Targeting and Assembly of Rat Mitochondrial Translocase of Outer Membrane 22 (TOM22) into the TOM Complex*

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Tom22 is a preprotein receptor and organizer of the mitochondrial outer membrane translocase complex (TOM complex). Rat Tom22 (rTOM22) is a 142-residue protein, embedded in the outer membrane through the internal transmembrane domain (TMD) with 82 N-terminal residues in the cytosol and 41 C-terminal residues in the intermembrane space. We analyzed the signals that target rTOM22 to the mitochondrial outer membrane and assembly into the TOM complex in cultured mammalian cells. Deletions or mutations were systematically introduced into the molecule, and the intracellular localization of the mutant constructs in HeLa cells was examined by confocal microscopy and cell fractionation. Their assembly into the TOM complex was also examined using blue native gel electrophoresis. These experiments revealed three separate structural elements: a cytoplasmic 10-residue segment with an acidic α-helical structure located 30 residues upstream of the TMD (the import sequence), TMD with an appropriate hydrophobicity, and a 20-residue C-terminal segment located 22 residues downstream of the TMD (C-tail signal). The import sequence and TMD were both essential for targeting and integration into the TOM complex, whereas the C-tail signal affected the import efficiency. The import sequence combined with foreign TMD functioned as a mitochondrial targeting and anchor signal but failed to integrate the construct into the TOM complex. Thus, the mitochondrial-targeting and TOM integration signal could be discriminated. A yeast two-hybrid assay revealed that the import sequence interacted with two intramolecular elements, the TMD and C-tail signal, and that it also interacted with the import receptor Tom20.

Integral membrane proteins of the mitochondrial outer membrane are synthesized by cytoplasmic ribosomes as mature precursors and post-translationally integrated into the membrane (1, 2). Unlike the matrix-targeted preproteins with cleavable presequences, the signals that target the outer membrane proteins are contained within the mature protein sequence. The import receptors of the preprotein translocase of the mitochondrial outer membrane (TOM complex; 3, Tom70 (4, 5), and Tom20 (6, 7)) are anchored to the membrane through the N-terminal α-helical transmembrane domain (TMD) in the N-in-C orientation. Tom22, which functions as the preprotein receptor and organizer of the TOM complex, is anchored to the outer membrane in the Nout-Cin orientation through a TMD in the middle portion of the molecule (8–11). Tom5, Tom6, and Tom7 are components of the TOM core complex and are anchored to the membrane through the C-terminal TMD (12–16). Tom40 (17–21) and porin (22, 23) are β-barrel proteins spanning the outer membrane by 12–14 antiparallel β-strands that function as the transport channels of preproteins (Tom40) or small molecules (porin).

In most outer membrane proteins that are anchored to the membrane through an α-helical TMD, the TMD and the flanking short segments function as the signal anchor for mitochondrial translocation. For example, Saccharomyces cerevisiae Tom70 targets and inserts into the mitochondrial outer membrane by a signal anchor composed of two domains: a positively charged N-terminal hydrophilic region (residues 1–10) followed by the TMD (residues 11–29). McBride et al. (5) demonstrated that the TMD functions as the signal anchor sequence that is both necessary and sufficient for targeting and insertion, whereas the N-terminal hydrophilic region enhanced import.

In Tom20, the N-terminal TMD and a net positive charge within five residues of the C-terminal flanking region function together as the mitochondrial targeting signal anchor (7). During translation, the signal recognition particle (24) recognizes the TMD of Tom20; basic amino acid residues in the C-terminal flanking region, however, interfere with the function of the signal recognition particle, thus preventing signal recognition particle-dependent endoplastic reticulum (ER) targeting (7).

The C-terminal positive charge and the TMD length are important determinants for the signal anchor of C-anchored proteins (25–28). In Tom5, an appropriate length TMD and a specific sequence containing proline in the TMD are required for functional targeting and assembly into the TOM complex (29). In contrast, the other C-tail-anchored proteins that are dispersed in the outer membrane depend on both an appropriate length TMD and positive charges of the C-terminal flanking segment (25–28).

