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Functional Analysis of Human Metaxin in Mitochondrial Protein Import in Cultured Cells and Its Relationship with the Tom Complex

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Metaxin is an outer membrane protein of mammalian mitochondria which is suggested to be involved in protein import into the organelle. RNA blot analysis showed that distribution of metaxin mRNA in human tissues differs from that of mRNA for the translocase component Tom20. Effect of overexpression of human metaxin on mitochondrial preprotein import and processing in COS-7 cells was studied. Overexpression of metaxin resulted in impaired mitochondrial import of natural and chimeric preproteins and in their accumulation. We previously reported that overexpression of Tom20 in cultured cells causes inhibition of import of mitochondrial preprotein. Coexpression of metaxin with Tom20 had no further effect on the preprotein import. Overexpression of the cytosolic domain of metaxin also caused inhibition of preprotein import, although less strongly than the full-length metaxin. In blue native PAGE, Tom40, Tom22, and a portion of Tom20 migrated as a complex of ~400 kDa, and the other portion of Tom20 migrated in smaller forms of ~100 and ~40 kDa. On the other hand, metaxin migrated at a position of ~50 kDa. These results confirm earlier *in vitro* results that metaxin participates in preprotein import into mammalian mitochondria, and indicates that it does not associate with the Tom complex.

Key Words: **metaxin; mitochondria; preprotein; protein import; Tom complex.**

Most mitochondrial proteins are initially synthesized on free ribosomes as precursors, many with NH_2-terminal presequences which function as mitochondrial targeting and import signals, and released into the cytosolic pool. Newly synthesized preproteins associate with cytosolic chaperones which keep them loosely folded in the cytosol, bind to a mitochondrial surface receptor(s), and are then transported into or across the outer and inner membranes. Many presequences of transported preproteins are then proteolytically cleaved in the mitochondrial matrix, and the mature portions are folded into their native conformations. The whole process of synthesis of mitochondrial preproteins, their translocation, processing, folding, and assembly involves many factors in the cytosol, mitochondrial membranes and matrix compartments (for reviews, see Refs. 1, 2). An important step in this process is the interaction of the preproteins with the outer membrane of mitochondria. A number of proteins in the outer membrane that are responsible for recognizing and translocating preproteins into the organelle have been identified in yeast and Neurospora (for reviews, see Refs. 3–5). 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 (6, 7), Tom22 (8, 9), and Tom70 (10, 11). In blue native PAGE, the yeast Tom complex migrates as a ~400 kDa complex containing Tom40, Tom22, and small Toms and a ~120 kDa complex containing Tom70 (12). Tom20 migrates in a band of 40 to 100 kDa and is partly associated with the ~400 kDa complex. A novel component, Tom37 has been reported for yeast (13), but this has not been found in association with the Tom complex.

Recent studies on the import process in mammalian mitochondria support the idea that the mechanisms and components of the mitochondrial import machin-

Abbreviations used: GFP, green fluorescent protein; ChMTX, human metaxin lacking the C-terminal hydrophobic region; OTC, ornithine transcarbamylase; pOTC, pre-ornithine transcarbamylase; pOTC-GFP, a fusion protein containing the presequence of human pOTC fused to GFP; Tom20, Tom22, and Tom40, translocases of the outer membrane of mitochondria of 20, 22, and 40 kDa; hTom20, human Tom20; Tom34, 34 kDa protein involved in mitochondrial protein import.

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ery are mostly conserved between fungi and mammals, but significant structural differences and potential mechanistic variations in the components exist (2). Human (14, 15) and rat (16) homologues of fungal Tom20 have been shown to function as an outer membrane receptor for mitochondrial preproteins. Rat Tom20 can complement both the growth and mitochondrial defects of the *tom20* mutation in yeast cells. However, there are subtle functional differences between fungal and mammalian Tom20s. Human Tom20, but not yeast Tom20, can prevent a cryptic matrix targeting sequence of outer membrane proteins from gaining access to the protein translocation machinery (17). The NMR structure of rat Tom20 in a complex of a presequence peptide was recently solved and showed that presequence recognition is mainly due to interactions between the hydrophobic face of the signal peptide and the receptor domain (18). Another novel component, human Tom34 was shown to be involved in mitochondrial protein import in mammalian cells (19, 20). Tom34 is largely cytosolic and partly associated with the mitochondria and has no apparent counterpart in fungi.

