclonal antibodies against human 1C9-2 because of poor reactivity with the rat counterpart. k, kilodaltons; MSF(L), mitochondrial import stimulation factor large subunit. B, submitochondrial localization of 1C9-2. The rat liver submitochondrial fractions obtained after sucrose density gradient centrifugation were resolved by SDS-PAGE and then subjected to Western blot analysis with the indicated antibodies. T, unfractionated membranes (2.5 µg) from rat liver mitochondria. C, topology of 1C9-2 in the mitochondrial outer membrane as probed by proteinase K susceptibility. C-terminal FLAG-tagged 1C9-2 was expressed in L cells as described under "Experimental Procedures." The mitochondrion-rich fraction was isolated; divided into aliquots; and treated with (lanes 2–4) or without (lanes 1) 20 µg/ml proteinase K at 0 °C for 30 min in isotonic buffer (lanes 2), in hypotonic buffer (lanes 3), or in isotonic buffer containing 1% Triton X-100 (lanes 4). Each reaction mixture was divided into two aliquots and resolved by SDS-PAGE. The gels were subjected to Western blot analysis using anti-FLAG monoclonal antibody (left panel) or anti-1C9-2 monoclonal antibody (right panel). Note that the anti-1C9-2 monoclonal antibody did not react with endogenous 1C9-2 in L cells.
identity of 93.6%. 1C9-2 cofractionated with rat TOM40, the central component of the protein import channel of the mitochondrial outer membrane, but not with microsomal cytochrome P450 (44) or with a large subunit of the mitochondrial import stimulation factor in the cytosol (40, 45) (Fig. 4A). Submitochondrial fractionation using sucrose density gradient centrifugation indicated that 1C9-2 cosedimented with rat TOM40, but not with rat TIM23, a component of the protein import machinery of the inner membrane (33) (Fig. 4B).

We then analyzed the topology of 1C9-2 within mitochondria using proteinase K as the probe (Fig. 4C). For this purpose, mouse L cells were used because anti-1C9-2 monoclonal antibody does not recognize rodent 1C9-2 (data not shown), but detects only human 1C9-2 expressed in L cells. Human 1C9-2 carrying a FLAG tag at the C terminus was expressed in L cells, and the mitochondrial-rich fraction was prepared. As shown in Fig. 4C (lanes 2), proteinase K treatment produced a 1C9-2 fragment with slightly increased mobility by removal of the small N-terminal region while the C-terminal FLAG tag remained attached to the fragment. The fragment that had lost the entire extramitochondrial segment could not be detected for unknown reasons. Under hypotonic conditions, which disrupt the outer membrane but maintain the integrity of the inner membrane (33), proteinase K digestion produced an ~17-kDa fragment that had lost the FLAG tag (Fig. 4C, lanes 3), and this fragment was completely digested by proteinase K in the presence of Triton X-100 (lanes 4). These results indicated that 1C9-2 is inserted into the outer membrane with the N-terminal portion protruding to the mitochondrial surface and the C-terminal portion protruding to the intermembrane space (Ncyt-Cin orientation). Thus, 1C9-2 has the same membrane topology as fungal Tom22. The ~17-kDa fragment seems to be protected against proteinase K within the TOM complex (see below) until the complex is dissociated by Triton X-100.

1C9-2 Is a Component of the ~400-kDa TOM Complex Containing Tom40—In fungal mitochondria, Tom22 forms the TOM core complex of ~400 kDa with Tom40 and three smaller Tom proteins (14, 18, 46). Because the results described above suggest that 1C9-2 is the mammalian counterpart of fungal Tom22, we examined whether it is contained in the complex associating with several mammalian TOM components identified so far. The outer membrane of rat liver mitochondria was solubilized by 2% digitonin and subjected to first dimension blue native PAGE and then to second dimension Tricine/SDS-PAGE. After electrophoresis, the gel was stained for proteins. The bands were excised from the gel and analyzed by mass spectrometry.

