Translocase Complex of the Outer Mitochondrial Membrane\*  

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 277, No. 44, Issue of November 1, pp. 42197–42204, 2002

This paper is available on line at http://www.jbc.org

Received for publication, June 6, 2002, and in revised form, July 30, 2002
Published, JBC Papers in Press, August 26, 2002, DOI 10.1074/jbc.M205613200

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Tom7 is a component of the translocase of the outer mitochondrial membrane (TOM) and assembles into a general import pore complex that translocates preproteins into mitochondria. We have identified the human Tom7 homolog and characterized its import and assembly into the mammalian TOM complex. Tom7 is imported into mitochondria in a nucleotide-independent manner and is anchored to the outer membrane with its C terminus facing the intermembrane space. Unlike studies in fungi, we found that human Tom7 assembles into an ~120-kDa import intermediate in HeLa cell mitochondria. To detect subunits within this complex, we employed a novel supershift analysis whereby mitochondria containing newly imported Tom7 were incubated with antibodies specific for individual TOM components prior to separation by blue native electrophoresis. We found that the 120-kDa complex contains Tom40 and lacks receptor components. This intermediate can be chased to the stable ~380-kDa mammalian TOM complex that additionally contains Tom22. Overexpression of Tom22 in HeLa cells results in the rapid assembly of Tom7 into the 380-kDa complex indicating that Tom22 is rate-limiting for TOM complex formation. These results indicate that the levels of Tom22 within mitochondria dictate the assembly of TOM complexes and hence may regulate its biogenesis.

Most proteins found in mitochondria are encoded by genes in the nucleus and following synthesis in the cytosol are imported into the organelle. Proteins are directed to their appropriate sub-mitochondrial location, the outer and inner membranes, the intermembrane space (IMS),\(^1\) and the matrix via targeting signals (1–3). Matrix-targeted proteins are synthesized with cryptic, internal targeting signals. Nevertheless, it seems that all proteins targeted to the mitochondria engage with members of the translocase of the outer membrane (TOM). Those precursors that are targeted to the inner membrane or matrix interact with one of two translocase complexes of the inner membrane (4).

Much of our knowledge of mitochondrial protein import has been derived from work with the fungal systems \textit{Saccharomyces} cerevisiae and \textit{Neurospora crassa}. In yeast, the following nine TOM subunits have been identified: Tom72, Tom70, Tom40, Tom37, Tom22, Tom20, Tom7, Tom6, and Tom5 (1). All of the TOM subunits have been found in \textit{N. crassa} apart from Tom37 and Tom5 (2, 5). Tom20 is the major import receptor, binding to matrix-targeted precursors (6), whereas Tom70 seems to show selectivity for precursor binding (7) and has been reported to interact functionally with Tom37 (8). Tom22 has a major receptor function (9–11) and is also required for assembly of the TOM components into a stable multimeric complex and therefore has been termed an “organizer” of the TOM complex (12). Tom40 forms the voltage-gated membrane channel (5, 13, 14) and associates with three small subunits, Tom7, Tom6, and Tom5, along with Tom22 in an ~400-kDa core complex (15). Tom70 and Tom20 also associate with this complex but are less tightly bound. Tom5 possesses receptor functions (16), whereas Tom6 and Tom7 seem to play counteractive roles in mediating TOM-TOM interactions. Tom6 is important for promoting the interactions between Tom20 and Tom70 with the TOM complex (17) and also for stimulating the assembly of Tom22 with Tom40 (15). In contrast, Tom7 seems to play a role in destabilizing the interactions between the TOM complex and the TOM receptors, and this may be important for insertion of proteins into the outer membrane (15, 18).

