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Characterization of the Novel Mitochondrial Protein Import Component, Tom34, in Mammalian Cells[1]

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Tom34 is a newly-found component of the mitochondrial protein import machinery in mammalian cells with no apparent counterpart in fungi. RNA blot and immunoblot analyses showed that the expression of Tom34 varies among tissues and differs from that of the core translocase component Tom20. In contrast to a previous report [Nuttal, S.D. *et al.* (1997) *DNA Cell Biol.* 16, 1067-1074], the present study using a newly-prepared anti-Tom34 antibody with a high titer showed that Tom34 is present largely in the cytosolic fraction and partly in the mitochondrial and membrane fractions after fractionation of tissues and cells, and that the membrane-associated form is largely extractable with 0.1 M sodium carbonate. The *in vitro* import of preproteins into isolated rat mitochondria was strongly inhibited by ΔhTom34 which lacks the NH_2-terminal hydrophobic region of human Tom34 (hTom34). Import was also strongly inhibited by anti-hTom34. In pulse-chase experiments using COS-7 cells, pre-ornithine transcarbamylase (pOTC) was rapidly processed to the mature form. Coexpression of hTom34 resulted in a stimulation of pOTC processing, whereas the coexpression of hTom34 antisense RNA caused inhibition. The results confirm that Tom34 plays a role in mitochondrial protein import in mammals, and suggest it to be an ancillary component of the translocation machinery in mammalian cells.

Key words: mitochondria, protein transport, tissue distribution, Tom20, Tom34.

Most mitochondrial proteins are encoded by nuclear genes, synthesized as preproteins in the cytosol, targeted to the mitochondria and imported into the organelle *via* translocation machinery located in the outer and inner mitochondrial membranes (for reviews, see Refs. *1-4*). An important step in this process is the interaction of the preproteins with the outer surface of the mitochondria. Genetic and biochemical studies in yeast and *Neurospora* have identified a number of proteins in the mitochondrial outer membrane that are responsible for recognizing and translocating preproteins into the organelle. They form a dynamic protein complex, termed the Translocase of the Outer membrane of Mitochondria (Tom) complex. Subunits of the complex that have been identified include the receptor components Tom20 (*5, 6*), Tom22 (*7, 8*), Tom37 (*9*), and Tom70 (*10, 11*). Among these subunits, Tom20 has been shown to bind NH_2-terminal, basic amphiphilic targeting sequences of preproteins through electrostatic interactions with the acidic receptor domain (*12*). Together with Tom22, Tom20 in yeast mediates the import of all preproteins known to use the general import machinery of the mitochondria (*13*). The Tom20 and Tom70 subunits of yeast mitochondria have been shown to interact *via* the tetratricopeptide repeat (TPR) motif in Tom20 (*14*). The COOH-terminus of yeast Tom70, including most of the TPR motifs, has been shown to be necessary for the function of the protein, but not for its targeting to the mitochondria (*15*).

On the other hand, much less is known about the Tom complex in animals (reviewed in Ref. *16*). Recently, the cDNA for a human homologue (hTom20) (*17-19*) and a rat homologue (*20*) of yeast and *Neurospora* Tom20 were isolated. Although sequence homology between fungal and mammalian Tom20 is low, the universality of the mitochondrial import process was demonstrated by the functional complementarity of Toms; rat Tom20 is able to complement both the growth and mitochondrial defects of *Δtom20* yeast cells (*20*). The *in vitro* import of preproteins into isolated mitochondria is inhibited by the soluble domain of hTom20 (ΔhTom20) (*19, 21*) and by anti-hTom20 (*18, 19, 21*). In addition to the *in vitro* assay of hTom20, we developed an *in vivo* assay method in which

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Abbreviations: ΔhTom20, hTom20 lacking the NH_2-terminal transmembrane domain; ΔhTom34, hTom34 lacking the NH_2-terminal hydrophobic region; hpOTC, human pOTC; hpOTC-GFP, a fusion protein containing the presequence of hpOTC fused to green fluorescent protein; hTom20, human Tom20; hTom34, human Tom34; pAAT, pre-aspartate aminotransferase; pOTC, pre-ornithine transcarbamylase; pSPT, pre-serine:pyruvate aminotransferase; rpOTC, rat pOTC; Tom20, translocase of the outer membrane of mitochondria of 20 kDa; Tom34, 34 kDa protein involved in mitochondrial protein import.