On the other hand, the signal for mitochondrial targeting and assembly of Tom6 and Tom22 is composed of the TMD and separately localized segments. The targeting signal of S. cerevisiae Tom6 is composed of a 10-residue internal segment in...
the cytoplasmic domain, TMD, and the following C-terminal 9-residue segment (30). In vitro import studies of *Neurospora crassa* TOM22 demonstrated that a segment of the cytosolic domain (encompassing residues 45–75) resembling matrix-targeted presequences is essential for import and assembly into the TOM complex (10). This segment is potentially amphipathic, carries a net positive charge, and is enriched in serine, tyrosine, and threonine residues, and it is referred to as the “import sequence.” The precise character of the internal import sequence, how the signal is recognized by the TOM components, and the mechanism by which the import signal and TMD determine the membrane topology during membrane disintegration, however, remain unclear.

Human TOM22 exhibited overall sequence identities of 19% and 20% to TOM22 from *S. cerevisiae* and *N. crassa*, respectively (31, 32). Although the sequence identity is low, these proteins share a structural similarity in the distribution of clusters of acidic amino acid residues along the cytoplasmic domain toward the N terminus and in the hydropathy profile. The distribution of acidic amino acid residues along the intermembrane segments, however, is distinct. The C-terminal tails of *N. crassa* and *S. cerevisiae* Tom22 have an overall negative charge with a net charge of −5. In marked contrast, human TOM22 has a C-terminal tail with a neutral net charge.

These differences led us to investigate the signal of rat TOM22 (rTOM22) that determines mitochondrial targeting and integration into the TOM complex using in vivo and in vitro systems. rTOM22 is composed of 142 amino acid residues and has an overall sequence identity of 93% to human TOM22.

**EXPERIMENTAL PROCEDURES**

**Materials**—Antibodies against rTOM20 and rTOM70 were prepared as previously described (31). Rat liver mitochondria and the mitochondrial outer membrane were prepared in 1 ml of homogenization buffer. The cells were disrupted by five-stroke aspiration through a 22-gauge needle and centrifuged at 10,000 *g* for 2 h. The OMV precipitates were collected and suspended in 1 ml of homogenization buffer. The cells were disrupted by five-stroke aspiration through a 22-gauge needle and centrifuged at 100,000 *g* for 2 h. The OMV precipitates were collected and suspended in 1 ml of homogenization buffer.

**Molecular cloning**—The internal import sequence in the cytoplasmic segment, TMD, and has an overall sequence identity of 93% to human TOM22.

**Plasmid DNA (1 *µg*) was transfected to HeLa cells, and the cells were incubated in 3.5-cm dishes at 37 °C for 24 h.**

**Fluorescence Microscopy**—HeLa cells were cultured on coverslips in 35-mm dishes in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum at 37 °C for 24 h under an atmosphere of 5% CO₂ in air. Transfection was performed as above, and the cells were incubated for 24 h. To locate mitochondria with MitoTracker, HeLa cells cultured for 24 h were incubated in the presence of 50 nM MitoTracker for 30 min at 37 °C. The cells were cultured on coverslips and washed twice with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde at room temperature for 30 min, washed twice with PBS, and treated with acetone/methanol (1:1) for 4 min. After washing sequentially with PBS and 2% bovine serum albumin in PBS, the cells were incubated for 2 h with anti-FLAG M2 monoclonal IgG (Sigma) or anti-FLAG IgG plus anti-calnexin IgGs (Stressgen) in PBS containing 1% bovine serum albumin, washed five times with PBS, and then immunostained using fluorescein-conjugated anti-mouse IgGs (for rTOM22 constructs) or Texas Red-conjugated anti-rabbit IgGs (for calnexin). Fluorescent images were taken and analyzed using a confocal laser microscope (Radiance 2000; Bio-Rad).