The TOM machinery is also required for its own biogenesis. TOM proteins are synthesized in the cytosol and engage with other pre-existing TOM subunits during their import, insertion into the outer membrane, and assembly (19). For example, yeast Tom40 undergoes an elaborate pathway for its own assembly. It first associates with TOM receptors and then translocates across the outer membrane where it is found in an ~250-kDa complex with Tom5 at the inner face of the outer membrane. It then integrates into the outer membrane where it associates in an ~100-kDa complex with Tom5, Tom6, and Tom7 and, in the presence of Tom22, assembles into the TOM core complex (20). In contrast, fungal Tom22, Tom5, and Tom7 precursors each appear to assemble directly into the TOM complex without forming detectable intermediates (15, 19, 20). Tom6 preferentially assembles into an intermediate complex with Tom40, Tom5, and Tom7 before assembling into the TOM complex through recruitment of Tom22 (15, 20, 21). Interestingly, the presence of Tom7 is required for the efficient assen-
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The basic processes of protein import into mitochondria seem to be well conserved from fungi to mammals, only partial information is available for higher eukaryotes (3, 22). Recently, a number of mammalian translocase components have been identified and include the following TOM subunits: Tom20 (23–25), Tom22 (26), Tom40 (27), and Tom70 (27). As yet no small TOM subunits have been identified and characterized. However, several novel mammalian outer membrane-associated proteins have also been suggested to play a role in import and include Tom34 (28), metaxin (29), and OM37 (30). Thus, some features of mitochondrial protein import may be unique to higher eukaryotes, indicating that further characterization is needed. Although some studies into the interactions between the various mammalian TOM subunits have been performed, little information is available on the molecular architecture of the core TOM complex. Furthermore, an analysis of the assembly pathway of any known mammalian component has not been studied in detail.

Here we report the identification of the human Tom7 ortholog and analyze its import and assembly into mitochondria. Unlike its fungal counterparts, human Tom7 assemblies into the TOM core complex via an intermediate that contains Tom40 but not Tom22. Interestingly, the formation of this intermediate was not detectable in all mitochondrial preparations tested, thus pointing to a difference in the regulation and/or expression of TOM subunits between cell types. This is supported by our findings that increasing the levels of Tom22 in mitochondria can accelerate the rate of Tom7 assembly into the mature TOM complex.

EXPERIMENTAL PROCEDURES

Cloning Procedures—The cDNA encoding human Tom7 (accession number AA186406) was obtained from the IMAGE Consortium. The Tom7 open reading frame (ORF) was amplified by the PCR and subsequently cloned into the vector pSP65 (Promega) at the restriction sites EcoRI and HindIII. For construction of a Tom7-GFP fusion protein, the human Tom7 ORF lacking its stop codon was cloned into the vector pE-GFP (Clontech) at EcoRI and BamHI sites and in-frame with the GFP coding region. A similar approach was employed for construction of a vector to encode GFP fused at the N terminus of Tom7. In this case, following PCR, the Tom7 cDNA was cloned into a modified form of the pE-GFP at HindIII and BamHI sites in-frame with the GFP ORF. For construction of a vector encoding a fusion protein containing a flexible linker between GFP and Tom7 (GFP-L-Tom7), two complementary primers coding for the sequence KL(GGS)3 (31) and containing BamHI sticky ends were annealed and ligated into the pE-GFP vector digested with BamHI. The correct sequences of all clones were confirmed by dideoxynucleotide sequencing.

Cell Culture and Transfection—COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% (v/v) fetal calf serum at 37 °C under an atmosphere of 5% CO2 and 95% air. Cells (9-cm plate) were transfected with 10 μg of plasmid DNA according to Chen and Okayama (52). At 24 h post-transfection, cells were trypsinized and seeded into separate dishes containing sterile coverslips. Following overnight incubation to allow for attachment, one plate of cells was incubated with 80 nM Mitotracker Red (Molecular Probes) for 30 min. Media were removed, and the cells were washed with phosphate-buffered saline prior to mounting coverslips face down onto microscope slides. Cells were visualized using an Olympus BX-50 fluorescence microscope.

In Vitro Transcription and Translation—Plasmid DNA or PCR products (32) were used for in vitro transcription using SP6 RNA polymerase (Promega). Two additional methionines were engineered at the C terminus of Tom7 to enhance detection of the radiolabeled product (18). In vitro translation of the RNA transscripts was performed according to Ryan et al. (32).