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cultured cells are cotransfected with cDNA plasmids for preproteins and import factors, followed by pulse-chase experiments (*21, 22*). Coexpression of exogenous hTom20 retards mitochondrial import and the processing of pre-ornithine transcarbamylase (pOTC) (*21*), whereas it stimulates the import of a fusion protein pOTC-GFP consisting of the presequence of human pOTC fused to green fluorescent protein (*23*). Metaxin, a mitochondrial outer membrane protein that shows weak sequence identity with yeast Tom37, has been reported to function in the import of mitochondrial preproteins into mammalian mitochondria (*24*). We have identified hTom34, which appears to be an outer membrane protein of 34 kDa with no sequence homology to any protein in the yeast genome; hTom34 is required for protein import into mitochondria from mammalian cells (*25*). Antibodies against hTom34 inhibit the *in vitro* import of pOTC into rat mitochondria.

Here, we report the tissue distribution of Tom34 and show that it differs from that of Tom20. Re-examination of the intracellular localization of Tom34 using a newly-prepared antibody shows it to be largely cytosolic and partly associated with the mitochondrial membrane. We also show that the *in vitro* mitochondrial import of several preproteins is strongly inhibited by ΔhTom34, which lacks the NH_2-terminal hydrophobic region of hTom34, and by affinity-purified anti-hTom34 antibody. Coexpression of hTom34 with pOTC in COS-7 cells enhances the processing of pOTC to the mature form, whereas the coexpression of hTom34 antisense RNA retards preprotein processing.

MATERIALS AND METHODS

Materials—The *in vitro* transcription plasmids for human pOTC (*21*), rat pOTC (*26*), pig pre-aspartate aminotransferase (pAAT) (*27*), rat pre-serine:pyruvate aminotransferase (pSPT) (*28*), and chimeric protein pOTC-GFP (*23*) (hpOTC-GFP in this paper) have been previously described. The cytosolic domain of human Tom20 (ΔhTom20) was prepared as described (*19*).

Expression of hTom34 Lacking the NH_2-Terminal Hydrophobic Region (ΔhTom34) and Antibody Production—The oligonucleotide primers flanking amino acid residues 109 to 309 of hTom34, 5′-GGCCTATGTTGGATCCAAGACTGTGCTGC-3′ and 5′-AAAAAAGTCGACTTAGTGTAGGTTCTG-3′, were used to amplify the ΔhTom34 fragment using recombinant plasmid pSVT7/hTom34 (*25*) as a template. The PCR product was digested with restriction enzymes *Bam*HI and *Sal*I and subcloned into the *Bam*HI/*Sal*I site of plasmid pQE-30 (QIAGEN, Chatworth, CA). His-tagged ΔhTom34 was expressed in *Escherichia coli* strain M15[pREP4] according to the manufacturer's protocol. ΔhTom34 was recovered in the inclusion bodies, solubilized in buffer A (20 mM Tris-HCl, pH 7.5/0.5 M NaCl/6 M guanidine hydrochloride) containing 4 mM imidazole and subjected to Ni^{2+}-NTA Sepharose column chromatography. The column was first washed with buffer A containing 20 mM imidazole, and then ΔhTom34 was eluted with buffer A containing 1.2 M imidazole. The eluate was dialyzed against 20 mM Tris-HCl, pH 7.5, and then the protein was further purified by DEAE-Sepharose (Pharmacia Biotech) chromatography. The purified protein was used to raise an antibody in a rabbit. Affinity purification of anti-hTom34 was done using *N*-hydroxy succimide-activated Sepharose HP (Pharmacia Biotech) conjugated with ΔhTom34.

RNA Blot Analysis—Human multiple tissue Northern blot and human RNA master blot were obtained from Clontech (Palo Alto, CA). Multi-primed ^{32}P-labeled cDNAs for hTom20 and hTom34 were used as hybridization probes.