To further confirm the mitochondrial protein import defect in Δtom22 yeast cells, we tested whether 1C9-2 complemented the mitochondrial protein import defect in Δtom22 yeast cells. As shown in Fig. 7B, when Δtom22 cells harboring pMD288 were grown in glucose-synthetic medium, the precursor form of Hsp60 accumulated in the cells. In marked contrast, the precursor form of Hsp60 was expressed at a significantly reduced level in Δtom22 cells harboring pMD288/h1C9-2-2 transformed cells (Fig. 7B). Endogenous Tom22 was undetectable under these conditions. We conclude that 1C9-2 complements the defects of mitochondrial function in Δtom22 yeast cells.

DISCUSSION

We have identified an ~22-kDa human mitochondrial outer membrane protein (1C9-2) as a component of the TOM complex. The amino acid sequence of 1C9-2 has an overall identity of 19% to S. cerevisiae Tom22. Despite the low identity of the primary sequence, they have several structural similarities: transmembrane orientation, hydrophathy profile, and distribution of highly acidic amino acid regions in the N-terminal segment. Consistent with these features, 1C9-2 complemented the defects of both growth and protein import in Δtom22 yeast cells. We thus conclude that 1C9-2 is the functional homologue of Tom22. To our knowledge, this is the first report on the identification of mammalian TOM22 as a subunit of the TOM complex, and we refer to this protein as human TOM22. We also cloned rat TOM22 cDNA, which codes for a 142-amino acid protein with a sequence identity of 93.6% to human TOM22. Blue native PAGE of the digitonin-solubilized outer membrane of rat liver mitochondria revealed that rat TOM22 is firmly associated with rat TOM40 and forms the ~400-kDa TOM complex. In addition to these components, several components ~5–10 kDa in molecular size, OM10, OM7.5, and OM5, have

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*H. Suzuki, K. Saeki, and K. Mihara, unpublished observations.*
been detected in the rat TOM complex. The TOM complex purified from potato mitochondria is ~230 kDa in size and has unique subunit compositions: Tom40, Tom20, Tom7, and four other components of smaller molecular size, but the counterpart of Tom22 was absent (47). Therefore, the mammalian TOM complex resembles the fungal TOM complex in size and composition, although the smaller molecular size components remain to be characterized.

The surface-exposed cis-site and the intermembrane space-exposed trans-site act in series to drive the translocation of preproteins across the outer membrane (48). Tom20 and Tom22 provide the cis-site, and preproteins bind to this site through weak electrostatic interactions (48–50). The intermembrane space-exposed segment of Tom40 and the C-terminal tail of Tom22 might contribute to the trans-site (51–53). Highly acidic segments are frequently observed in many components of the mitochondrial import system, and it has been speculated that a preprotein with a basic N-terminal mitochondrion-targeting signal is transported across the outer membrane by sequential binding to a relay of acidic receptor sites (the acid chain hypothesis) (54, 55). In fact, the N-terminal cytoplasmic domain of Tom22 is conserved among species carrying highly abundant acidic amino acid residues: the contents of acidic residues in this region are 22.6, 25.2, and 25.6% for N. crassa, S. cerevisiae, and humans, respectively. The conserved highly acidic region might participate as the cis-binding site in the recognition and transfer of the basic N-terminal mitochondrion-targeting signal of the preprotein (54, 55), although this issue is still controversial (51, 56). The intermembrane space-exposed C-terminal tail of fungal Tom22 is rich in acidic amino acid residues: the contents of acidic residues are 18.4% (net charge, $-5$) and 21.2% (net charge, $-5$) for N. crassa and S. cerevisiae, respectively. The significance of the acidic amino acid residues in this region in protein translocation, however, remains controversial (43, 51, 53, 57). In contrast, the C-terminal tail of mammalian TOM22 does not carry net negative charges: the acidic amino acid content is 7.31% (net charge, 0). It should be noted, in this relation, that the C-terminal tail of C. elegans TOM22 carries a net positive charge. Thus, the importance of the C-terminal tail of Tom22 in preprotein import into mitochondria should be further examined. More extensive studies using the purified domain or liposome-reconstituted vesicles with defined TOM components are required.

In summary, we have identified TOM22 in organisms other than S. cerevisiae and N. crassa. These results, in conjunction with the fact that several mammalian homologues (TOM20, TOM40, and several TIM proteins) have been identified, indicate that the mitochondrial import machineries of the outer and inner membranes are evolutionarily conserved among eukaryotic organisms. Subtle variations do exist, however, among different species. Future work is required to clarify the functional significance of these differences among species.

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