Functional Analysis of Human Mitochondrial Receptor Tom20 for Protein Import into Mitochondria*

(Received for publication, May 13, 1998, and in revised form, July 25, 1998)

Masato Yano, Masaki Kanazawa‡, Kazutoyo Terada, Motohiro Takeya§, Nicholas Hoogenraad¶, and Masataka Mori

From the Department of Molecular Genetics and the §Second Department of Pathology, Kumamoto University School of Medicine, Kumamoto 862, Japan and the ¶Department of Biochemistry, La Trobe University, Bundoora, Victoria 3083, Australia

The mitochondrial import receptor translocase of the outer membrane of mitochondria (Tom20) consists of five segments, an N-terminal membrane-anchor segment, a linker segment rich in charged amino acids, a tetratricopeptide repeat motif, a glutamine-rich segment, and a C-terminal segment. To assess the role of each segment, four C-terminally truncated mutants of the human receptor (hTom20) were constructed, and the effect of their overexpression in COS-7 cells was analyzed. Expression of a mutant lacking the tetratricopeptide repeat motif inhibited preornithine transcarbamylase (pOTC) import to the same extent as the wild-type receptor. Thus, overexpression of the membrane-anchor and the linker segments is sufficient for the inhibition of import. Expression of either the wild-type receptor or a mutant lacking the C-terminal end of 20 amino acid residues stimulated import of pOTC-green fluorescent protein (GFP), a fusion protein in which the presequence of pOTC was fused to green fluorescent protein. On the other hand, expression of mutants lacking either the glutamine-rich segment or larger deletions inhibited pOTC-GFP import. In vitro import of pOTC was inhibited by the wild-type hTom20 and the mutant lacking the C-terminal end, but much less strongly by the mutant lacking the glutamine-rich segment. On the other hand, import of pOTC-GFP was little affected by any of the forms of hTom20. In binding assays, pOTC binding to hTom20 was only moderately decreased by the deletion of the glutamine-rich segment, whereas pOTC-GFP binding was completely lost by this deletion. Binding of pOTCN-GFP, a construct that contains an additional 58 N-terminal residues of mature OTC, resembled that of pOTC. All of these results indicate that the region 106–125 containing the glutamine-rich segment of hTom20 is essential for binding and import stimulation in vivo of pOTC-GFP and for inhibition of in vitro import of pOTC. The results also indicate that this region is important for mitochondrial aggregation. The different behaviors of pOTC and the pOTC-GFP chimera toward hTom20 mutants is explicable on the basis of the conformation of the precursor proteins.

* This work was supported by Grants-in-aid 08457040 and 0725321 (to M. M.) from the Ministry of Education, Science, Sports and Culture of Japan, and from the Australian Research Council (to N. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Department of Pediatrics, Chiba University School of Medicine, Chiba 280, Japan.

§ To whom correspondence should be addressed: Dept. of Molecular Genetics, Kumamoto University School of Medicine, Kuronjo 4-24-1, Kumamoto 862, Japan. Tel.: 81-96-373-5140; Fax: 81-96-373-5145; E-mail: massa@gpo.kumamoto-u.ac.jp.

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 Refs. 1–4). They form a dynamic protein complex, termed the translocase of the outer membrane of mitochondria. 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 was shown to bind to the basic amphiphilic targeting sequences of preproteins through electrostatic interactions with the acidic receptor domain (12). Together with Tom22, Tom20 of 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 mitochondrial were shown to interact via the tetratricopeptide repeat (TPR) motif in Tom20 (14).