Subcellular Fractionation—Rat tissues or HeLa cells were homogenized with a Dounce homogenizer in 20 mM K-Hepes, pH 7.2, containing 0.25 M sucrose, 3 mM $MgCl_2$, 1 mM PMSF, 50 μM antipain, 50 μM leupeptin, 50 μM chymostatin, and 50 μM pepstatin, and mitochondrial and cytosolic fractions were prepared as described (*29*). Soluble and membrane fractions of rat tissues or HeLa cells were prepared by centrifugation of the tissue or cell homogenates at $140,000 \times g$ for 60 min at 4°C. The membrane fraction was extracted with 0.1 M sodium carbonate, pH 11.5, as described (*30*).

Cell Culture and DNA Transfection—A mammalian expression vector pCAGGS (*31*) was provided by J. Miyazaki (Osaka University, Osaka). pCAGGS/hTom34 was constructed by inserting the *Bam*HI/*Sal*I-excised and blunt-ended cDNA fragment for hTom34 into the blunted *Xho*I site of pCAGGS. The plasmid containing hTom34 cDNA in the antisense orientation was named pCAGGS/hTom34(R). COS-7 cells were cultured in 10-cm dishes and transfected with 10 μg of pCAGGS, pCAGGS/hTom34, or pCAGGS/hTom34(R) plasmid using TransIT LTI polyamine (Pan Vera, Madison, WI) as described (*23*). After 24 h, the cells were harvested and disrupted by sonication in 10 mM Tris-HCl, pH 7.4, containing 5 mM $MgCl_2$, 1 mM DTT, and 1 mM PMSF. The homogenate was separated into the soluble and membrane fractions by centrifugation at $100,000 \times g$ for 10 min in a refrigerated ultra-centrifuge. Pulse-chase experiments were performed as described (*21, 22*).

Other Methods—Immunoblot analysis was performed using the biotin/avidin system (ABC kit; Vector Laboratories, Burlingame, CA) and a chemiluminescence kit (ECL kit; Amersham) as described (*32*). *In vitro* protein import into isolated rat liver mitochondria was performed as described (*21*). Protein was determined with the protein assay reagent (Bio-Rad) using bovine serum albumin as a standard.

RESULTS

Distribution of Tom34 and Tom20 mRNAs in Human Tissues—The tissue distribution of hTom34 was examined by RNA blot and dot blot analyses and compared with that of hTom20, which is a core mitochondrial import receptor (Fig. 1). The Tom20 mRNA of about 4.1 kb was found to be expressed ubiquitously and at similar levels in human adult and fetal tissues and brain regions. The Tom34 mRNA of about 2.3 kb was also found to be expressed ubiquitously in all tissues. However, the level of expression of Tom34 mRNA differs from that of Tom20 mRNA in being enriched in the testis and some regions of the brain and much less abundant in some other tissues.

Tissue Distribution and Intracellular Localization of Tom34 and Tom20—The anti-Tom34 IgY used in the previous study (*25*) was not potent and a faint band for the Tom34 polypeptide was detected only in the mitochondrial

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outer membrane fraction of rat liver, which is enriched in Tom34, but not in the mitochondrial or cytosolic fractions. Thus, a new antibody with a higher titer was prepared. ΔhTom34 was expressed in *E. coli* and purified, and antibody against it was raised in a rabbit. The distribution of Tom20 and Tom34 proteins in rat tissues was examined by immunoblot analysis (Fig. 2A). Antibodies against the human proteins cross-reacted strongly with the rat proteins. Tom20 with a molecular mass of about 16 kDa was present in all tissues tested, but its amount varied among tissues and did not correlate well with the amount of mRNA. This difference may be due to a species difference, the translation efficiencies of mRNA in different tissues or differences in protein turnover in different tissues. Tom34 with a molecular mass of about 34 kDa was also present in all tissues, however, its tissue distribution differed from that of Tom20. Tom34 was found to be most abundant in the testis followed by brain and some other tissues.

