Participation of the import receptor Tom20 in protein import into mammalian mitochondria: analyses in vitro and in cultured cells

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Abstract Requirement of the mitochondrial import receptor Tom20 in protein import into mammalian mitochondria was studied in vitro and in cultured cells. Import of human and rat pre-ornithine transcarbamylase (pOTC), pig pre-aspartate aminotransferase (pAAT) and rat serine: pyruvate aminotransferase (pSPT) was inhibited by ΔhTom20 that lacks the NH_2-terminal transmembrane domain of human Tom20 (hTom20). Import of these preproteins was also inhibited by anti-Tom20. The inhibitions by ΔhTom20 and anti-hTom20 were the strongest for human pOTC, followed by rat pOTC, pAAT and pSPT. Coexpression of human pOTC and hTom20 in COS-7 cells followed by immunoblot analysis showed that overexpression of hTom20, but not ΔhTom20, decreases production of mature OTC. In pulse-chase experiments, pOTC was synthesized and rapidly processed to the mature form. Coexpression of hTom20, but not ΔhTom20, resulted in a decrease of pOTC processing, probably due to an imbalance of the normal stoichiometry of the receptor complex. These results show that both in vitro and in intact cells, Tom20 is involved in mitochondrial protein import in higher animals and that the requirement for Tom20 is different for different preproteins.

Key words: Human Tom20; Import receptor; Mitochondria; Protein transport; Ornithine transcarbamylase

1. Introduction

Most mitochondrial proteins are encoded by nuclear genes, synthesized as preproteins in the cytosol, targeted to the mitochondria and imported into the organelle. 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 (reviewed in [1–3]). They form a dynamic protein complex, termed the mitochondrial import receptor complex. Subunits of the receptor complex that have been identified include Tom20 [4,5], Tom22 [6,7], Tom37 [8] and Tom70 [9,10]. Among these subunits, Tom20 was shown to bind to the basic amphiphilic targeting sequence of preproteins through electrostatic interactions with the acidic receptor domain [11]. Together with Tom22, Tom20 of yeast mediates the import of all preproteins known to use the general import machinery of the mitochondria [12]. The Tom20 and Tom70 subunits of yeast mitochondria were shown to interact via the tetratricopeptide repeat motif in Tom20 [13].

On the other hand, little is known about the import receptor of animals. A protein of 52 kDa that binds to the presequence of pre-ornithine aminotransferase has been purified [14]. Recently, cDNA for a human homolog (hTom20) of yeast and *Neurospora* Tom20 was isolated [15–17]. This protein was shown to be the human counterpart of yeast and *Neurospora* Tom20, because it can assemble with yeast receptor complex [15] and complements the respiratory defect of the *tom20* deficient yeast cells [16]. Furthermore, in vitro import of preproteins into isolated mitochondria was inhibited by the soluble domain of hTom20 (ΔhTom20) [17] and by anti-hTom20 [16,17]. In addition to the in vitro assay of hTom20, it is important to assess its role in cultured animal cells (called in vivo assay here). However, no functional in vivo assay method has so far been developed.

Here, we report that the import of several preproteins are inhibited by ΔhTom20 and by anti-hTom20, and that the inhibition varies among the preproteins. We developed an in vivo assay method in which cultured cells were cotransfected with plasmids for a preprotein and hTom20, followed by pulse-chase experiments, and show that coexpression of hTom20, but not ΔhTom20, retards mitochondrial import and processing of a preprotein.

2. Materials and methods

2.1. Materials

A mammalian expression vector pCAGGS [18] was provided by J. Miyazaki (Osaka University, Japan). pCAGGS/pOTC was constructed by inserting the *Eco*RI fragment of human pOTC cDNA [19] into the *Eco*RI site of pCAGGS. For construction of pCAGGS/hTom20, the *Bam*HI/*Not*I fragment for hTom20 was excised from pGEMT/hMas20 [17], blunt-ended and cloned into the blunt-ended *Xho*I site of pCAGGS. For construction of pCAGGS/ΔhTom20 encoding hTom20 lacking the NH_2-terminal anchor sequence, polymerase chain reaction (PCR) was performed. The upstream primer to introduce a new translation initiation codon was 5′-GCCACCATGGACCCCAACTTCAAGAACAGG-3′, and the downstream primer was 5′-GATTTAGGTGACACTATAG-3′. The plasmid used as a template was pGEMT/hMas20 [17]. The PCR fragment was inserted into the blunt-ended *Xho*I site of pCAGGS.