On the other hand, little is known about the import receptors of animals. Recently, cDNA for a human homolog (hTom20) of yeast and Neurospora Tom20 was isolated (15–17). In vitro import of preproteins into isolated mitochondria was inhibited by the soluble domain of hTom20 (ΔhTom20) (17) and by anti-hTom20 (16, 17). We also showed that the import of several preproteins was inhibited by ΔhTom20 and by anti-hTom20, and found that the inhibition varied among preproteins (18). In addition to the in vitro assay of hTom20, we have assessed its role in cultured animal cells, which more closely resembles the in vivo situation. We developed an in vivo assay method in which cultured cells were cotransfected with plasmids for a preprotein and for hTom20 and showed that in pulse-chase experiments coexpression of exogenous hTom20 retarded mitochondrial import and processing of preornithine transcarbamylase (pOTC) (18). On the other hand, overexpression of hTom20 resulted in stimulated mitochondrial import of a fusion protein pOTC-GFP that consists of the presequence of human pOTC fused to green fluorescent protein (GFP) (19). Surprisingly, overexpression of hTom20 resulted in the perinuclear aggregation of mitochondria (19). In each of these assays, ΔhTom20 had no effect.

Here, we report that the overexpression in COS-7 cells of the...
Materials and Methods

Antibodies—The NcoI/blunt-ended XhoI fragment encoding Aequorea victoria GFP was excised from pGFP-S65T (CLONTECH, Palo Alto, CA), and cloned into the NcoI/blunt-ended HindIII site of the pET30a (Novagen, Madison, WI). Histidine- and 5'-peptide-tagged GFP was expressed in Escherichia coli from the resulting plasmid, pET30a-GFP, purified by metal chelation chromatography and used for making anti-GFP antibody. Anti-human OTC and anti-human Tom20 antibodies were prepared as described previously (19).

Construction of Plasmids—Polymerase chain reaction was employed for construction of the plasmids expressing the full-length (145 amino acid residues) and C-terminally truncated forms of human mitochondrial import receptor, hTom20. The plasmid pCAGGS-hTom20 (19) was used as the template. The common upstream primer used for all five derivatives was 5'-AAAAAGATCTGGACATGGTTGTTGCGGAAGCTTACCATACATCTCAGCC-3', and the downstream primers used for each expression plasmid were 5'-TTTCTTCTCGAGTCTACCATCTCTTTTCTTCTTTTCTTCTACCCAC-3' for pCAGGS-(1-145)hTom20 (wild-type), 5'-TTTTTTCCTCACTGTTCTTTTCTTCTTCTTCTTCTTTTCTTCTACCCAC-3' for pCAGGS-(1-105)hTom20, 5'-TTTTTTCCTCTGACTCGACCCCTCTCTATCTTCTACCCAC-3' for pCAGGS-(1-89)hTom20 and 5'-TTTTTTCCTCTGACTCGACCCCTCTCTATCTTCTACCCAC-3' for pCAGGS-(1-73)hTom20. Site-directed mutagenesis was used to construct the plasmid pCAGGS-OTC (Amersham Pharmacia Biotech, Uppsala, Sweden). The resulting plasmids pCAGGS-hTom20, pCAGGS-pOTC, and the plasmid pCAGGS-(1-125)hTom20, 5'-TTTTTTCCTTACGTTACTTGGCTGCTCCAC-3' for pCAGGS-(1-105)hTom20, 5'-TTTTTTCCTCTGACTCGACCCCTCTCTATCTTCTACCCAC-3' for pCAGGS-(1-89)hTom20 and 5'-TTTTTTCCTCTGACTCGACCCCTCTCTATCTTCTACCCAC-3' for pCAGGS-(1-73)hTom20 were shown in Fig. 1A. Construction of pCAGGS-pOTC (21) and pCAGGS-pOTC-GFP (19) were previously described. For constructing the plasmid expressing GST-fused hTom20 derivatives, the hTom20 gene was amplified by PCR. The common upstream primer used for all three derivatives was 5'-AAAAAGATCTGGACATGGTTGTTGCGGAAGCTTACCATACATCTCAGCC-3', and the downstream primers used for each expression plasmid were the same as used for construction of pCAGGS-(1-145)hTom20, pCAGGS-(1-125)hTom20, and pCAGGS-(1-105)hTom20. The structure of these plasmids is described in Fig. 1A.