In animals, most studies have been performed in an *in vitro* system in which the preproteins synthesized in reticulocyte lysate were imported into isolated mitochondria. It is important to test the *in vitro* results *in vivo* and there is a need for procedures that will enable protein import to be investigated in intact cells. We developed a procedure of cotransfecting cDNAs for preproteins and those for import factors in cultured cells and of analyzing their effects by immunoblotting or pulse-chase experiments (20–22).

Metaxin, which is a mammalian mitochondrial outer membrane protein, was discovered as a novel gene located between the glucocerebrosidase and thrombospondin 3 genes in the mouse (23). Metaxin contains a putative mitochondrial outer membrane signal anchor domain at its C-terminus and shares a weak sequence identity with yeast Tom37 at its N-terminus. Antibodies against metaxin inhibited the import of preadrenodoxin into mitochondria (24), leading to the suggestion that metaxin also functions in the import of preproteins into mammalian mitochondria.

We report here that coexpression of metaxin in cultured cells inhibits the import of several preproteins into the mitochondria and results in their accumulation. Coexpression of the cytosolic domain of metaxin inhibited preprotein import, although the inhibition was much less than that observed with the intact metaxin. Different tissue distribution of metaxin from that of Tom20 and its separation from the Tom complex in blue native PAGE suggests that metaxin is not involved in the Tom complex.

MATERIALS AND METHODS

Plasmids. Polymerase chain reaction (PCR) was employed for construction of the plasmid expressing the full-length human metaxin of 317 amino acid residues. Human cDNA library (Multi choice cDNA, OriGene, Rockville, MD) was used as template. The upstream primer used was 5′-AGAGGGTGGGCAAGATGGCG-3′ and the downstream primer was 5′-CTTGG-GAGCGTGAG-GACAAATC-3′. The PCR product was cloned into the *Hinc*II site of pGEM-3zf(+) (Promega, Madison, WI) to yield pGEM-3zf(+)-hMTX. The sequence was confirmed by sequencing. The *Nco*I fragment encoding the cytosolic domain of human metaxin (ChMTX), lacking the C-terminal hydrophobic region (40 amino acid residues) was excised from pGEM-3zf(+)-hMTX and cloned into the same site of pET-30a(+) (Novagen, Madison, WI) to yield pET-30a(+)-ChMTX. A mammalian expression vector pCAGGS (25) was provided by J. Miyazaki (Osaka University, Osaka). To construct pCAGGS-hMTX encoding full-length metaxin, the *Bam*HI/*Hin*dIII fragment was excised from pGEM-3zf(+)-hMTX, blunted with T4 DNA polymerase and cloned into the blunted *Xho*I site of pCAGGS. PCR was performed for the construction of an expression plasmid for ChMTX using pGEM-3zf(+)-hMTX as template. The upstream primer was 5′-AAAAAACTCGAGATGGCGGCGCCCATG-3′ and the downstream primer was 5′-TTTTTTCTCGAGTCAGTTCCGGCGCCG-3′. The PCR fragment was digested with *Xho*I, and cloned into the same site of pCAGGS yielding pCAGGS-ChMTX. Construction of pCAGGS-hTom20 (26), pCAGGS-pOTC (22), pCAGGS-pOTC-GFP (22) and pCAGGS-hAII (27) were described previously.

Preparation of ChMTX and antibody production. The recombinant plasmid pET-30a(+)-ChMTX was transformed into DE3 cells (Stratagene, CA) and His-tagged ChMTX was expressed with 1 mM IPTG at 37°C for 4 h. ChMTX was recovered in the inclusion bodies, washed with 1% Triton X-100, solubilized in buffer A (20 mM Tris–HCl, pH 7.9, 0.5 M NaCl, 8 M urea) and subjected to Ni^{2+}-NTA Sepharose (Pharmacia Biotech, UK) column chromatography. The column was first washed with buffer A containing 16 mM imidazole, and then ChMTX was eluted with buffer A containing 1 M imidazole. The eluate was dialyzed against 50 mM Tris–HCl, pH 7.8 containing 0.5 M NaCl and concentrated with Centricon-10 (Amicon, Beverly, MA). The purified ChMTX was used to raise an antibody in a rabbit.