When rat tissues and HeLa cells were fractionated into soluble and membrane fractions, Tom20 was recovered almost exclusively (rat tissues) or largely (HeLa cells) in the membrane fraction (Fig. 2B). In contrast, Tom34 was recovered largely in the soluble fraction and partly in the membrane fraction. Tom20 in the membrane fractions from rat brain and HeLa cells was only partially extractable with 0.1 M sodium carbonate, whereas Tom34 could be largely extracted (Fig. 2C). When HeLa cells were fractionated, a large proportion of the Tom34 was recovered in the cytosolic fraction and a small proportion was recovered

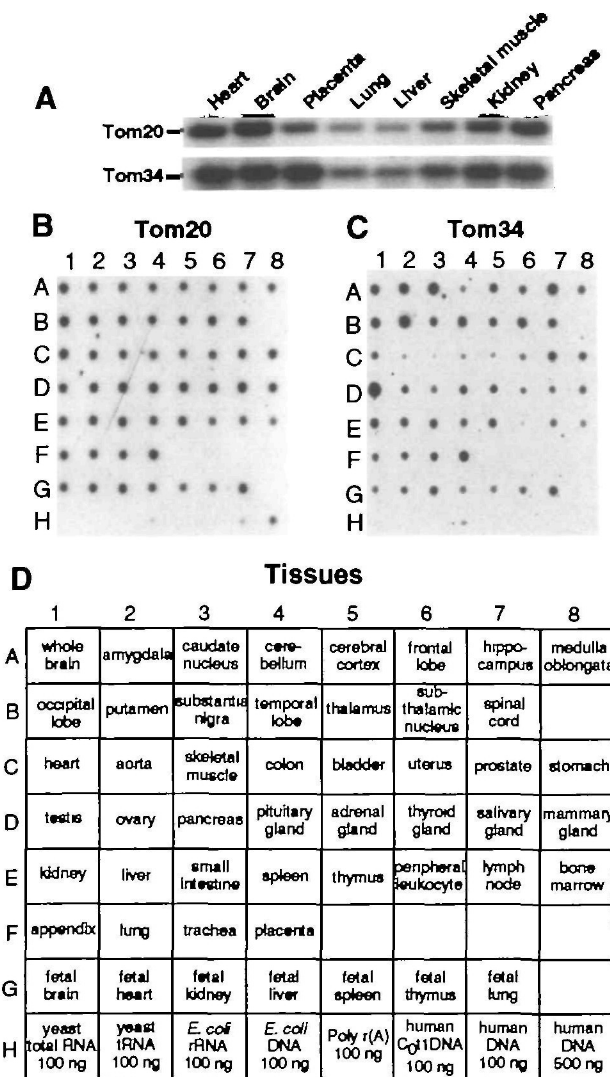

Fig. 1. **Distribution of Tom20 and Tom34 mRNAs in human tissues.** A: Human multiple tissue Northern blot (2 μg of polyA$^+$ RNA) was probed with ^{32}P-labeled cDNAs for hTom20 or hTom34. The same blot was used for hTom20 and hTom34. B and C: A human RNA master blot was probed with amounts of polyA$^+$ RNA samples that were normalized to the mRNA expression levels of eight different housekeeping genes. The same blot was used in B and C. D shows the tissue diagram of the human RNA master blot.