In Vitro Import into Isolated Mitochondria—Mitochondria were isolated from rat organs using differential centrifugation as described previously (33). Mitochondria were resuspended in import buffer (250 mM sucrose, 5 mM magnesium acetate, 80 mM potassium acetate, 10 mM sodium succinate, 1 mM dithiothreitol, 0.1 mM ADP, 20 mM Hepes-KOH, pH 7.4) and used within 6 h following isolation. For the isolation of mitochondria from human cells, HeLa cells were grown until confluent, washed in phosphate-buffered saline, and harvested using a rubber policeman. Cells (∼2 × 106) were pelleted at 500 × g for 5 min at room temperature and resuspended in 30 ml of isolation buffer (220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 0.5 mM PMSF, 2 mg/ml bovine serum albumin, 20 mM Hepes-KOH, pH 7.4) before mitochondrial isolation. Mitochondria were stored in 0.5 mg/ml aliquots in storage buffer (500 mM sucrose, 10 mM Hepes-KOH, pH 7.4) at −80 °C prior to use. These mitochondria retained a membranous potential after thawing and had a functional TOM complex. Yeast mitochondria were prepared from the S. cerevisiae strain YPH499 (34) according to Daum et al. (35).

In vitro protein import studies, 35S-labeled proteins were incubated with isolated mitochondria at 37 °C for various times as indicated in figure legends. Samples were subjected to various treatments according to Ryan et al. (32).

BN-PAGE Supershift Assays—Following import, mitochondria were pelleted at 12,000 × g for 5 min at 4 °C and subsequently washed in import buffer prior to re-isolation. Mitochondria were resuspended in 80 μl of digitonin extraction buffer (1% (w/v) digitonin, 50 mM NaCl, 10% (v/v) glycerol, 20 mM BisTris, pH 7.0) followed by the addition of 1–5 μl of polyclonal antiserum. Following incubation at 4 °C for 20 min, samples were clarified by centrifugation (18,000 × g at 4 °C for 5 min) before addition of 8 μl of BN-PAGE loading dye (5% (w/v) Coomassie Brilliant Blue G-250, 500 mM e-amino-n-caproic acid, 180 mM BisTris, pH 7.0). Samples were then subjected to BN-PAGE (32, 38), destained, dried, and subjected to PhosphoImager analysis (Amersham Biosciences).

Antibodies—Antibodies specific for Tom20 and Tom22 were raised in rabbits (28). Antibodies to mouse VDAC were raised against hexahistidine-tagged recombinant protein, expressed in Escherichia coli, and purified using nickel-nitritotriacetic acid-agarose (Qiagen) chromatography under denaturing conditions. VDAC in 8 M urea was precipitated by a 50-fold dilution in phosphate-buffered saline. The precipitate was pelleted and washed with phosphate-buffered saline prior to injection into rabbits using Freund’s complete adjuvant and (for subsequent boosts) Freund’s incomplete adjuvant. Polyclonal antibodies specific for Tom40 were prepared using a histidine-tagged form of recombinant human Tom40.

Miscellaneous—Tris-Tricine SDS-PAGE was performed as described previously (37). Radiolabeled preproteins were detected using PhosphorImager storage technology and quantitated using ImageQuant software (Amersham Biosciences). Western blotting was performed using a semi-dry transfer method (38). Immunoreactive proteins from blots were detected on Hyperfilm ECL (Amersham Biosciences) using horseradish peroxidase-coupled secondary antibodies and SuperSignal West Pico chemiluminescent substrate (Pierce).

RESULTS

A BLAST search of the nucleotide data base revealed a human cDNA with significant homology (47% similarity and 28% identity) with yeast Tom7 (18). The human cDNA contains a 165-bp ORF encoding a protein of 55 amino acids with a predicted molecular mass of 6.2 kDa. An amino acid alignment of the human ORF with known Tom7 orthologs from the yeast S. cerevisiae, (18), N. crassa (39), and potato (Solanum tuberosum (40)) along with putative Tom7 proteins from Caenorhabditis elegans (GenBank accession number P43660) and mouse (Mus musculus, GenBank accession number W74865) revealed that they all contain the conserved motif aGaXpXXXXG where α is an aromatic residue and X is any residue (Fig. 1A). The conserved Pro residue has recently been shown to be essential for the targeting of yeast Tom7 to the mitochondrial outer membrane (41).