**Subcellular Fractionation**—The transfected HeLa cells were cultured in 10-cm dishes for 24 h and washed twice with PBS. The collected cells were washed twice with the homogenization buffer (10 mM HEPES-KOH (pH 7.4) containing 250 mM sucrose and 1 mM EDTA (35) containing protease inhibitor (Complete, EDTA Free; Roche Molecular Biochemicals)) and suspended in 1 ml of homogenization buffer. The cells were disrupted by five-stroke aspiration through a 27-gauge needle and centrifuged at 800 × *g* for 5 min. The supernatant was centrifuged at 100,000 × *g* for 30 min to separate the ER-enriched fraction (P1). The supernatant was centrifuged at 100,000 × *g* for 30 min to separate the ER-enriched fraction (P2) and the supernatant. The subcellular fractions were subjected to SDS-PAGE and then immunoblotting using anti-FLAG antibodies. The immunobots were visualized by electrochemiluminescence (Amersham Biosciences), and the images were analyzed by LAS-1000 (Fuji Film).

**Preparation of Rat Liver Mitochondrial Outer Membrane**—Rat liver mitochondrial outer membrane vesicles (OMV) were prepared essentially as described by Nishimoto and Ito (34). The mitochondrial (33) was suspended in 5 mM potassium phosphate buffer (pH 7.4) containing 2 mM diithiothreitol, 5 µM/ml each leupeptin and pepstatin, incubated on ice for 20 min, and then sonicated with a Branson sonifier at 150 watts for 1 s, six times at 30-s intervals. The mixture was layered over 1.25 M sucrose in 10 mM HEPES-KOH buffer (pH 7.4) containing 2 mM MgCl₂, 2 mM dithiothreitol, and 5 µg/ml each leupeptin and pepstatin and centrifuged at 100,000 × *g* for 2 h. The 1.25 M sucrose layer was collected and diluted with 5 mM potassium phosphate buffer (pH 7.4) containing 2 mM dithiothreitol, 5 µg/ml each leupeptin and pepstatin, and centrifuged at 10,000 × *g* for 2 h. The OMV precipitates were suspended in the homogenization buffer.

**Binding of the GPF Constructs Converting the rTOM22 Elements to OMV**—The internal import sequence in the cytoplasmic segment, TMD, C-tail signal, or their combinations was fused to the N terminus of GFP (Roche Applied Science).
buffer (pH 7.4) containing 50 μg of OMV, 0.25 M sucrose, and 0.1 mg/ml α2-macroglobulin at 30 °C for 60 min. The reaction mixture was mixed with 120 μl of 10 mM HEPES-KOH (pH 7.4) containing 2.5 M sucrose, placed at the bottom of discontinuous layers of 0.25 M sucrose (150 μl) and 1.25 M sucrose (150 μl) in 10 mM HEPES-KOH buffer (pH 7.4), and centrifuged at 100,000 × g for 90 min. The OMV, which floated to the upper 240 μl of the tube, was trichloroacetic acid-precipitated and subjected to SDS-PAGE followed by digital autoradiography (Fuji BAS-2000).

Blue Native PAGE—Blue native PAGE (BN-PAGE) was performed essentially as described previously (36). The mitochondria isolated from HeLa cells expressing the rTOM22 mutants were solubilized in 30 μl of homogenization buffer containing 2% digitonin, and insoluble material was removed by centrifugation for 15 min at 100,000 g. The supernatant was mixed with 2 μl of sample buffer (5% Coomassie Brilliant Blue G-250, 100 mM bis-Tris (pH 7.0), 500 mM 6-aminocaproic acid) and electrophoresed through 5–41% sucrose gradient gels. The gels were subjected to immunoblotting using antibodies against HA for TOM22 constructs or against the indicated proteins. Tom40, PDI, and H450 were used as markers for mitochondria, microsomes, and supernatant fractions, respectively. Assembly of the rTOM22 mutants into the TOM complex as revealed by BN-PAGE. Mitochondria were prepared from HeLa cells expressing the indicated constructs and were solubilized by 2% digitonin. The isolated mitochondria were fractionated into low speed supernatant (S1) and high speed precipitate (P2; microsome-enriched fraction), and the supernatant (S) as described under “Experimental Procedures.” The isolated fractions were subjected to SDS-PAGE followed by immunoblotting using antibodies against HA for TOM22 constructs or against the indicated proteins. Tom40, PDI, and H450 were used as markers for mitochondria, microsomes, and supernatant fractions, respectively. Assembly of the rTOM22 mutants into the TOM complex as revealed by BN-PAGE.