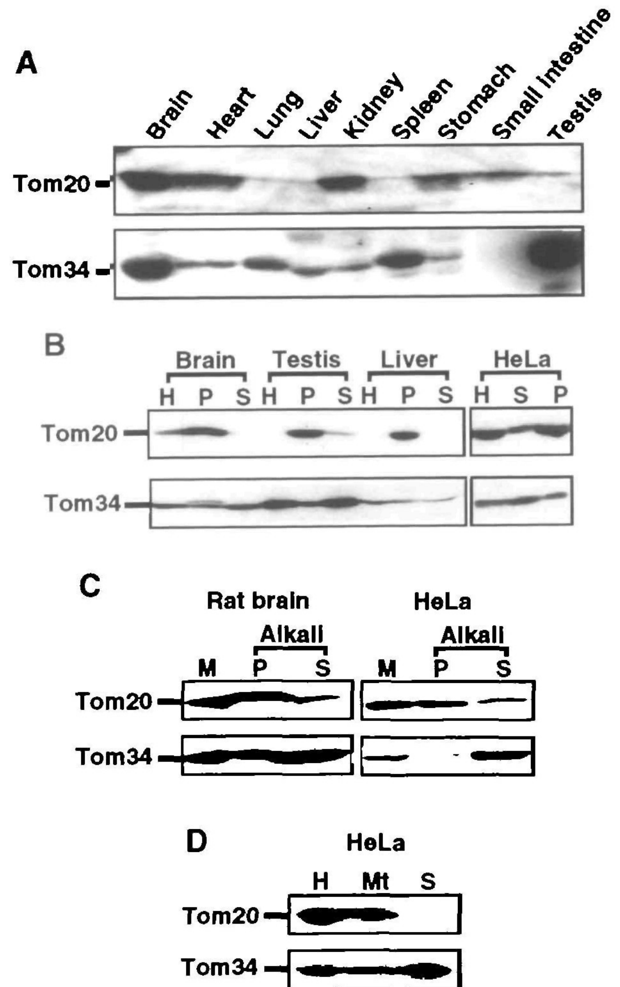

Fig. 2. **Tissue distribution and subcellular localization of Tom20 and Tom34.** A: Cell extracts (50 μg of protein) from rat tissues were subjected to immunoblot analysis for Tom20 (upper panel) and Tom34 (lower panel) using anti-hTom20 and anti-hTom34 sera (1:1,000 dilution), respectively, as primary antibodies. B: Cell homogenates (H), membrane fractions (P), and cytosolic fractions (S) of rat tissues and HeLa cells (40 μg of protein) were subjected to immunoblot analysis. C: The membrane fractions from rat brain and HeLa cells were extracted with 0.1 M sodium carbonate. The membrane fractions (M) and the alkali-extracted pellet (P) and supernatant (S) fractions (40 μg of protein) were subjected to immunoblot analysis. D: HeLa cell homogenate (H) (13 μg of protein), mitochondrial fraction (Mt) (9 μg of protein), or cytosolic fraction (S) (9 μg of protein) were subjected to immunoblot analysis.

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in the mitochondrial fraction (Fig. 2D).

Intracellular Concentrations of Tom20 and Tom34—The concentrations of Tom20 and Tom34 in human hepatoma HepG2 cells and human cervical carcinoma HeLa cells were measured by immunoblot analysis using purified ΔhTom20 and ΔhTom34 as standards (Fig. 3). The concentrations of Tom20 in both HepG2 and HeLa cells were about 0.2 μg/mg of total protein or 12 pmol/mg of total protein. On the other hand, the concentrations of Tom34 in these cells were about 1.0 μg/mg of total protein or 30 pmol/mg of total protein.

Inhibition of Preprotein Import into Isolated Mitochondria by ΔhTom34—The effect of ΔhTom34 on the import of preproteins into isolated rat mitochondria was studied by assessing the processing of the preproteins to the mature forms (Fig. 4). The import of human and rat pOTCs, pig pAAT, rat pSPT, and fusion protein hpOTC-GFP containing the presequence of human pOTC fused to green fluorescent protein was inhibited by increasing amounts of ΔhTom34. Inhibition was similar among the preproteins and 50% inhibition was observed with 6-10 μg of ΔhTom34. Nearly complete inhibition was observed at higher amounts of ΔhTom34. Bovine serum albumin had little effect on import.

Inhibition of Preprotein Import into Isolated Mitochondria by Anti-hTom34—The effect of anti-hTom34 on the import of preproteins into isolated mitochondria is shown in Fig. 5. Because unfractionated serum or total IgG often gives nonspecific effects, affinity-purified antibody was used. The import of all preproteins was inhibited by increasing amounts of the antibody. Again, the inhibition did not vary much among the preproteins and 50% inhibition was observed with 1-2 μg IgG for all preproteins except hpOTC-GFP whose import was inhibited by 50% at much lower amounts (0.1 μg) of IgG. Inhibition was nearly complete with higher amounts of IgG. Non-immune rabbit IgG had little effect on import. Tom34 was shown to be present in the cytosolic fractions of rat tissues and human cells (see above). In contrast, Tom34 was not detected in rabbit reticulocyte lysates (data not shown). Therefore, the inhibition of *in vitro* mitochondrial import by anti-Tom34 appears to be due to its binding to the mitochondria-associated Tom34.