To identify the subcellular location of the putative Tom7 protein, we transiently expressed Tom7-GFP and GFP-Tom7 in COS-7 cells and subjected those cells to fluorescence microscopy. The fluorescence pattern observed for GFP-Tom7 resembled that of mitochondria (Fig. 1B, III). Counterstaining of cells expressing GFP-Tom7 with Mitotracker Red confirmed that the fusion protein was indeed localized to mitochondria because of the presence of the yellow signal after merging of red and green fluorescence in transfected cells (Fig. 1B, IV). Interestingly, when GFP was fused to the C terminus of Tom7, the

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fusion protein showed the same cytosolic and nuclear localization as cells expressing GFP alone (Fig. 1B, compare I and II). The lack of mitochondrial localization of this construct may be due to GFP either sterically hindering insertion of Tom7 into the outer membrane or masking mitochondrial targeting information that may reside at the C-terminal end of the protein.

Fungal Tom7 is part of an ~400-kDa general import pore complex where it is tightly associated with the channel-forming protein Tom40 (15, 18, 20, 21, 39). We asked whether human Tom7 could be imported into mammalian mitochondria and assembled into the TOM complex in the outer membrane. The Tom7 precursor was synthesized in vitro in rabbit reticulocyte lysate in the presence of L-[35S]methionine/cysteine (Fig. 2A, lane 1) and incubated with mitochondria isolated from rat kidney. As expected, the protein bound to mitochondria and was resistant to externally added proteinase K, resulting in its shift to a lower molecular weight (Fig. 2B, lanes 2 and 4). The assembly pattern of the Tom7 precursor was identical to the assembly of in vitro imported 35S-Tom22. The matrix imported 35S-pre-Hsp60, while assembled into a complex of ~440-kDa, consistent with it assembling into a heptamer (42). This complex could only be formed in the presence of Δψ, whereas a broader complex at a lower molecular weight most likely represents the precursor form of Hsp60 bound to the mitochondrial surface (Fig. 2B, lanes 10–12). Western analysis of mitochondrial extracts using antibodies against the IMS domain of Tom22 (26) revealed that both radiolabeled Tom7 and Tom22 precursors assembled into a complex indistinguishable in molecular weight from the endogenous TOM complex (Fig. 2B, lanes 13 and 14). In order to compare the size of the mammalian TOM complex with that of the well characterized yeast complex,
we also imported yeast Tom7 into mitochondria isolated from wild-type yeast (lanes 15 and 16). The yeast TOM complex migrated more slowly than the mammalian TOM complex indicating that some TOM homologs may be absent in mammals or the stoichiometry of the various TOM subunits may differ.

We wondered what other known TOM components were present in the TOM complex with Tom7. We employed a supershift analysis whereby mitochondria containing assembled \(^{35}S\)-Tom7 were first solubilized in digitonin-containing buffer followed by the addition of polyclonal antisera specific for either Tom40, Tom22, or Tom20. As a negative control, we used antibodies specific to voltage-dependent anion channel (VDAC), which is an abundant protein of the mitochondrial outer membrane that does not associate with TOM subunits. The binding of antibody molecules to subunits within the complex is predicted to result in the complex shifting to a higher molecular weight. As can be seen (Fig. 2C), the complex containing \(^{35}S\)-Tom7 is shifted upon the addition of antibodies to Tom40 and Tom22 but not to Tom20 and VDAC. Antibodies directed against Tom7 also failed to shift the Tom7-containing complex (data not shown). The formation of two higher molecular weight complexes following incubation with antibodies specific for either Tom40 or Tom22 (Fig. 2C, lanes 3 and 5) is most likely due to the presence of TOM complexes containing either one or two antibody molecules bound (where one IgG is \(~150\text{-kDa}\)).

A number of precursor proteins, including the precursor of Tom40, are dependent on external ATP for their import into mitochondria (45). This is most likely due to their dependence on molecular chaperones such as Hsp70 and MSF (22, 46–48). Tom7 contains no obvious mitochondrial targeting information and, given its hydrophobic nature, may also bind to cytosolic factors. In order to address whether the precursor of Tom7 requires ATP for its targeting or assembly into the TOM complex, imports were conducted in the presence or absence of ATP. As can be seen (Fig. 3A), \(^{35}S\)-Tom7 was imported and assembled into the TOM complex independent of ATP. As a
control, the import and subsequent assembly of Hsp60 into a heptameric complex was reduced to ~20% when ATP was depleted from the import reaction (Fig. 3, A and B).