Yeast Two-hybrid Assay—Yeast two-hybrid assays were performed using the MATCHMAKER GAL4 system 3 (Clontech) according to the manufacturer’s protocol. The cytosolic domains of rTOM20 (Asp25–Glu49), rTOM22 (Met1–Arg40), rTOM70 (Arg64–Leu80), and three internal sequence elements of rTOM22 (the import sequence (residues 41–60), TMD (residues 83–101), and C-tail signal (residues 123–142)) were amplified by PCR, and the PCR fragments were inserted separately downstream of the GAL4 DNA-binding domain (BD) on the pAS2-1 plasmid (TRP1) or into the GAL4-activating domain (AD) on the pACT2 plasmid (LEU2). Two-hybrid interactions were assayed using the HIS3 reporter system. Co-transformation of two hybrid vectors into S. cerevisiae AH109 (MATa, leu2–3, 112, ura3–52, his3–200, gal4Δ, gal80Δ, lys2::GAL1UAS-GAL1TATA-HIS3, GAL2UAS-GAL2TATA-ADE2, URA3::MEL1UAS-MEL1TATA-lacZ) was performed according to the manufacturer’s instructions. The transformants were screened for their potential to grow on synthetic complete medium lacking adenine, tryptophan, leucine, and histidine.

RESULTS

The Internal Sequence with an Acidic Amphiphilic Helical Structure in the Cytosolic Segment Is Required for Mitochondrial Targeting of rTOM22—To define the mitochondrial targeting signal of rTOM22 (the primary sequence shown in Fig. 1), consecutive 10-residue deletions were introduced into rTOM22 carrying FLAG and HA tags at the N and C terminus, respectively, and expressed in HeLa cells, and their intracellular...
Acidic amphiphilic α-helicity is critical for the function of the import sequence. A, rTOM22 constructs carrying the import sequence with a proline replacement or alanine insertions were expressed in HeLa cells, and their immunofluorescence images were taken. Merged images of immunofluorescence (green) and with MitoTracker (red) are shown. B, rTOM22 mutants in which leucine residues in the import sequence were replaced by alanine residues in the indicated combinations were expressed in HeLa cells, and their intracellular localization was examined as in A. C, HeLa cells expressing the indicated rTOM22 mutants were subfractionated into the mitochondria-enriched low speed precipitate (P1), microsome-enriched high speed precipitate (P2), and supernatant (S) fractions, which were then subjected to SDS-PAGE followed by immunoblotting using anti-HA antibodies. D, rTOM22 mutants in which glutamic acid residues in the import sequence were replaced by glutamine residues in the indicated combinations were expressed in HeLa cells, and their intracellular localization was examined as in A. E, α-helical plot of amino acid residues 41–58.
lar localization was examined by confocal microscopy and cell fractionation. Merged images of FLAG tag and MitoTracker immunofluorescence are shown in Fig. 2A. The mutant with a deletion in the cytoplasmic domain, Δ41–50, exhibited clear mislocalization to the cytoplasm, which was also confirmed by cell fractionation (Fig. 2B). The TMD-deleted construct (deletion of residues 83–101; ΔTM) also mislocalized to the cytoplasm (Fig. 2, A and B). In contrast, the other constructs were all correctly targeted to mitochondria (fractionation data are shown for Δ21–30, Δ51–60, and Δ133–142 and are typical for the constructs that displayed mitochondrial localization in Fig. 2A).