RNA blot analysis. Human multiple tissue Northern blot was obtained from Clontech (Palo Alto, CA). Multi-primed ^{32}P-labeled cDNAs for human Tom20 and hMTX were used as hybridization probes.

Cell culture and DNA transfection. COS-7 cells were cultured in 10 cm dishes in Dulbecco's modified Eagle's medium supplimented with 10% fetal calf serum at 37°C under an atmosphere of 5% CO_2 and 95% air. The cells were transfected with plasmids at 37°C for 4 h by the use of TransIT LT1 polyamine (Pan Vera, Madison, WI) and cultured at 37°C for 24 h to allow expression.

Cell fractionation. The cells were harvested with trypsinization, washed twice with phosphate-buffered saline, and then suspended in ice-cold hypotonic buffer (10 mM Tris–HCl, pH 7.4, containing 5 mM magnesium chloride, 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride). After sonication, the homogenate was used as whole cell extract. The homogenate was separated into the soluble and membrane fractions by centrifugation at 100,000*g* for 10 min in a refrigerated ultracentrifuge. The membrane fraction was extracted with 0.1 M sodium carbonate, pH 11.5, as described (28).

Preparation of mitochondria from COS-7 cells. COS-7 cells were harvested with PBS plus 1 mM EDTA, and washed twice with PBS. The cells were suspended in the mitochondria isolation buffer (3 mM Hepes-KOH, pH 7.4, 0.21 M mannitol, 0.07 M sucrose, 0.2 mM EGTA), homogenized with a Dounce homogenizer (Wheaton, USA), and then centrifuged at 500*g* for 5 min at 4°C. The supernatant was

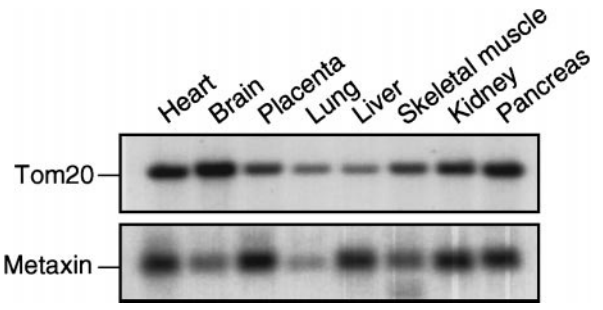

FIG. 1. Distribution of Tom20 and metaxin mRNAs in human tissues. Human multiple tissue Northern blot (2 μg of polyA$^+$ RNA) was probed with ^{32}P-labeled cDNAs for Tom20 or metaxin. The same blot was used for Tom20 and metaxin.

further centrifuged at 8000*g* for 5 min at 4°C, and the precipitated mitochondria were washed twice with the same buffer.

Immunoblot analysis. Proteins were separated by SDS–PAGE and electrotransferred onto nitrocellulose membranes. Antisera against human OTC (20), gelly fish GFP (22), human arginase II (29), hTom20 (22), human Tom22 (30), Tom40, and human metaxin were used as primary antibodies. Immunodetection was performed by the use of the chemiluminescence kit (ECL kit, Amersham).

Blue native PAGE. Blue native PAGE was performed essentially as described (12, 31, 32). Briefly, COS-7 mitochondrial pellets (50–100 μg of protein) were lysed in 50 μl of ice-cold digitonin buffer (1% digitonin, 20 mM Tris–HCl, pH 7.4, 0.1 mM EDTA, 50 mM NaCl, 10% glycerol, 1 mM phenylmethyl sulfonyl fluoride). After a clarifying spin, 5 μl of sample buffer (5% Coomassie brilliant blue G, 100 mM Bis-Tris, pH 7.0, 500 mM 6-aminocaproic acid) was added to the supernatant, and electrophoresis was performed for about 6 h in a 6–16.5% polyacrylamide gradient gel in a cold room (4–6°C). For two dimensional gel analysis, individual lanes were excised from the first-dimension blue native gel and layered on top of the stacking gel of a second dimension SDS–PAGE.