Next we examined the topology of human Tom7 in the mitochondrial outer membrane. By taking advantage of our earlier finding that the GFP-Tom7 construct was targeted to mitochondria, we translated both Tom7 and GFP-Tom7 precursors in the presence of [35S]methionine/cysteine and added these to isolated mitochondria. Both forms assembled into the TOM complex with the GFP-Tom7 precursor imparting an overall increase into the molecular weight of the complex thereby resulting in its decreased mobility relative to the assembled, authentic Tom7 precursor (Fig. 4A, compare lanes 8 and 9 in A). A sample containing premixed [35S]-GFP-L-Tom7 and [35S]-Tom7 translation products was also subjected to SDS-PAGE. The radiolabeled band in lane 1 marked Hg is hemoglobin present in the rabbit reticulocyte lysate.

The complex contained a radiolabeled protein that co-migrated with GFP-L-Tom7 (compare lanes 1 and 2), whereas following protease treatment, the radiolabeled proteolytic fragment retained in the complex co-migrated with Tom7 (lane 3). We conclude that the N terminus of human Tom7 faces the cytosol, consistent with the proposed topology of yeast Tom7 (41). Furthermore, these data indicate that the N-terminal region of Tom7 is most likely buried within the membrane region of the complex or, alternatively, is very protease-resistant.

Fig. 4. Tom7 assembles into the TOM complex with its C terminus facing the intermembrane space. A, radiolabeled Tom7, GFP-Tom7, and GFP-L-Tom7 were incubated with HeLa cell mitochondria for 20 min. Samples were treated to carbonate extraction (Carb. Ext.) or proteinase K (Prot. K) treatment (100 μg/ml) prior to solubilization with digitonin and analysis by BN-PAGE. P, pellet following carbonate extraction. B, complexes corresponding to lanes 8 and 9 in A were excised from the Blue Native gel and subjected to SDS-PAGE (lanes 2 and 3, respectively). A sample containing premixed [35S]-GFP-L-Tom7 and [35S]-Tom7 translation products was also subjected to SDS-PAGE. The radiolabeled band in lane 1 marked Hg is hemoglobin present in the rabbit reticulocyte lysate.

Fig. 5. Tom7 assembles into a 120-kDa complex. A, [35S]-Tom7 was incubated with mitochondria isolated from various rat organs and HeLa cells for 20 min as indicated and treated with or without 25 μg/ml proteinase K (Prot. K). Samples were subjected to BN-PAGE and PhosphorImager analysis. B, [35S]-Tom7 and [35S]-Tom22 precursors were incubated with mitochondria from HeLa cells for 30 min and subjected to BN-PAGE and PhosphorImager analysis and immuno-decoration using antibodies specific for Tom22 and Tom40. The 120-kDa complex is marked with an asterisk.

same molecular weight (Fig. 4A, compare lanes 3 and 6). This suggests that either the GFP portion of the fusion protein was facing the IMS, and was therefore inaccessible to external protease, or the protease-resistant GFP domain faced the cytosolic side but pressed against the outer membrane and could not be proteolytically released from Tom7. In order to address the latter possibility, an 11-amino acid flexible linker (31) was inserted between GFP and Tom7 (GFP-L-Tom7) and the fusion protein imported into mitochondria. This fusion protein showed the same assembly pattern as GFP-Tom7 and was also carbonate-resistant (Fig. 4A, lanes 7 and 8); however, protease treatment did indeed lead to a reduction in both the overall signal and in the size of the complex (lane 9), most likely because of the release of the GFP domain. This was confirmed by excising the gel slices containing the complexes following BN-PAGE and subjecting them to SDS-PAGE in the second dimension (Fig. 4B).