We then examined whether the deletion constructs targeted to the mitochondria were correctly assembled into the TOM complex using BN-PAGE. The constructs with deletions encompassing residues 1–40 were efficiently integrated into the ~400-kDa TOM complex (36) (Fig. 2C). The constructs with deletions in the C-terminal intermembrane segment (residues 103–142) were integrated into the TOM complex with slightly reduced efficiency, and unassembled products were detected, suggesting that the deletion of the C-terminal segment affected assembly into the TOM complex. The immunoblot signals were not detected either in the TOM complex or in the position of the unassembled species of the constructs with deletions encompassing residues 51–80 (Fig. 2C, upper panel), notwithstanding their presence in the digitonin-solubilized fraction (Fig. 2C, lower panel). Confocal microscopy and cell fractionation clearly indicated that these constructs were targeted to mitochondria, and, as such, the proteins were detected in the mitochondria by SDS-PAGE and subsequent immunoblotting (Fig. 2A; Fig. 2B for Δ51–60). We have no explanation for this phenomenon.

Secondary structure prediction indicated that residues 41–58 potentially formed an acidic amphiphilic α-helical structure, in which four glutamic acid residues were placed on one side of the helix and hydrophobic residues including leucine on the other side; the critical segment in the cytosolic domain as assigned above (residues 41–50) was contained in this helix (Fig. 3E). We therefore addressed the importance of the predicted helical structure as well as the acidic or hydrophobic residues in this segment. First, an increasing number of alanine residues were inserted in the middle portion of this segment to disturb the topology of authentic amino acid residues in the helical wheel.

All of the constructs except for the four-alanine insertions (+4A) failed to produce correct mitochondrial targeting; they were mislocalized to the cytoplasm, indicating that some defined amino acid residues must be placed in the helix with the correct topology (Fig. 3A; cell fractionation data in Fig. 3C). Furthermore, when amino acid residues in this segment were replaced by a helix-breaking proline, the constructs (E46P and W49P in Fig. 3A) were also mislocalized to the cytoplasm. These results indicated that the α-helical structure of this segment is essential for correct mitochondrial targeting. We then addressed the importance of leucine residues in this segment. For this purpose, three leucine residues in this segment were replaced by alanine in various combinations (Fig. 3B). When at least two leucine residues were replaced by alanine, the correct mitochondrial targeting was lost (Fig. 3, B and C), indicating the importance of hydrophobicity of the face in the predicted helix where the leucine residues are placed. Because four glutamic acid residues at 42, 46, 53, and 57 are on the same face of the helix (see Fig. 3E), we examined the importance of these residues by replacing them with glutamine in different combinations. A single mutation from glutamic acid to glutamine at residue 42 (1Q in Fig. 3D; E42Q), but not at the other positions, induced partial mislocalization of the con-
targeting; therefore, the segment encompassing residues 123–142 was important for proper targeting. Of note, the constructs \( \text{H9004}^{123-132} \) and \( \text{H9004}^{133-142} \) were efficiently targeted to mitochondria (Fig. 2, A and B, and Fig. 4 A). These results indicated that either half of the segment (residues 123–132 or 133–142) in residues 123–142 was sufficient for maintaining the targeting efficiency of the constructs.

The segment encompassing residues 123–142 is rich in proline and glycine residues and potentially forms a \( \text{H9252} \)-turn structure, based on secondary structure prediction. We therefore examined whether proline and glycine residues were required for maintaining the targeting efficiency of the constructs.

The Authentic TMD of rTOM22 Is Required for Assembly into the TOM Complex

We then examined the importance of the TMD structure. When hydrophilic residues in the TMD were replaced by 2 or 4 valine residues without changing the length, the constructs localized to the ER; i.e., the mutants colocalized with the ER marker calnexin (Fig. 5, A and B). Thus, a TMD with appropriate hydrophobicity is essential for mitochondrial targeting. The importance of a proline residue in the TMD of Tom22 was demonstrated in yeast (37), and was also confirmed in rTOM22. When Pro-98 was replaced with alanine (TM(P/A)) in Fig. 5A), the mutant was mistargeted to the ER (Fig. 5, A and B).