Expression and Purification of GST-fused hTom20s—The plasmids, pGEX-2T and two types of its derivatives were transformed into TOPP 2 cells (Stratagene, La Jolla, CA). Expression of GST-fused hTom20s and absorption onto glutathione-agarose (Amersham Pharmacia Biotech) were performed as described previously (17). The absorbance of the beads was measured at 10° C and no significant change was observed during the period of storage.

In Vitro Translational and Translation—In vitro translation of the N-terminal hTom20 was performed for 90 min at 30 °C in a rabbit reticulocyte lysate system (Promega, Madison, WI; 60% (v/v) lysate) in the presence of Pro-mix containing l-[35S]methionine and l-[35S]cysteine (Amersham Pharmacia Biotech), as described previously (18).

Expression and Purification of GST-fused hTom20s—The plasmids, pGEM3Zf (+) + pOTC (18), pGEM3Zf (+) + pOTC-GFP, and pGEM3Zf (+) + pOTC-EGFP (19), were used for in vitro transcription. In vitro translation of the RNA transcripts was performed for 90 min at 30 °C in a rabbit reticulocyte lysate system (Promega, Madison, WI; 60% (v/v) lysate) in the presence of Pro-mix containing l-[35S]methionine and l-[35S]cysteine (Amersham Pharmacia Biotech), as described previously (18).

Expression and Purification of GST-fused hTom20s—Purified GST or GST-hTom20 derivatives (7 mM) were absorbed onto glutathione-agarose in 1.25 ml of binding buffer (20 mM Hepes-KOH (pH 7.4), 50 mM potassium chloride, 1 mM magnesium chloride, 0.1 mg/ml bovine serum albumin) containing 62.5 μl of 50% slurry of glutathione-agarose (Amersham Pharmacia Biotech, Uppsala, Sweden). The agarose beads were washed three times and resuspended in 250 μl of binding buffer. For assay of GST derivative-bound agarose was diluted in 270 μl of binding buffer, and then mixed with 10 μl of reticulocyte lysate containing 35S-labeled translation products for 30 min at 25 °C by gentle shaking. After centrifugation, the supernatant was removed and the beads were washed once with binding buffer. Fifty μl of 50 mM Tris-HCl (pH 8.0) containing 15 mM reduced glutathione were added to the wet agarose beads, the mixture was shaken gently for 1 h at 25 °C, and 40 μl of the supernatant was recovered after centrifugation. Twenty μl of the supernatant was subjected to 10% SDS-PAGE, and the proteins were stained with Coomassie Brilliant Blue R-250 to check the amount of eluted protein.

Proteinase Sensitivity Assay—Reticulocyte lysate (4 μl) containing 35S-labeled translation products was incubated in 16 μl of 20 mM Heps-KOH (pH 7.4) containing 50 μg/ml cycloheximide and various amounts of trypsin for 10 min on ice. The reaction was stopped by adding 20 μl of 20 mM Heps-KOH (pH 7.4) containing 2 mM phenylmethylsulfonyl fluoride. Ten μl of the mixture (total 40 μl) were subjected to 10% SDS-PAGE, and the radioactivity in the gels was visualized.

RESULTS

Expression of C-terminally Truncated Mutants of hTom20—cDNAs encoding C-terminally truncated mutants of hTom20 (1–125)hTom20, (1–105)hTom20, (1–89)hTom20, and (1–73)hTom20 as well as wild-type, (1–145)hTom20 (Fig. 1A),
were transfected into COS-7 cells in a potent mammalian expression system. The cells were fractionated as described under “Materials and Methods.” The whole cell extracts (W) (about 30 μg), soluble fractions (S) (about 20 μg), and membrane fractions (P) (about 10 μg) were subjected to Tris-Tricine PAGE and immunoblot analysis using the antiserum to hTom20. Molecular mass markers are lysozyme (14.3 kDa) and aprotinin (6.5 kDa).