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The import experiments performed in this study and described up to now employed mitochondria isolated from rat kidney. We next investigated whether equivalent TOM complexes are found in mitochondria isolated from different rat organs or from mitochondria isolated from human (HeLa) cells in culture. Although the Tom7 precursor assembled into the TOM complex in all mitochondrial preparations, it was also observed in an additional ~120-kDa complex in mitochondria isolated from both rat liver and spleen (Fig. 5A). Whereas the appearance and intensity of this 120-kDa complex was variable in different mitochondrial isolates (data not shown), this complex was consistently observed in HeLa cell mitochondria (lanes 11 and 12). In all cases, treatment with proteinase K led to only a slight change in the mobility of this smaller complex and no significant loss in radiolabeled signal, suggesting that the 120-kDa Tom7 precursor was not associated with receptors on the outer mitochondrial surface nor was it in a complex with subunits containing substantial cytosolic domains. Furthermore, this complex was not visible by immuno-decoration with antibodies against Tom40 or Tom22 (Fig. 5B). In addition, 35S-Tom22 imported efficiently and assembled exclusively into the TOM complex (Fig. 5B). In yeast mitochondria, Tom7 directly assembles into the 400-kDa complex, whereas Tom6 and Tom40 assemble via intermediates (15, 19, 20, 49). We therefore addressed whether this 120-kDa complex was an assembly intermediate for newly imported human Tom7. An import time course revealed that after 5 min of incubation at 37 °C, 35S-Tom7 was found in the 120-kDa complex, whereas its integration into the 380-kDa complex required longer incubation times (Fig. 6A). Quantitation of the 120- and 380-kDa complexes showed that the 120-kDa complex rapidly formed and then decreased over time, while the Tom7 precursor assembled into the TOM complex in a linear fashion (Fig. 6A, right panel). The assembly of Tom7 into the 380-kDa complex was not entirely concomitant with an equivalent reduction in the level of the 120-kDa complex over time, most likely due to the presence of constant pools of 35S-Tom7 precursor being imported into mitochondria during the assay. To verify that the 120-kDa complex is a true assembly intermediate, 35S-Tom7 was imported into HeLa cell mitochondria for 5 min to accumulate the 120-kDa complex, followed by isolation of mitochondria to remove unbound precursor prior to a chase period at 37 °C for various times. Samples were then split and treated with or without proteinase K followed by BN-PAGE. As can be seen (Fig. 6B),
all 35S-Tom7 shifted from the initial 120-kDa complex to the 380-kDa TOM complex. Because protease treatment did not result in significant changes in the 120-kDa complex, it is likely that this complex does not contain the receptors Tom70, Tom22, or Tom20 because they have cytosolic domains that are removed following protease treatment. In order to assess this more directly, the 120-kDa complex was formed, solubilized in digitonin, and then incubated with antibodies against Tom40, Tom22, Tom20 and the control VDAC (Fig. 6C). Only Tom40 antibodies could readily shift the Tom7-containing 120-kDa complex. We therefore conclude that Tom7 inserts into the outer membrane by first assembling into a 120-kDa complex that contains Tom40 but not Tom70, Tom22, or Tom20. Because the 120-kDa complex is not detectable by Western blotting with Tom40 antibodies, this intermediate complex is of low abundance and suggests that Tom7 does not directly assemble into the relatively abundant pre-existing 380-kDa TOM complexes.

In yeast, the lack of Tom22 in tom22Δ cells or the impairment in its assembly in mitochondria lacking Tom6 results in the accumulation of Tom40 in a 100-kDa complex together with the small TOM subunits (12, 15). This raised the question whether HeLa cell mitochondria contained limited Tom22 to facilitate the efficient assembly of the Tom7 intermediate complex into the mature 380-kDa TOM complex. To investigate this, we overexpressed Tom22 in HeLa cells (Tom22Δ) before isolating mitochondria. Western blot analysis confirmed that the levels of Tom22 were increased in Tom22Δ mitochondria relative to wild-type mitochondria, whereas the levels of Tom40 and VDAC remained the same (Fig. 6D). 35S-Tom7 precursor was incubated with wild-type and Tom22Δ mitochondria for various times, and its assembly was monitored by BN-PAGE. As can be seen (Fig. 6E, left panel), mitochondria containing higher levels of Tom22 led to the assembly of Tom7 into the fully assembled TOM complex with very little intermediate visible. Furthermore, the rate of assembly of Tom7 into the TOM complex was increased in mitochondria containing excess Tom22 (Fig. 6E, right panel). We conclude that the human Tom7 precursor follows an assembly pathway whereby it first integrates into the outer membrane, associates with free Tom40 and, following its interaction with Tom22, forms the 380-kDa TOM core complex. Tom22 is therefore rate-limiting for the assembly of the TOM complex.