We next examined whether the TMD of other membrane proteins is functional for mitochondria targeting or assembly into the TOM complex. For this purpose, the TMD of rTOM22 (19 residues; average hydrophobicity 2.05) was replaced by that of rTOM20 (18 residues; average hydrophobicity 2.00; Fig. 5 A). When expressed in HeLa cells, the construct TM(Tom20) was targeted to mitochondria (Fig. 5, A and B). BN-PAGE, however, revealed that it failed to be integrated into the TOM complex (Fig. 5C).

Taken together, these results indicated that the import sequence in conjunction with the TMD of other membrane proteins with an appropriate hydrophobicity functioned as the

**Fig. 5.** Mitochondrial targeting and assembly into the TOM complex of rTOM22 depend on the TMD structure. A, schematic presentation of rTOM22 constructs carrying TMD mutations or heterologous TMD from TOM22. The indicated rTOM22 mutants were expressed in HeLa cells, and their intracellular localization was examined as in Fig. 2A. Immunostaining with anti-calnexin antibodies (red) was used to localize the ER. B, HeLa cells expressing the indicated constructs were subfractionated into P1, P2, and supernatant as in Fig. 2B. C, the mitochondria isolated from HeLa cells expressing the indicated rTOM22 constructs were subjected to BN-PAGE and subsequent immunoblotting using anti-FLAG antibodies.
mitochondria targeting signal. For integration of rTOM22 into the TOM complex, however, cooperation of the authentic TMD and the import sequence was required. A specific sequence containing Pro-98 in the TMD was probably critical for the function. Thus, the signals required for mitochondrial targeting and for integration into the TOM complex could be differentiated.

The Import Sequence Is Recognized by the Import Receptor rTOM20—We then addressed whether the components of the TOM complex recognized any of the three sequence elements in rTOM22 that are required for mitochondrial targeting and TOM assembly. The three elements identified were fused, separately or in combinations, to the N terminus of GFP (Fig. 6A), and binding of the fusion constructs to the isolated OMV was measured by sucrose floatation centrifugation. The construct carrying only the import sequence (N-GFP) did not bind to the OMV (Fig. 6B). In contrast, the constructs carrying the import sequence and TMD together (NTM-GFP and NTMC-GFP) bound to the OMV to a significant extent. Moreover, their binding was significantly decreased when trypsin-pretreated OMV was used. These results indicated that the import sequence in conjunction with TMD functioned as the mitochondrial-targeting signal, which was probably recognized by the trypsin-sensitive import receptors of the mitochondrial surface.

We then addressed whether the import sequence in the cytosolic domain interacted with the TMD or C-tail signal using a yeast two-hybrid assay. The import sequence fused to the GAL4 DNA-binding domain (BD-N) interacted with the TMD fused to the GAL4 activator domain (AD-TM) (Fig. 6C). There was also an interaction of the import sequence (BD-N) with the C-terminal signal (AD-C), whereas no interaction was detected between TMD and the C-tail signal (left panel in Fig. 6C). These results suggested cooperation of three segments in TOM22 during membrane targeting and integration. It is possible that the import sequence cooperates with the TMD to function as an import signal with a hairpin loop structure.

We further examined whether the import sequence interacted with the cytoplasmic segment of the import receptors Tom20, Tom70, or Tom22, using a yeast two-hybrid assay. As controls, AD-Tom20 interacted with BD-Tom22, and vice versa (Fig. 6E), confirming a genetic as well as physical interaction of Tom20 and Tom22 (38, 39). The import sequence fused to BD (BD-N) interacted with both the cytoplasmic segment of Tom20 and Tom22, which were fused to AD (AD-20 and AD-22, respectively), suggesting that the import signal was recognized by Tom20 and Tom22 in the initial import processes. This is consistent with the results of the import inhibition by anti-Tom20 antibodies (see below).