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Abbreviations: OTC, ornithine transcarbamylase; pOTC, pre-OTC; pAAT, pre-aspartate aminotransferase; pSPT, pre-serine:pyruvate aminotransferase; Tom20, translocase of the outer membrane of mitochondria; hTom20, human Tom20; ΔhTom20, hTom20 lacking the transmembrane domain

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ΔhTom20 was expressed in *Escherichia coli* and was purified as described [17]. Antibodies against human OTC and hTom20 were raised in rabbits by injecting the *E. coli*-expressed and purified human OTC and ΔhTom20 [17], respectively. Anti-hTom20 was affinity-purified with ΔhTom20 coupled to *N*-hydroxysuccimide-activated Sepharose HP.

2.2. In vitro import

mRNAs for human pOTC, rat pOTC, pig pre-aspartate aminotransferase (pAAT) and rat pre-serine:pyruvate aminotransferase (pSPT) were synthesized by in vitro transcription of the recombinant plasmids pGEM3Zf/hpOTC, pSPT/pOTC [20], pSP65/pmAAT [21] and pAS321 [22], respectively. pGEM3Zf/hpOTC was constructed by inserting the *Eco*RI fragment of human pOTC cDNA [19] into the *Eco*RI site of pGEM3Zf plasmid (Promega). In vitro translation of the RNA transcripts was performed in a rabbit reticulocyte lysate system (Promega, 40% (v/v) lysate) in the presence of Pro-mix (Amersham) containing L-[^{35}S]methionine and L-[^{35}S]cysteine, as described [23,24]. The import mixture (50 μl) containing 4.0 μl of the lysate and ^{35}S-labeled preproteins (8.8–65 kBq) was incubated with isolated rat liver mitochondria (100 μg protein) at 25°C for 12 min. The reaction was stopped by diluting the import mixture into the ice-cold mitochondrial isolation buffer containing 0.1 mM dinitrophenol [23]. The mitochondria were reisolated by centrifugation and subjected to 10% SDS-PAGE. The radioactive polypeptides were visualized by fluorography and quantitated by imaging plate analysis using a FUJIX BAS2000 analyzer (Fuji Film Co.).

2.3. Cell culture and DNA transfection

COS-7 cells were cultured in 10 cm culture dishes in DMEM plus 10% fetal calf serum. Subconfluent cells were transfected with expression vectors by a calcium phosphate precipitation procedure.

2.4. Immunoblot analysis

After culture for 24 h, the whole cell extracts or fractionated cell extracts were separated by SDS-PAGE and electrotransferred onto nitrocellulose membrane. Cell fractionation into the soluble fraction and the particulate fraction containing mitochondria with digitonin was performed as described previously [25]. Antisera against OTC or hTom20 were used as primary antibodies. Enhanced detection was performed by the use of the ABC-PO kit (Vector Laboratories).

2.5. Pulse and pulse-chase experiments

Twenty-four hours after transfection, the COS-7 cells in a 10 cm culture dish were harvested with trypsinization, washed twice with phosphate-buffered saline, suspended in 3 ml of methionine-free DMEM, preincubated at 37°C for 1 h in a 50 ml conical tube, and radiolabeled with 8 MBq of Pro-mix. Aliquots (0.6 ml) were removed at the indicated times and mixed with 0.4 ml of ice-cold 25 mM Tris-HCl (pH 7.4) containing 5 mM EDTA, 0.25% SDS, 0.25% Triton X-100, 125 μM chymostatin, 125 μM pepstatin, 125 μM leupeptin and 125 μM antipain. The cell lysates were clarified at 10000×*g* for 10 min. Radiolabeled proteins were immunoprecipitated with antisera and 200 μl of 10% suspension of protein A-Sepharose as described [25], subjected to 10% SDS-PAGE and fluorography and quantitated by imaging plate analysis using a FUJIX BAS2000 analyzer. In pulse-chase experiments, the cells were suspended in 1 ml of methionine-free medium, preincubated for 1 h, radiolabeled for 5 min, and then chased by adding 2 ml of medium containing 20 mM methionine.

3. Results and discussion

3.1. Inhibition by ΔhTom20 of preprotein import into isolated mitochondria

The effect of ΔhTom20 on import of preproteins into isolated mitochondria was studied by assessing processing to the mature form [25] (Fig. 1). Import of human and rat pOTCs, pig pAAT and rat pSPT was inhibited by increasing amounts of ΔhTom20. However, the inhibition varied among the preproteins, being 69% for human pOTC, 54% for rat pOTC, 38% for pAAT and 37% for pSPT at 12.5 μg of ΔhTom20. Bovine serum albumin had little effect on the import.