Effect of the Overexpression of hTom34 and Antisense RNA on pOTC Import in COS-7 Cells—COS-7 cells were transfected with expression plasmids and short-time pulse-chase experiments were performed using suspended cells (Fig. 6). In immunoblot analysis, Tom34 was barely detected in non-transfected COS-7 cells. When the hTom34 cDNA plasmid was transfected, the hTom34 polypeptide of about 34 kDa was readily detected in the total cell extracts and was recovered largely in the soluble fraction and partly in the membrane fraction (total protein in the soluble fraction was several times that of the membrane fraction) (Fig. 6A). In pulse-chase experiments using cells transfected with the pOTC plasmid alone, 37% of the newly-synthesized pOTC was processed to the mature form by pulse-labeling for 5 min, and pOTC was

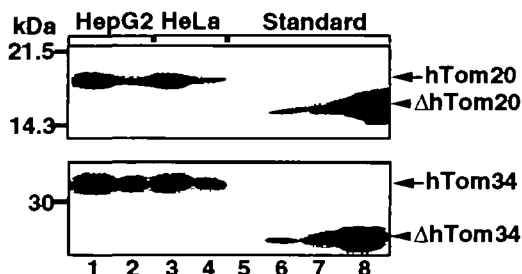

Fig. 3. **Quantitation of Tom20 and Tom34 in human cell lines.** Upper panel; 40 μg (lanes 1 and 3) or 10 μg of protein (lanes 2 and 4) from HepG2 or HeLa cell extracts was subjected to immunoblot analysis for Tom20 using anti-hTom20 serum (1:1,000 dilution) as a primary antibody. Purified ΔTom20 (lanes 5-8; 2, 4, 8, and 16 ng) was used as a standard. Lower panel; 8 μg (lanes 1 and 3) or 4 μg of protein (lanes 2 and 4) from HepG2 or HeLa cell extracts was subjected to immunoblot analysis for Tom34 using anti-hTom34 serum (1:1,000 dilution) as a primary antibody. Purified ΔTom34 (lanes 5-8; 2, 4, 8, and 16 ng) was used as a standard.

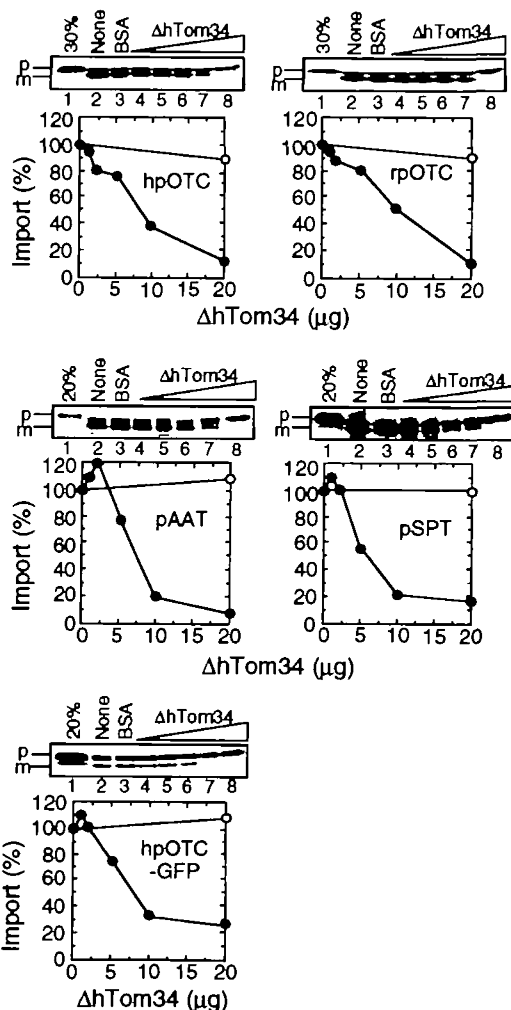

Fig. 4. **Effect of ΔhTom34 on the import of preproteins into isolated mitochondria.** Rabbit reticulocyte lysate (4 μl) containing the newly-synthesized and ^{35}S-labeled human pOTC (hpOTC), rat pOTC (rpOTC), pig pAAT, rat pSPT, or hpOTC-GFP was subjected to *in vitro* import assay (50 μl in total) in the absence (None) or presence of the indicated amounts (1-20 μg) of ΔhTom34 (●) (lanes 4-8) or 20 μg of bovine serum albumin (○) (lane 3) as described in "MATERIALS AND METHODS." Portions of the fluorograms and the results quantitated by imaging plate analysis are shown. p, preproteins; m, mature proteins. Lanes 1, 30% or 20% of input preproteins.