Effect of Overexpression of Truncated hTom20s on Mitochondrial Import of pOTC—When human pOTC is transiently expressed in COS-7 cells, it is imported efficiently into the mitochondria and processed to the mature form, as revealed by cell fractionation and immunoblot analysis (18, 21). The effect of overexpressing C-terminally truncated hTom20s on pOTC import and processing was analyzed by pulse-chase experiments, and the results were quantitated (Fig. 2). When the COS-7 cells expressing pOTC alone were labeled for 5 min with [35S]methionine, 45% of the newly synthesized pOTC was converted to the mature form (line a). The cells were then chased with cold methionine, the labeled pOTC was converted to the mature form much more slowly in the chase (line b). Very similar results were obtained when the four truncated mutants were coexpressed with pOTC. COS-7 cells were cultured in 10-cm dishes. Five μg of pCAGGS-pOTC was co-transfected with 5 μg of pCAGGS (line a, ○○○○), pCAGGS-1–145hTom20 (line b, ●●●●), pCAGGS-1–125hTom20 (line c, □□□□), pCAGGS-1–105hTom20 (line d, ■■■■), pCAGGS-1–89hTom20 (line e, ▲▲▲▲), or pCAGGS-1–73hTom20 (line f, ▼▼▼▼). After 16 h culture, the cells were harvested and subjected to pulse-chase experiments as described under “Materials and Methods.” Immunoprecipitation was performed with 20 μl of the antiserum to human OTC and 200 μl of a 10% suspension of protein A-Sepharose, and the immunoprecipitated proteins were subjected to 10% SDS-PAGE. The radioactive pOTC and mature OTC on the SDS-polyacrylamide gel were quantitated by image plate analysis, and the ratios of mature OTC (mOTC) versus pOTC plus mOTC are shown. Values are represented by means ± S.E. of three independent experiments for lines a and b.

Effect of Overexpression of Truncated Tom20s on Mitochondrial Import of pOTC-GFP—When the fusion protein pOTC-GFP, consisting of the presequence of human pOTC fused to green fluorescent protein (GFP), is expressed in COS-7 cells, it is imported into mitochondria and processed to the mature form, as revealed by cell fractionation and immunoblot analysis (18, 21). The effect of overexpressing C-terminally truncated hTom20s on pOTC-GFP import and processing was analyzed by pulse-chase experiments (Fig. 3). When the cells expressing pOTC-GFP alone were labeled for 5 min, about 4% of newly synthesized pOTC-GFP was imported and processed (line a) and was gradually processed to mature GFP in the chase. The import and processing of pOTC-GFP appears to be rate-limiting under these conditions, probably due to the

Fig. 1. Structure and expression of C-terminally truncated mutants of hTom20. A, the primary structures of truncated hTom20s are shown. Filled boxes show the predicted transmembrane domain (TM), gray boxes show the sequence corresponding to the TPR motif, and striped boxes show the predicted transmembrane domain (TM). Gray boxes show the sequence corresponding to the TPR motif, and striped boxes show the predicted transmembrane domain (TM). B, COS-7 cells were transfected with 10 μg of pCAGGS-(1–145)hTom20 (wild-type), pCAGGS-(1–125)hTom20, pCAGGS-(1–105)hTom20, pCAGGS-(1–89)hTom20, or pCAGGS-(1–73)hTom20, and incubated for 24 h. The cells were harvested, and fractionated as described under “Materials and Methods.” The whole cell extracts (W) (about 30 μg), soluble fractions (S) (about 20 μg), and membrane fractions (P) (about 10 μg) were subjected to Tris-Tricine PAGE and immunoblot analysis using the antiserum to hTom20. Molecular mass markers are lysozyme (14.3 kDa) and aprotinin (6.5 kDa).

Fig. 2. Co-expression of C-terminally truncated hTom20s and pOTC. COS-7 cells were cultured in 10-cm dishes. Five μg of pCAGGS-pOTC was co-transfected with 5 μg of pCAGGS (line a, ○○○○), pCAGGS-1–145hTom20 (line b, ●●●●), pCAGGS-1–125hTom20 (line c, □□□□), pCAGGS-1–105hTom20 (line d, ■■■■), pCAGGS-1–89hTom20 (line e, ▲▲▲▲), or pCAGGS-1–73hTom20 (line f, ▼▼▼▼). After 16 h culture, the cells were harvested and subjected to pulse-chase experiments as described in Fig. 2 except for changing the antiserum to that for GFP. The radioactive pOTC-GFP and mature OTC-GFP on the SDS-polyacrylamide gel were quantitated by image plate analysis, and the ratios of mature OTC (mOTC) versus pOTC plus mOTC are shown. Values are represented by means ± S.E. of three independent experiments for lines a and b.