**DISCUSSION**

We report here the identification of human Tom7 and its import and assembly into the mammalian TOM complex. Tom7 seems to be one of the most highly conserved TOM proteins in evolution (50), and its function can therefore be predicted to be similar to that found for the originally characterized yeast Tom7. Furthermore, like its fungal equivalents, human Tom7 is found in tight association with the channel-forming protein Tom40 and the TOM organizer/receptor Tom22. When analyzed by BN-PAGE, these proteins assemble into an ~380-kDa complex. Comparison with the yeast TOM complex revealed that the mammalian complex migrates slightly faster on BN-PAGE. This may be simply due to a difference in the monomeric molecular weights between the various TOM proteins in yeast and mammals; however, comparison of the calculated monomeric molecular weights does not reveal significant size differences. An alternative proposal is that either or both Tom6 and Tom5 are not present in mammalian mitochondria. Indeed, data base searches have not revealed their presence, whereas a Tom5 subunit has so far only been identified in yeast (16). However, the presence of a number of small molecular weight subunits co-immunoprecipitating with antibodies against mammalian Tom40 has been recently shown (27), and so their presence cannot be ruled out. In addition to the stable TOM complex observed by BN-PAGE, mammalian Tom70 and Tom20 have been shown to interact with members of the TOM machinery, although their associations, like that of their yeast counterparts, are not as strong (15, 28, 27, 51), leading to their displacement from the complex on BN-PAGE.

The precursor of Tom7 assemble into an ~120-kDa complex without the import of any other TOM precursors, indicating that Tom7 assembles with pre-existing Tom40 subunits. The presence of such an intermediate has not been reported previously. Fungal Tom7, on the other hand, assembles into pre-existing 400-kDa TOM complexes (15, 19, 20, 39). The assembly of human Tom7 via this intermediate resembles that observed for the import of yeast Tom6. Both subunits first assemble with free Tom40 prior to the binding of Tom22, which leads to formation of the fully assembled TOM complex (15, 20). Because human Tom7 does not directly assemble into pre-existing 380-kDa TOM complexes and that free Tom40 subunits are not detectable by Western blot analysis, it seems that TOM subunits are in flux between free and assembled forms, such that free Tom40 can bind to newly imported Tom7 precursors. Alternatively, the Tom7 precursor may transiently interact with the fully assembled TOM complex, and its association results in a destabilization of the complex and the resultant formation of the Tom7-Tom40 intermediate. Such a scenario remains to be tested, although this model would be consistent with the role of Tom7 in destabilizing TOM-TOM interactions (18, 20). The 120-kDa complex is then assembled into the TOM complex through the association of Tom22 which leads to oligomerization of these intermediate complexes. The results presented here strengthen the previous proposal (12) that Tom22 is required as an organizer of the TOM complex through its role in multimerization of smaller Tom40-containing complexes. Whereas human Tom7 is the first component of the mammalian TOM core complex whose assembly has been studied, our results point to the possibility that the assembly pathways of other members of the TOM machinery may also differ from the previously reported assembly pathways of their fungal counterparts.

Interestingly, whereas a visible Tom7 assembly intermediate is consistently seen in mitochondria isolated from HeLa cells, its presence in mitochondria from other tissues varies (Fig. 5A). Because overexpression of Tom22 in HeLa cells decreases the amount of detectable 120-kDa complex and leads to the more efficient assembly of Tom7 into the 380-kDa TOM complex, we conclude that mitochondria from different sources most likely contain different levels of Tom22 capable of assembly into new TOM complexes. It will be of interest to examine the regulation of the TOM genes in various tissues and to investigate the abundance and turnover of TOM proteins in order to understand further the complexities of the biogenesis of the mammalian TOM complex. Nevertheless, it seems that the constant import of new TOM subunits leads to their assembly into pre-existing TOM complexes and the subsequent freeing of endogenous older subunits. Whether these older subunits are turned over or become the seeds for the formation of new TOM complexes remains to be established.

**Acknowledgments**—We thank D. Stojanovski for critically reading the manuscript and L. Ward for experimental advice.

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