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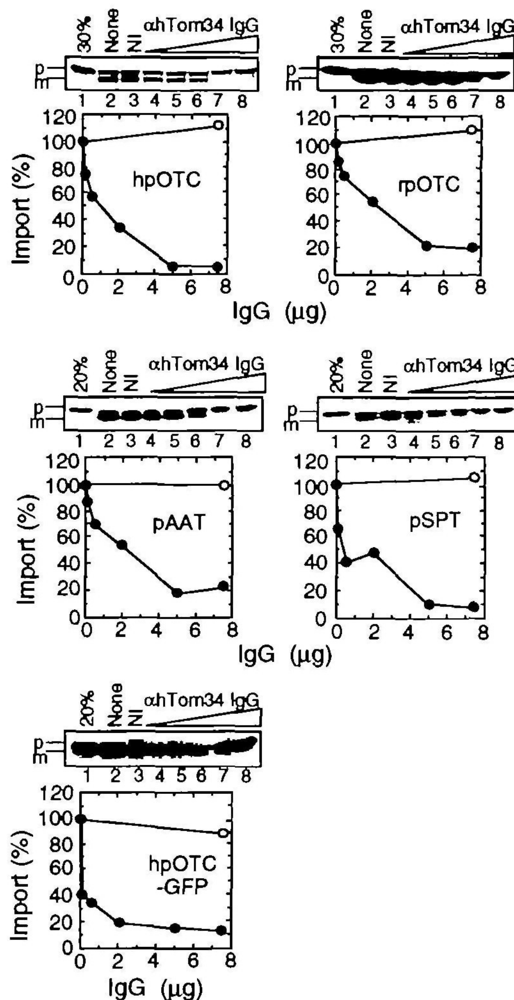

Fig. 5. **Effect of anti-hTom34 on the import of preproteins into isolated mitochondria.** Rabbit reticulocyte lysate (4 μl) containing the newly-synthesized and ^{35}S-labeled human pOTC (hpOTC), rat pOTC (rpOTC), pig pAAT, rat pSPT, or hpOTC-GFP was subjected to *in vitro* import assay (50 μl in total) in the presence of the indicated amounts (0.1-7.5 μg) of affinity-purified anti-hTom34 IgG (●) (lanes 4-8) or 7.5 μg of non-immune rabbit IgG (○) (lane 3) as described in "MATERIALS AND METHODS." Portions of the fluorograms and the results quantitated by imaging plate analysis are shown. p, preproteins; m, mature proteins. Lane 1, 30% or 20% of input preproteins. Lane 2, import without addition.

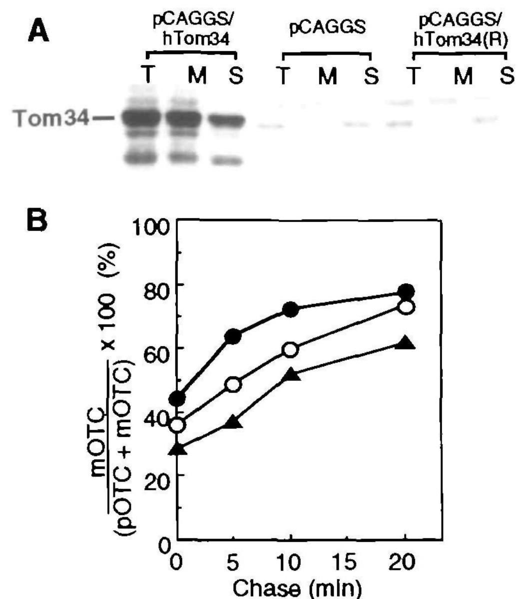

Fig. 6. **Effect of the overexpression of hTom34 and its antisense RNA on the processing of human pOTC in COS-7 cells.** A: COS-7 cells in a 10-cm culture dish were transfected with 10 μg of pCAGGS, pCAGGS/hTom34, or pCAGGS/hTom34(R). After 24 h, cell extracts (T) were fractionated into the soluble (S) and membrane (M) fractions. Thirty micrograms of protein was subjected to immunoblot analysis. B: COS-7 cells were transfected with 5 μg of pCAGGS/hpOTC plus 5 μg of pCAGGS (○), pCAGGS/hTom34 (●), or pCAGGS/hTom34(R) (▲). After 24 h, the cells were pulse-labeled with 8 MBq of Pro-mix (Amersham) for 5 min, and chased for the indicated periods. The results were quantitated by imaging plate analysis and values of mature OTC (mOTC) expressed as percent of pOTC plus mOTC are shown.

further processed during the chase (Fig. 6B). When hTom34 was coexpressed, 44% of the newly-synthesized pOTC was processed within the 5-min pulse, and pOTC was processed more rapidly during the chase. When hTom34 antisense RNA was coexpressed, only 29% of the newly-synthesized pOTC was processed within the 5-min pulse and pOTC was processed more slowly during the chase. Similar results were obtained when hpOTC-GFP was used instead of pOTC (data not shown).