Other methods. Protein was determined with the protein assay reagent (Bio Rad, CA) using bovine serum albumin as standard. Cloning of human Tom40 cDNA and preparation of an antibody against it will appear elsewhere.

RESULTS

Distribution of metaxin and Tom20 mRNAs in human tissues. The tissue distribution of metaxin was examined by RNA blot analysis and compared with that of Tom20, which is a core mitochondrial import receptor (Fig. 1). Tom20 mRNA of about 4.1 kb was expressed ubiquitously in various tissues, but not uniformly. Metaxin mRNA of about 1.3 kb was also found to be expressed ubiquitously, but not uniformly. However, there were a number of significant differences in the distribution of metaxin mRNA from that of Tom20 mRNA. For example, Tom20 mRNA but not metaxin mRNA, was abundant in the brain, whereas metaxin mRNA but not Tom20 mRNA, was abundant in the liver.

Intracellular concentration and localization of metaxin. The concentrations of metaxin in monkey kidney-derived COS-7 cells and human cervical carcinoma HeLa cells were measured by immunoblot analysis using purified his-tagged ChMTX as standard (Fig. 2A). The purified his-tagged ChMTX (molecular size calculated from the sequence, 39.7 kDa) always gave two polypeptides; the faster-migrating one was presumably a degradation product. By using an antibody against ChMTX, a metaxin polypeptide of 35 kDa was detected in COS-7 and HeLa cells. The concentration of metaxin in COS-7 cells was 0.25 μg per mg of total protein, assuming that the antibody cross-reacted equally with the monkey protein, and that in HeLa cells was 0.30 μg per mg of total protein.