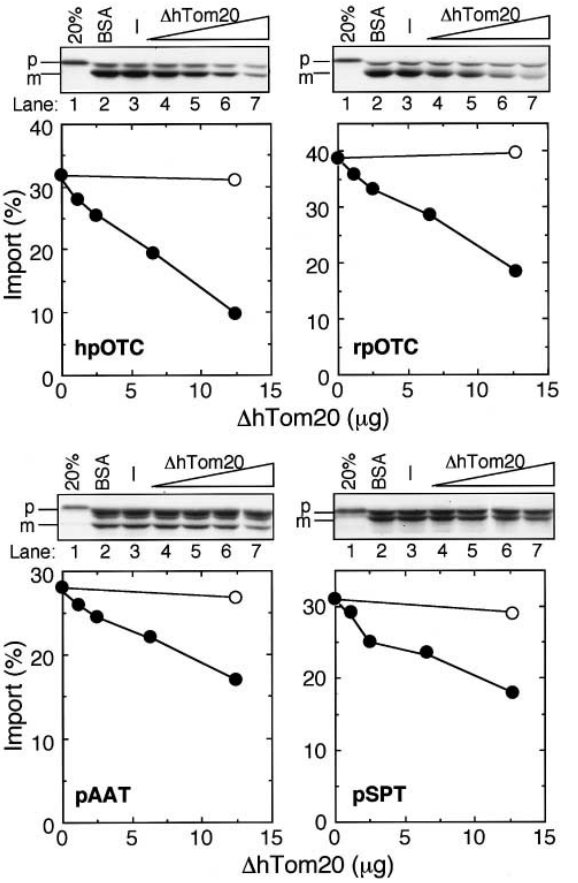

Fig. 1. Effect of ΔhTom20 on import of preproteins into isolated mitochondria. Rabbit reticulocyte lysate (4 μl) containing the newly synthesized and ^{35}S-labeled human pOTC (hOTC), rat pOTC (rpOTC), pig pAAT or rat pSPT was subjected to in vitro import assay (50 μl in total) in the presence of indicated amounts of ΔhTom20 (●) or bovine serum albumin (○) as described in Section 2. Portions of the fluorograms and the quantitated results by imaging plate analysis are shown. p, preproteins; m, mature proteins. Lane 1, 20% of input preproteins. Lane 2, import in the presence of 12.5 μg bovine serum albumin. Lane 3, import without addition. Lanes 4–7: import in the presence of 1.3–13 μg of ΔhTom20.

3.2. Inhibition by anti-hTom20 of preprotein import into isolated mitochondria

The effect of anti-hTom20 on import of preproteins into isolated mitochondria is shown in Fig. 2. Because unfractionated sera often give nonspecific effects, affinity-purified antibody was used. Import of all preproteins was inhibited by increasing amounts of the antibody and reached close to maximum levels at the highest concentrations used. Again, the inhibition varied among the preproteins, being 80% for human pOTC, 55% for rat pOTC, 53% for pAAT and 40% for pSPT at 4.8 μg of IgG. The extent to which the four preproteins were inhibited by anti-hTom20 coincided with that of inhibition by ΔhTom20 (Fig. 1). Residual import which was not inhibited by anti-Tom20 may be due to either import by another receptor(s) or receptor-independent import which was observed with trypsin-treated mitochondria [17].

3.3. Decreased production of mature OTC in cultured cells by overexpression of hTom20

Effects of overexpression of hTom20 on synthesis of pOTC and mature OTC in COS-7 cells were studied by immunoblot