Fig. 3. Co-expression of C-terminally truncated hTom20s with pOTC-GFP. COS-7 cells were cultured in 10-cm dishes. Five μg of pCAGGS-pOTC-GFP was co-transfected with 5 μg of pCAGGS (line a, ○○○○), pCAGGS-1–145hTom20 (line b, □□□□), pCAGGS-1–125hTom20 (line c, ▲▲▲▲), pCAGGS-1–105hTom20 (line d, ■■■■), pCAGGS-1–89hTom20 (line e, ▼▼▼▼), or pCAGGS-1–73hTom20 (line f, ▼▼▼▼). After 16 h culture, the cells were harvested and subjected to pulse-chase experiments as described in Fig. 2 except for changing the antiserum to that for GFP. The radioactive pOTC-GFP and mature OTC-GFP on the SDS-polyacrylamide gel were quantitated by image plate analysis, and the ratios of mature OTC-GFP (mOTC-GFP) versus pOTC-GFP plus mOTC-GFP are shown. Values are represented by means ± S.E. of three independent experiments for lines a and b, and by means of two independent experiments for lines c–f. These results indicate that insertion of the N-terminal half of hTom20, containing the membrane-anchor and the linker segments (region 1–73) into mitochondria, is sufficient to inhibit pOTC import and processing and therefore the domain containing the TPR motif, glutamine-rich segment, and the C-terminal segment are not essential for inhibition of import and processing in cultured cells.

Effect of Overexpression of Truncated Tom20s on Mitochondrial Import of pOTC-GFP—When the fusion protein pOTC-GFP, consisting of the presequence of human pOTC fused to GFP, is expressed in COS-7 cells, it is imported into mitochondria and processed to mature GFP (19). The effect of overexpressing C-terminally truncated hTom20s on pOTC-GFP import and processing was analyzed by pulse-chase experiments (Fig. 3). When the cells expressing pOTC-GFP alone were labeled for 5 min, about 4% of newly synthesized pOTC-GFP was imported and processed (line a) and was gradually processed to mature GFP in the chase. The import and processing of pOTC-GFP appears to be rate-limiting under these conditions, probably due to the

Effect of Overexpression of Truncated Tom20s on Mitochondrial Import of pOTC-GFP—When the fusion protein pOTC-GFP, consisting of the presequence of human pOTC fused to GFP, is expressed in COS-7 cells, it is imported into mitochondria and processed to mature GFP (19). The effect of overexpressing C-terminally truncated hTom20s on pOTC-GFP import and processing was analyzed by pulse-chase experiments (Fig. 3). When the cells expressing pOTC-GFP alone were labeled for 5 min, about 4% of newly synthesized pOTC-GFP was imported and processed (line a) and was gradually processed to mature GFP in the chase. The import and processing of pOTC-GFP appears to be rate-limiting under these conditions, probably due to the
large amount expressed. In contrast to the case with pOTC, when wild-type hTom20 was coexpressed, import and processing was stimulated about 2-fold (line b). (1–125)hTom20 was also stimulatory (line c). On the other hand, coexpression of the shorter mutants resulted in slight inhibition of the import and processing (lines d–f). These results show that the region 106–125 containing the glutamine-rich segment of hTom20 is essential for stimulation of pOTC-GFP import and processing, but the C-terminal end (region 126–145) is not. The results also show that insertion of the N-terminal half of hTom20, containing the membrane-anchor and linker segments (region 1–73), into the mitochondrial membrane, inhibits import and processing of pOTC-GFP.