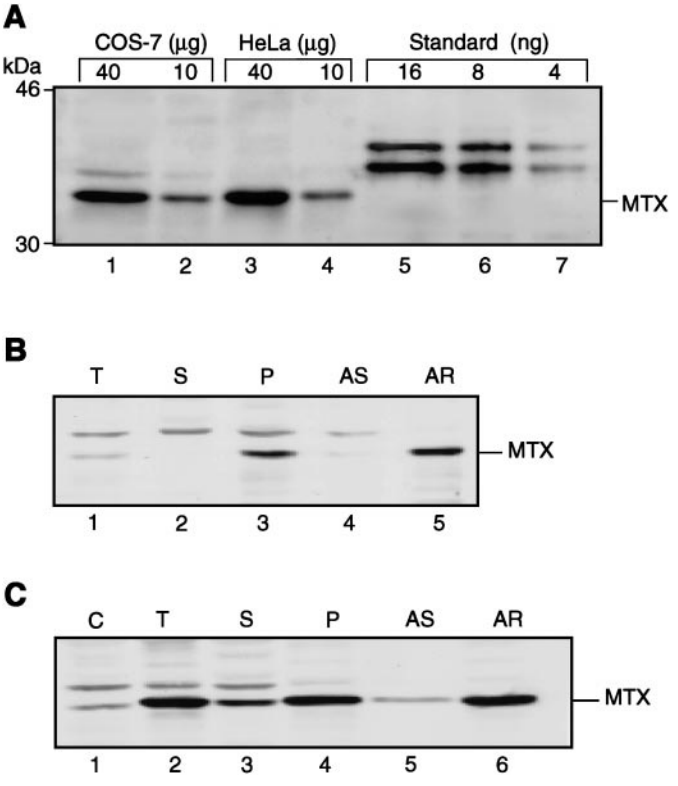

FIG. 2. Content (A) and subcellular localization (B and C) of metaxin in cultured cells. (A) 40 μg (lanes 1 and 3) or 10 μg of protein (lanes 2 and 4) from monkey COS-7 or human HeLa cell extracts were subjected to immunoblot analysis for metaxin using anti-human metaxin serum (1:1000 dilution) as a primary antibody. Purified ChMTX (lanes 5–7; 16, 8 and 4 ng) were used as standard. (B and C) COS-7 cells (B) or COS-7 cells transfected with pCAGGS-hMTX (C) were fractionated into the soluble (S) and membrane (P) fractions. Protein distribution in these fractions was 80 and 20%, respectively. Membrane fractions were further extracted with 0.1 M sodium carbonate and separated into alkali-soluble (AS) and alkali-resistant (AR) fractions. Protein distribution in these fractions was 40 and 60%, respectively. Whole cell extract (T) and fractionated samples were subjected to immunoblot analysis for metaxin using anti-human metaxin serum (1:1000 dilution) as primary antibody. In (B), 30 μg of protein was applied in each lane. In (C), protein applied was 30 μg (lane 2), 24 μg (lane 3), 6 μg (lane 4), 2.4 μg (lane 5), and 3.6 μg (lane 6). Protein was distributed in these fractions at the same ratios. Lane 1, extract of non-transfected COS-7 cells (30 μg of protein).