The C-tail-deleted rTOM22 Is Imported into Mitochondria via the Import Receptor-dependent Pathway—Mitochondrial import of N. crassa Tom22 depends on both Tom70 and Tom20, and trypsin-treatment of mitochondria significantly reduces the import (8, 10, 40). Mutants with a deletion of the C-terminal intermembrane space domain are efficiently imported through a receptor-independent bypass import pathway (10, 40). Because the sequence similarity of mammalian TOM22 to N. crassa Tom22 is as low as 23%, we examined whether the import of rTOM22 had the same requirement of surface receptors as N. crassa Tom22. rTOM22 has a Nout-Cin orientation in the outer membrane, and a C-terminal 6-kDa fragment is produced by proteinase K treatment of mitochondria (31). We therefore assessed correct membrane integration of rTOM22 using the proteinase K-produced 6-kDa fragment as a marker. In vitro synthesized 35S-TOM22 was integrated into the energized mitochondria and formed the 6-kDa proteinase K-resistant fragment (Fig. 7A, 22f). The import was significantly inhibited by trypsin pretreatment of the mitochondria; only a small fraction (~5%) of rTOM22 was imported via the surface receptor-independent “bypass” pathway (Fig. 7, A and B). Furthermore, import was inhibited by anti-rTOM20 IgGs but not by anti-rTOM70 IgGs (Fig. 7, A and B). Thus, import of rTOM22 depended on rTOM20.
Because the C-tail-deleted *N. crassa* Tom22 is efficiently imported into mitochondria via the bypass route, we examined this in *rTom22* by measuring assembly of the *rTom22* constructs into the −400-kDa TOM complex (Fig. 7C). BN-PAGE revealed that 35S-labeled *rTom22* (wild type) was integrated into the −400-kDa complex, and trypsin treatment of mitochondria strongly inhibited the integration (Fig. 7C, lanes 1 and 2). 35S-rTom22(A103–142) was imported into the intact mitochondria, but with the efficiency of −50% of the activity of rTom22 (wild type), confirming the results shown in Fig. 4 that the C-terminal segment affected import efficiency. In marked contrast to *N. crassa* Tom22, only a small fraction (−1%) of 35S-rTom22(A103–142) was imported into trypsin-pretreated mitochondria (Fig. 7C, lanes 3 and 4). The fast migrating bands in Fig. 7C (indicated by dots and a circle) corresponded to the C-terminal segments-trimmed rTom22 fragments as judged by immunoblot analysis of the in vitro translated and mitochondria-imported products of rTom22 carrying N-terminal FLAG and C-terminal HA tags (data not shown). We concluded that the precursor of the C-terminal segment-deleted rTom22 was imported into the mitochondria mainly through a TOM20-dependent pathway; only a small fraction (less than 5%) was imported via the receptor-independent bypass pathway. Thus, the C-tail segment of rTom22 did not affect the bypass import pathway in a mammalian mitochondrial system.

**DISCUSSION**

Tom22 is anchored to the outer membrane by an internal TMD in the Nout-Cin orientation. In contrast to the mitochondrial outer membrane proteins that are anchored to the membrane through the N- or C-terminal signal anchor sequences, rTom22 requires three internal elements for efficient targeting and integration into the TOM complex: the TMD, a short segment in the cytoplasmic domain localizing separately from the TMD (the import sequence), and the C-tail signal.

The importance of the sequences localizing in the cytoplasmic and intermembrane space has been reported for *N. crassa* Tom22, although the properties and presumed functions are distinct from those of mammalian TOM22. In *N. crassa* Tom22, the cytoplasmic segment encompassing residues 45–75 functions as an essential internal import signal. This segment resembles matrix-targeted presequences in that it is enriched in hydroxylated amino acid residues, possesses a net positive charge, and is potentially amphipathic. In contrast, the segment that functions as the internal import sequence of rTom22 is restricted to a narrow 10-residue region (residues 41–50) in the cytoplasmic domain. Acidic and amphipathic α-helicity is important for the function of this segment. In this sense, this signal is distinct from the classical matrix-targeting signal. This segment cooperates with an appropriate TMD as the mitochondrial targeting and membrane anchor signal.