Effect of Overexpression of Truncated hTom20s on Mitochondrial Organization in the Cell—Mitochondria-targeted pOTC-GFP gives organelle-associated fluorescence and overexpression of hTom20 induces perinuclear aggregation of fluorescent mitochondria (19). The effect of C-terminal truncation of hTom20 on the alteration of the mitochondrial organization was studied (Fig. 4). When wild-type hTom20 or (1–125)hTom20 was coexpressed with pOTC-GFP in COS-7 cells, aggregation of fluorescent mitochondria was observed in almost all transfected cells (panels c–f). On the other hand, when Tom20 was truncated to (1–105)hTom20, such mitochondrial aggregation was almost completely reversed, and fluorescent mitochondria were distributed throughout the cytoplasm (panels g and h). The mitochondrial distribution with the shorter mutants (panels i–l) appeared the same as the control (panels a and b). Therefore, the region 106–125 containing the glutamine-rich segment is essential for perinuclear mitochondrial aggregation, but the C-terminal end (region 126–145) is not.

Effect of Overexpression of hTom20 on Mitochondrial Morphology—The effect of hTom20 overexpression on morphological alterations of mitochondria was analyzed by electron microscopy (Fig. 5). In control cells, mitochondria of various shapes were distributed throughout the cytoplasm (a and b). In the hTom20-transfected cells, on the other hand, large aggregates of mitochondria were seen adjacent to the nucleus (c and d). The mitochondrial aggregation was seen in about 10% of cells, this value being consistent with the transfection efficiency obtained in these experiments. Such mitochondrial aggregation was not seen in cells transfected with the control plasmid pCAGGS. These results are in accord with those obtained by fluorescence microscopy.

The aggregates are composed of many round mitochondria of various sizes, and practically no mitochondria were seen outside the aggregates. Although all the mitochondria were confined to the aggregates, not all appeared to be in physical contact. No evidence was seen for fusion of mitochondria. The mitochondrial aggregates were close to nucleus but was not in direct contact. No prominent structures other than mitochondria were found in the aggregates.

Effect of GST-hTom20s on in Vitro Import into Isolated Mitochondria—To analyze the roles of hTom20 in mitochondrial protein import in vitro, we expressed a GST fusion protein containing the entire cytosolic domains of hTom20 (GST-(25–145)hTom20) and two containing deletions (GST-(25–105)hTom20 and GST-(25–125)hTom20). In all fusions, the predicted transmembrane region (region 1–24) was omitted. When in vitro synthesized pOTC was incubated with isolated rat liver mitochondria, it was efficiently imported into the mitochondria and processed to the mature form with time (Fig. 5).
Fig. 5. Electron microscopy of aggregated mitochondria. COS-7 cells were grown in 35-mm culture dishes and transfected with 2 μg of pCAGGS (a and b) or pCAGGS-(1–145)hTom20 (c and d). After 24-h culture, the cells were fixed with 2.5% glutaraldehyde, postfixed with 1% osmium tetroxide, dehydrated, and embedded in epoxy resin. Ultrathin sections stained with uranyl acetate and lead nitrate were observed electron microscopically. Bars, 1 μm.

pOTC import was not inhibited by GST, but was markedly inhibited by 20 μM GST-(25–145)hTom20. GST-(25–125)hTom20 was as effective as GST-(25–145)hTom20, whereas GST-(25–105)hTom20 was much less inhibitory (Fig. 6, B and C). Therefore, the region 106–125 which includes the major part of the glutamine-rich segment of hTom20 is critical for pOTC import. On the other hand, there was no measurable effect of GST-(25–145)hTom20 or the other deleted proteins on pOTC-GFP import (Fig. 6, B and C). These results suggest that not only the presequence portion but also the mature portion of the precursor protein is involved in the interaction with hTom20.