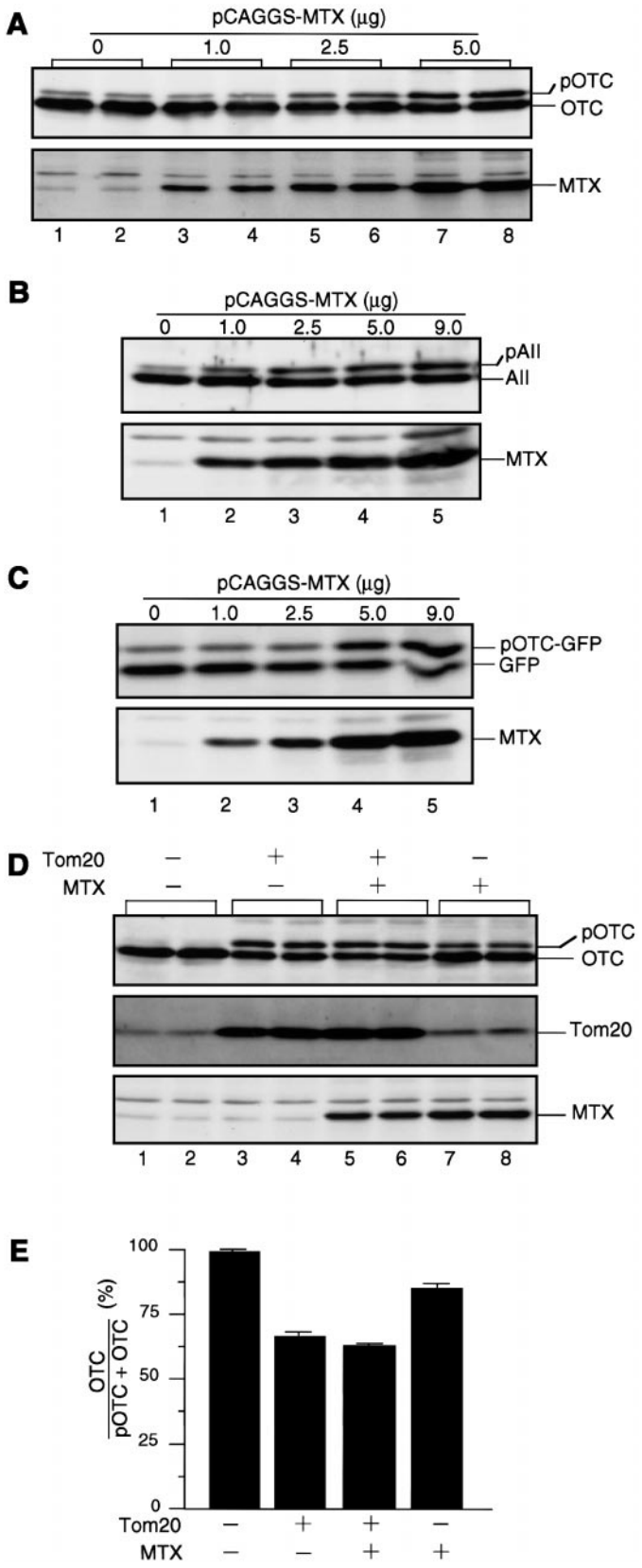

FIG. 3. Effect of overexpression of metaxin (A–C) and metaxin plus Tom20 (D and E) on mitochondrial import of preproteins in COS-7 cells. (A–C) COS-7 cells in 10-cm culture dishes were transfected with 5 μg of pCAGGS-pOTC (A), 1 μg of pCAGGS-hAII (B) or 1 μg of pCAGGS-pOTC-GFP (C) with indicated amounts of pCAGGS-hMTX. Total amount of plasmids was adjusted to 10 μg with pCAGGS. Twenty four h after transfection whole cell extracts (5 μg of protein) were subjected to immunoblot analysis for metaxin (MTX), pOTC/OTC, pre-arginase II (pAII)/arginase II (AII) and pOTC-GFP/GFP by using antisera (1:1000) against metaxin, OTC, arginase II and GFP, respectively, as primary antibodies. (D) pCAGGS-pOTC (5 μg) was cotransfected with indicated combinations of pCAGGS-hMTX (5 μg), pCAGGS-hTom20 (5 μg) in COS-7 cells cultured on 10-cm dishes. The total amount of transfected plasmids was adjusted to 15 μg with pCAGGS. Whole cell extracts (5 μg of protein) were subjected to immunoblot analysis for metaxin (MTX), pOTC/OTC and Tom20 by using antisera (1:1000) against metaxin, OTC and Tom20, respectively. (E) The results in (D) were quantified and percent processing of pOTC is shown as means ± ranges (n = 2).

When COS-7 cells were fractionated into the soluble and membrane fractions, endogenous metaxin was recovered exclusively in the membrane fraction and was not extracted with 0.1 M Na_2CO_2 (Fig. 2B). When human metaxin cDNA was transfected in COS-7 cells, human metaxin was highly expressed (Fig. 2C). When the transfected COS-7 cells were fractionated, most metaxin (about 90%) was recovered in the membrane fraction and was not alkali-extractable. These results together with a previous report (24) show that exogenously-expressed human metaxin is integrated into the mitochondrial membrane, where the endogenous protein is localized.

Effect of overexpression of metaxin on import of mitochondrial preproteins. Effect of overexpression of human metaxin on mitochondrial import and processing of preproteins in COS-7 cells were studied by immunoblot analysis (Fig. 3). When pOTC alone was expressed, it was imported and processed to the mature form almost completely and only a small amount of unprocessed pOTC was detected (Fig. 3A). When increasing amounts of metaxin was coexpressed, pOTC import was progressively inhibited and increasing amounts of pOTC accumulated. Similar results were obtained for another natural preprotein, prearginase II, and a chimeric preprotein pOTC-GFP (Figs. 3B and 3C).

In order to gain insight into the relationship between Tom20 and metaxin, we examined the effect of coexpression of these two proteins on pOTC import (Figs. 3D and 3E). Overexpression of Tom20 caused impaired import of pOTC and its accumulation, as we reported previously (26). Overexpression of metaxin also resulted in impaired import of pOTC (also see above). When Tom20 and metaxin were coexpressed, pOTC import was inhibited to an extent similar to that by Tom20 alone.

Effect of overexpression of ChMTX on pOTC processing. The cytosolic domain of human metaxin (ChMTX) was overexpressed and its effect on pOTC