DISCUSSION

The outer mitochondrial membrane contains components of the translocase machinery for the import of proteins. These consist of receptor components, which ensure the fidelity of protein import and restrict import to proteins with appropriate targeting information, and components comprising the general insertion pore that allows access to the inside of the mitochondrion (*3*). Recently, Künkele *et al.* (*33*) reconstituted the Tom components of *Neurospora* into liposomes and showed that this complex forms a cation-selective, high-conductance ion channel that can translocate preproteins across the lipid bilayer. This functional complex consists of Tom70, Tom40, Tom22, and Tom20 in the ratio of 1.5:8:3.1:2, and the small Toms 6 and 7. The complex in the outer mitochondrial membrane may also contain other components such as Tom5 (*34*). It would appear from these reconstitution experiments that there are several Tom40 subunits in the translocase and that Tom70 is a more loosely associated component.

The only essential components of the translocase are Tom40 and Tom22 (*35-39*), raising the question as to the specific roles of each subunit in the complex. Additionally, the finding of an apparently novel component, Tom34 (*25*), with no obvious homologue in fungi, raises further questions about the role of this component in mammalian cells. The demonstration here that the *in vitro* import of a number of preproteins into mitochondria is inhibited by both ΔhTom34 and antibodies against this domain suggests that this protein is involved in the delivery of preproteins to the Tom complex in mammalian cells. However, the functional properties of Tom34 differ from those of Tom20. The inhibition of the *in vitro* import by ΔhTom20 and anti-hTom20 differs for different preproteins (*21*), whereas

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the effects of ΔhTom34 and anti-hTom34 on import were similar. Likewise, the overexpression of hTom20 in COS-7 cells was found to inhibit preprotein import by about 2-fold (*21*), whereas the overexpression of hTom34 resulted in a small stimulation of import.

The functional role of Tom34 in protein import remains enigmatic. The expression of Tom20 mRNA in different tissues is uniform, as might be expected for a core component of the translocation machinery. In contrast, the expression of Tom34 mRNA varies considerably, being high in brain and testis and low in several other tissues. Perhaps this difference in distribution reflects a special role in these tissues, such as the regulation of protein import. Tom20, a transmembrane protein, associates tightly with the outer mitochondrial membrane. In contrast, careful re-examination of Tom34 localization with a newly-prepared antibody shows that it is present largely in the cytosol and partly associated with mitochondria, and that the membrane-associated form is largely extractable with 0.1 M sodium carbonate. When the hydropathy profile of Tom34 was carefully analyzed, the sequence which was speculated to be a transmembrane domain (*25*), is apparently not sufficiently hydrophobic. Therefore, Tom34 most likely associates with mitochondria as a peripheral membrane protein. The relationship between the cytosolic form and the mitochondria-associated form of Tom34 and the roles of these two forms remain to be studied. A similar dual localization of import machinery components has been reported for other organelles including the signal recognition particle (SRP) for endoplasmic reticulum (*40*) and the import receptors for peroxisomes, Pex5p (*41, 42*) and Pex7p (*43, 44*). It is noted that Pex5p, like Tom34, has multiple TPR sequences. It is tempting to speculate that Tom34 shuttles between the cytosol and the mitochondria. The present study does not show that Tom34 is a factor specific for mitochondrial protein import. It is possible that, like molecular chaperones, Tom34 is involved in protein traffic to other organelles. This hypothesis remains to be tested. Interestingly, Young *et al.* (*45*) found that the 90-kDa heat shock protein (hsp90) that is involved in the regulation of certain signal transduction molecules, can interact with Tom34. We are currently investigating other proteins associated with Tom34 and the effects of deletion of Tom34 on protein import.

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