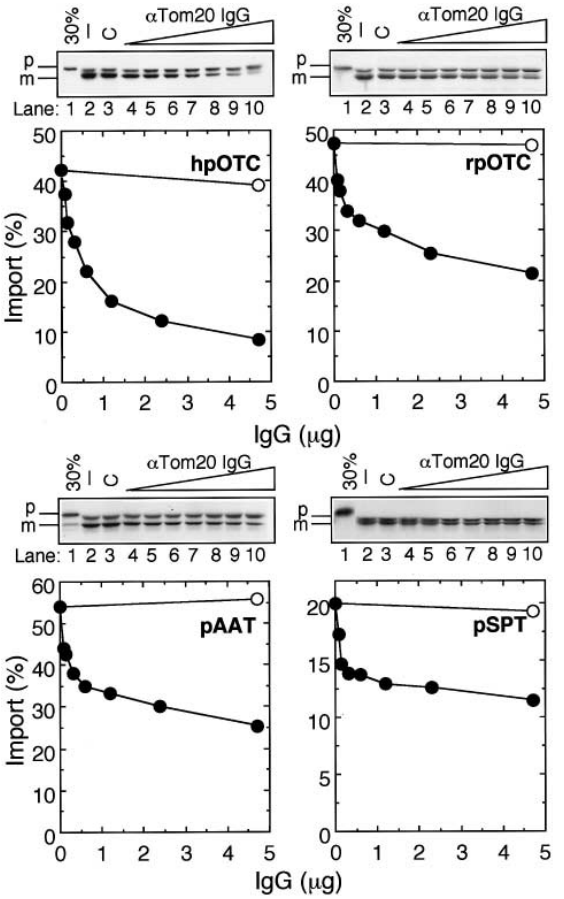

Fig. 2. Effect of anti-hTom20 on import of preproteins into isolated mitochondria. Rabbit reticulocyte lysate (4 μl) containing the newly synthesized and ^{35}S-labeled human pOTC (hOTC), rat pOTC (rpOTC), pig pAAT or rat pSPT was subjected to in vitro import assay (50 μl in total) in the presence of indicated amounts of affinity-purified anti-hTom20 IgG (●) or nonimmune IgG (○) as described in Section 2. Portions of the fluorograms and the quantitated results by imaging plate analysis are shown. p, preproteins; m, mature proteins; 30%, 30% of input preproteins. Lane 1, 30% of input preproteins. Lane 2, import without addition. Lane 3, import in the presence of 4.8 μg normal rabbit IgG. Lanes 4–10, import in the presence of 0.08–4.8 μg of affinity-purified anti-hTom20 IgG.

analysis (Fig. 3). When pOTC was expressed alone, a small amount of unprocessed pOTC as well as processed mature OTC was detected (Fig. 3A). After fractionation with digitonin, pOTC was recovered mostly in the soluble fraction, whereas mature OTC was recovered mostly in the particulate fraction. When increasing amounts of hTom20 were coexpressed, the amount of mature OTC decreased dose-dependently (Fig. 3B). The amount of pOTC remained unchanged up to 1 μg of pCAGGS/hTom20 and decreased at 5 μg. On the other hand, overexpression of ΔhTom20 had little effect on the amount of OTC. We speculate that hTom20 overexpression inhibits the step of mitochondrial import rather than the step of pOTC synthesis (see below). Lack of accumulation of pOTC in cells which overexpress hTom20 may be due to degradation in the cytosol [26].

3.4. *Retardation of pOTC import into mitochondria by overexpression of hTom20*

An in vivo assay system to analyze mitochondrial import and processing of preproteins in cultured cells was developed. COS-7 cells were transfected with expression plasmids and short-time pulse-chase experiments were performed on suspended cells. In labeling experiments, using cells transfected with the pOTC plasmid alone, newly synthesized pOTC of 40 kDa appeared within 1 min, increased up to about 5 min when it reached a plateau (Fig. 4A). On the other hand, mature OTC of 36 kDa appeared with a lag time of 2 min and increased up to 10 min. In pulse-chase experiments (Fig. 4B), after a pulse of 5 min, 65% of the newly synthesized OTC was processed to the mature form (Fig. 4, B-a and C). When the cells were chased with cold methionine after the 5 min pulse, pOTC decreased with an apparent half-life of 2–3 min and mature OTC increased concomitantly up to 8 min. These results accord with previous ones obtained using isolated rat hepatocytes [25] and HeLa cells transfected with pOTC cDNA [27]. When hTom20 was coexpressed, only 38% of the newly synthesized OTC was processed in 5 min pulse, and pOTC decreased more slowly in the chase (Fig. 4, B-b and C). On the other hand, coexpression of ΔhTom20 had little effect on pOTC processing (Fig. 4, B-c and C). Cell fractionation and immunocytochemical analyses showed that the expressed hTom20 was localized in the mitochondria, whereas ΔhTom20 was in the cytosol (data not shown). Because total radioactivity in pOTC plus mature OTC somewhat decreased in chase experiments, the decrease in pOTC may partly be due to its degradation in the cytosol. There was no apparent effect on the synthesis of pOTC by overexpression of hTom20 and ΔhTom20. The retardation of mitochondrial import of pOTC by overexpression of intact hTom20, and not ΔhTom20, is likely to be due to a disruption of the