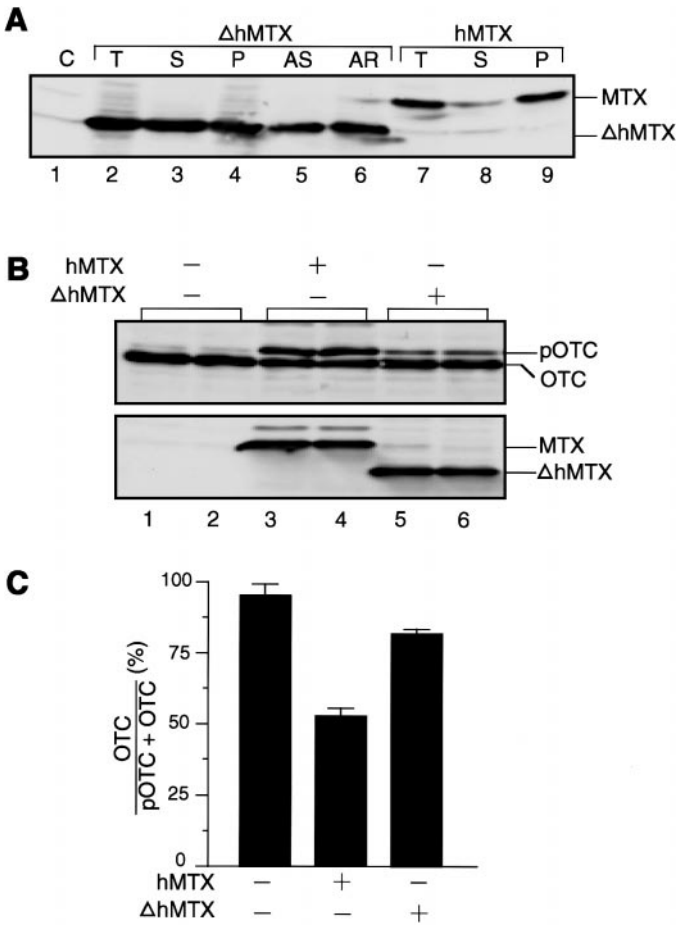

FIG. 4. Subcellular localization of ChMTX (A) and effect of its overexpression on pOTC transport (B and C) in COS-7 cells. (A) COS-7 cells in 10-cm culture dishes were transfected with 5 μg pCAGGS-hMTX or pCAGGS-ChMTX and total cell extracts (T) were fractionated into the soluble (S) and membrane (P) fractions. The membrane fraction was further extracted with 0.1 M sodium carbonate and separated into the alkali-soluble (AS) and alkali-resistant (AR) fractions. These fractions were subjected to immunoblot analysis using an antiserum against metaxin (MTX) (1:1000 dilution). Protein applied was 30 μg (lanes 2 and 7), 24 μg (lanes 3 and 8), 6 μg (lanes 4 and 9), 2.4 μg (lane 5), and 3.6 μg (lane 6). Protein was distributed in these fractions at the same ratios. Lane 1, total extract of non-transfected cells (30 μg of protein). (B) 5 μg of pCAGGS-pOTC was cotransfected with 5 μg of pCAGGS-hMTX or pCAGGS-ChMTX in COS-7 cells cultured on 10-cm dishes. Total amount of transfected plasmids was adjusted to 15 μg with pCAGGS. Whole cell extracts (5 μg of protein) were subjected to immunoblot analysis using anti-OTC antiserum (1:1000 dilution) or anti-metaxin antiserum (1:1000 dilution). (C) The results in (B) were quantified and percent processing is shown as means ± ranges (n = 2).

processing was studied. When COS-7 cells expressing ChMTX were fractionated into the soluble and membrane fractions, about 90% of ChMTX was recovered in the soluble fraction and the remaining 10% was recovered in the membrane fraction (Fig. 4A, see the legend for calculation). ChMTX associated with the membrane fraction was largely extracted with alkali.

When ChMTX was coexpressed with pOTC, the preprotein import was inhibited and its accumulation was evident (Figs. 4B and 4C). However, the inhibition by overexpression of ChMTX was weaker than that by overexpression of full-length metaxin; despite the similar level of expression of full-length metaxin and that of the truncated metaxin.

Separation of the Tom complex and metaxin in blue native PAGE. COS-7 mitochondria were isolated, solubilized with digitonin, and subjected to blue native PAGE followed by second dimension SDS–PAGE. Tom40 and Tom22 migrated as a large complex of ~400 kDa. A small portion of Tom22 migrated in a small form of ~70 kDa. Tom20 migrated partly as a complex of ~400 kDa and partly in smaller forms of ~40 kDa and ~100 kDa with a smear up to 200 kDa (Fig. 5). On the other hand, metaxin was found solely in a form of ~50 kDa. Thus, metaxin is apparently not associated with the Tom complex, and appears to exist in the mitochondrial outer membrane as a monomer or dimer.