What are the components that recognize these structural characteristics? Import of rTom22 clearly depends on the major import receptor Tom20 (Fig. 7). A yeast two-hybrid assay revealed that the import sequence interacted with the cytoplasmic domains of TOM20 and rTom22. In *N. crassa*, a Tom22 mutant lacking the C-terminal intermembrane space domain is efficiently imported into mitochondria in a surface receptor-independent “bypass” pathway (40), whereas the import is strongly compromised when the import sequence in the cytoplasmic domain is deleted (10), indicating that the import se-
quence functions in the absence of surface receptors. Our experiments revealed that, in marked contrast to *N. crassa* Tom22, only a small fraction of rTom22 (−5%) was imported into trypsin-treated mitochondria, indicating that the C-terminal tail-deleted rTom22 was also imported into mitochondria mainly through the import receptor-dependent pathway. These results suggested that the 10-amino acid residue import sequence is required somewhere in the import receptor-dependent pathway. The yeast two-hybrid assay and antibody inhibition experiments suggested that the import sequence could be recognized by rTom20. Because this sequence assumes an acidic amphiphilic α-helix, the helix might be accommodated into the shallow hydrophobic presequence-binding groove of rTom20 (41) through an interaction between hydrophobic residues aligned on one face of the helix and the hydrophobic groove of rTom20. It should be noted, in this context, that the GFP fusions carrying both the import sequence and TMD (NTM-GFP and NTMC-GFP) bind to the OMV, depending on the import receptor(s), whereas the construct carrying only the import sequence (N-GFP) did not (Fig. 6A). These results together suggest an auxiliary function of the TMD for the recognition; rTom20 recognizes the signal produced by cooperation of the import sequence and TMD. The component that recognizes the acidic residues aligned on the opposite side of the helix remains to be identified.

BN-PAGE revealed that the rTom22 mutant whose authentic TMD was replaced by the rTom20 TMD was targeted and integrated into the mitochondrial membrane but failed to be integrated into the TOM complex. These results indicated that the authentic rTom22 TMD is required for correct assembly into the TOM complex. As reported for *S. cerevisiae* Tom22 (37), a proline residue in the TMD (Pro-98) is required for the recognition. In *S. cerevisiae*, Tom22 lacking the intermembrane space domain is assembled into the 400-kDa TOM complex (42). When both domains were removed by proteinase K, the TOM complex was stably maintained. In the absence of Tom22, on the other hand, the 400-kDa TOM complex dissociated to produce a 100-kDa core complex. Therefore, the TMD of Tom22 is required to maintain the stability of the 400-kDa TOM complex (42). Our results, together with the above report, suggest that the rTom22 TMD is involved in both integration into the TOM complex and regulation of the assembly of the TOM complex, but the cytoplasmic segment is not involved in these processes.

How is the transbilayer orientation of simple bitopic membrane proteins determined on the outer mitochondrial membrane? In the ER and bacterial inner membranes, the net positive charge on the *cis*-side of the membrane is an important topologic determinant (43, 44) (“positive inside rule”). Rodríguez-Cousino et al. (10), however, demonstrated for *N. crassa* Tom22 that its orientation is not influenced by the charges flanking the TMD. We also confirmed this for rTom22. Its TMD is flanked by three positive charges on the cytoplasmic side and by one net negative charge on the intermembrane space side, and reversal of this charge distribution did not affect the orientation; the rTom22 mutant carrying two negative charges on the cytosolic side and two positive charges on the intermembrane space side was imported to the mitochondrial outer membrane in the correct N-out-C-in orientation. Using a fusion protein in which the signal anchor of Tom70 was fused to dihydrofolate reductase such that the upstream segment of the signal anchor was replaced by the matrix-targeting signal, Li and Shore (1, 45) demonstrated that the construct was inserted into the outer membrane in the inverted orientation, indicating that retention of the N terminus of the signal anchor on the cytoplasmic side of the outer membrane is an important factor for establishing the N-out-C-in orientation. Considering that the import sequence of rTom22 interacted with the cytoplasmic domain of the import receptor TOM20, it might function as the retention signal. It is possible that if the three topogenic sequences in rTom22 were independently recognized by the mitochondrial import components, transplanta-

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