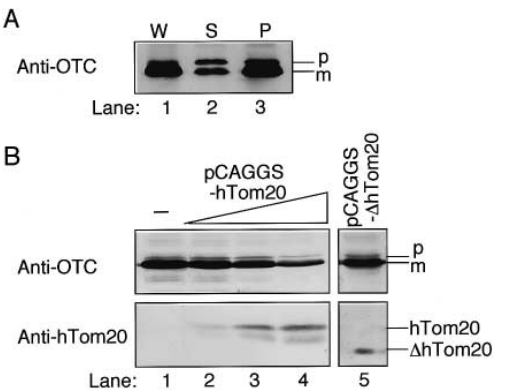

Fig. 3. Effects of overexpression of hTom20 and ΔhTom20 on the production of human OTC in COS-7 cells. A: COS-7 cells were transfected with 10 μg of pCAGGS/pOTC. After 24 h, the cells were fractionated, and the whole cell extracts (lane 1) (40 μg of protein), the supernatant fraction (lane 2) (18 μg of protein) and the particulate fraction (lane 3) (22 μg of protein) were subjected to 10% SDS-PAGE and immunoblot analysis. B: COS-7 cells were transfected with pCAGGS/pOTC (5 μg) and indicated plasmids. Lane 1, pCAGGS (5 μg). Lanes 2–4, pCAGGS/hTom20 added were 0.2 μg (lane 2), 1 μg (lane 3), and 5 μg (lane 4), respectively. An appropriate amount of a carrier plasmid pCAGGS was also added to make up a total DNA amount of 5 μg. Lane 5, pCAGGS/ΔhTom20 (5 μg). After 24 h, the whole cell extracts (40 μg of protein) were subjected to 10% SDS-PAGE and immunoblot analysis with anti-OTC serum (1:1000 dilution) or anti-hTom20 serum (1:1000 dilution). Increasing the amount of pCAGGS/hTom20 plasmid resulted in an increase in both number of transfectant cells and extent of expression per transfectant cells.

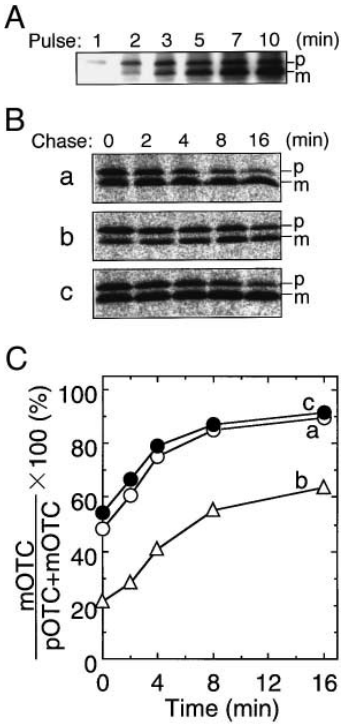

Fig. 4. Effects of overexpression of hTom20 and ΔhTom20 on mitochondrial import of human pOTC in COS-7 cells. A: COS-7 cells in a 10 cm culture dish were transfected with 10 μg of pCAGGS/pOTC and 10 μg of pCAGGS. After 24 h, the cell suspension (3 ml) were labeled with 8 MBq of Pro-mix. Aliquots (0.6 ml) were removed at indicated times and pOTC (p) and mature OTC (m) were immunoprecipitated with 20 μl of anti-OTC serum and 200 μl of 10% suspension of protein A-Sepharose, and subjected to 10% SDS-PAGE and fluorography. B: COS-7 cells were transfected with pCAGGS/pOTC plus pCAGGS (a), pCAGGS/pOTC plus pCAGGS/hTom20 (b) or pCAGGS/pOTC plus pCAGGS/ΔhTom20 (c). After 24 h, the cell suspension (1 ml) was labeled for 5 min with 8 MBq of Pro-mix and chased for the indicated periods as described in Section 2. C: The results in B were quantitated by imaging plate analysis and values of mature OTC (mOTC) expressed as a percent of pOTC plus mOTC are shown. The amount of mature form increased up to 8 min in all cases.

normal stoichiometry of subunits of the import receptor complex.

The present results both in vitro and in vivo demonstrate that Tom20 is involved in protein import into the mitochondria in mammalian cells. The in vitro studies also indicate that dependence of preprotein import on Tom20 varies from one preprotein to another. Among the preproteins tested, a major portion of human pOTC import appears to depend on Tom20, whereas a major portion of pSPT import appears to be Tom20-independent. Rat pOTC and pAAT are between the two. This accords with the yeast case where Tom20 and Tom70 work in parallel [1–3]. These two receptor molecules have different preference among various preproteins. More detailed analysis of mammalian Tom20 and identification of other subunits of the import receptor complex remain to be performed.

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