DISCUSSION

The outer mitochondrial membrane contains components of the translocation 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,

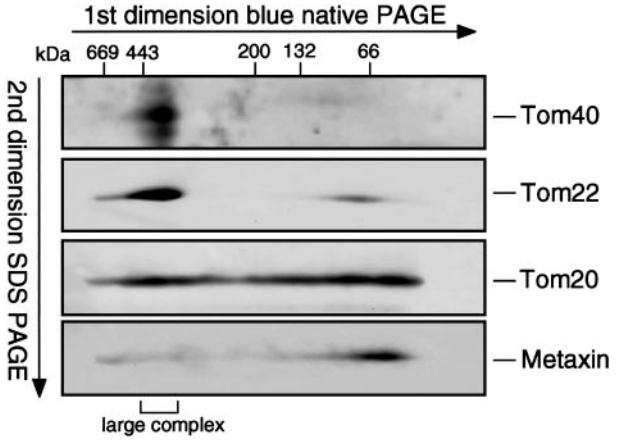

FIG. 5. Blue native PAGE of the Tom components and metaxin. Isolated COS-7 mitochondria (50–100 μg of protein) were lysed in digitonin buffer and subjected to blue native PAGE in the first dimension and SDS–PAGE in the second dimension as described under Materials and Methods. After electrophoresis, proteins were blotted and then immunodecorated with antisera (1:1000 dilution) against Tom40, Tom22, Tom20, or metaxin. The position of large complex (~400 kDa) is indicated. Molecular size markers in blue native PAGE are thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa) and albumin (132 and 66 kDa). Estimated molecular sizes of Toms and metaxin in blue native PAGE in the text are approximate, because in SDS–PAGE, the proteins migrated far from the blue native PAGE markers.

and components comprising the general insertion pore that allows access to the inside of the mitochondrion (1).

The only essential components of the translocase in fungi are Tom40 and Tom22 (for reviews, see Ref. 5), raising the question as to the specific roles of each subunit in the complex. Additionally, the finding of an apparently novel component metaxin (23), with no obvious homologue in fungi, raises further question about the role of this component in mammalian cells. Antibodies against metaxin inhibited the import of preadrenodoxin into isolated mitochondria (24). In the present study, we showed that overexpression of human metaxin in COS-7 cells resulted in inhibition of mitochondrial import and processing of preproteins including pOTC, pre-arginase II and a fusion preprotein pOTC-GFP, and in their accumulation in the cell. The cytosolic domain of human metaxin had similar but less marked effects. As shown previously, overexpression of Tom20 in COS-7 cells also inhibited mitochondrial import of pOTC, whereas the cytosolic domain of Tom20 had little effect (26). Among the other mammalian transport factors, overexpression of Tom34 stimulated the import of pOTC (20), and that of HSDJ (hdj-2/dj2), a cytosolic DnaJ homologue which participates in mitochondrial protein import, inhibited the pOTC import (21). Overexpression of an unrelated cytosolic protein, phenylalanine hydroxylase, had no effect (21). We speculate that if a factor is limiting the preprotein import, its overexpression may result in stimulation of the import. In contrast, when a factor is not limiting the import, its overexpression may result in inhibition due to an imbalance of the import factors or disruption of complexes containing these factors which are required for the import pathway.

The functional role of metaxin in mitochondrial protein import is enigmatic. Metaxin is expressed ubiquitously, but its tissue distribution differs from that of Tom20 which is a receptor of the transport machinery. In blue native PAGE, the yeast Tom complex consisting of Tom40, Tom22, and small Toms migrates as a 400 kDa complex (12). Tom20 exists mainly in a dissociated form of about 40 to 100 kDa and partly with the 400 kDa complex. Here, we performed blue native PAGE of mammalian Tom components and showed that Tom40 and Tom22 migrate as a complex of ~400 kDa, like fungal Toms. Tom20 was partly associated with this Tom complex and migrated partly in smaller dissociated forms. These results show that the mammalian Tom complex is similar in structure to the yeast complex. On the other hand, metaxin migrated separately in a form of ~50 kDa. These results indicate that metaxin is not a component of the Tom complex. The fact that the cytosolic domain of metaxin inhibits the preprotein import, suggests that it binds with the preprotein and inhibits the productive transport. Binding studies are in progress.

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