Binding of Precursor Proteins to GST-hTom20s—Recently, Schleiff et al. (23) measured the direct interaction between hTom20 and precursor proteins by using the cytosolic portion of hTom20 fused to GST. We employed their system to analyze the binding of pOTC and pOTC-GFP to the C-terminally truncated hTom20 s. 35S-Labeled pOTC or pOTC-GFP synthesized in vitro was incubated with glutathione-agarose beads pre-bound with GST or GST-hTom20 proteins, and the precursor proteins, and GST fusions were then eluted with reduced glutathione. About 80% of GST and GST-hTom20 fusions that were applied to the binding assay, were recovered in the eluate (data not shown). Eight percent of applied pOTC was bound to GST-(25–145)hTom20 (Fig. 7). pOTC binding was decreased as hTom20 was C-termionally truncated from 145 to 125. It was further decreased as hTom20 was further truncated from 125 to 105, but a moderate amount of binding still remained (about one-fourth of that to (25–145)hTom20). Slightly less (about 6%) of pOTC-GFP bound to GST-(25–145)hTom20. pOTC-GFP binding decreased as hTom20 was truncated. However, in sharp contrast with pOTC, the binding of pOTC-GFP was almost completely lost when hTom20 was deleted to (25–105)hTom20. When pOTCN-GFP in which the presequence plus 58 residues of mature OTC was fused to GFP, was used, it behaved very similarly to pOTC. We have previously shown that this construct is imported more efficiently than pOTC-GFP, but upon processing it does not become fluorescent, suggesting that, unlike pOTC-GFP, this construct remains unfolded after removal of the presequence (19). These results indicate that both the C-terminal segment and the glutamine-rich region are involved in precursor binding, but the region 106–125 containing the glutamine-rich segment is critical for the binding of pOTC-GFP, but was less critical for binding of pOTC and pOTCN-GFP.

Protease Sensitivity of Precursor Proteins—Although pOTC and pOTC-GFP have the same presequence, they differ in mitochondrial import both in vivo and in vitro, and in binding to the hTom20 mutants. In order to determine whether this difference is due to a different tendency of the precursor proteins to fold, we compared protease sensitivity of these two proteins (Fig. 8). When pOTC (about 40 kDa) was treated with increasing concentrations of trypsin, pOTC was digested without formation of a trypsin-resistant fragment. On the other hand, when pOTC-GFP (about 31.5 kDa) was digested under the same conditions, a fragment of 28 kDa, that apparently corresponded to mature GFP, was formed. These results suggest that the mature portion of pOTC remained unfolded, whereas that of pOTC-GFP (namely GFP) was folded into a trypsin-resistant folded conformation.

DISCUSSION

The results obtained from the present experiments with cultured cells show that amino acids 106–125 of hTom20, which contain the glutamine-rich segment, is essential for stimulation of pOTC-GFP import, but the C-terminal end (region 126–145) seems not to be. The essential region has no “consensus” sequence, but is moderately homologous with the corresponding regions of yeast and Neurospora Tom20 (15–17). Overexpression of the N-terminal half of hTom20 (region 1–73), containing the membrane-anchor and charged linker segments, and its insertion into the mitochondrial outer membrane, inhibits import and processing of both pOTC and pOTC-GFP, suggesting that this portion of hTom20 can disrupt the import machinery. The linker segment of 45 amino acid residues contains 17 positively charged residues (Arg plus Lys) and 6 negatively charged residues (Glu plus Asp). This linker segment may be important for the observed dominant-negative effect in transfected cells. However, this effect may require that the linker segment be attached to the membrane-anchor segment.
so that it can associate with other components of the translocation machinery and disrupt its function, since overexpression of the soluble domain of hTom20 (domain 30–145) containing the linker segment does not inhibit pOTC import in cultured cells (18). Although the TPR motif of Tom20 of yeast was reported to increase interaction of Tom20 with the Tom70-Tom37 complex, its mutation did not inactivate the receptor function of Tom20 (14). Likewise, in this study, we found that the TPR motif of hTom20 was not essential for the dominant negative effect.

We have previously shown that overexpression of hTom20 results in perinuclear aggregation of mitochondria in addition to affecting mitochondrial import and processing (19). Whether the effect on import and perinuclear mitochondrial aggregation are related in this analysis is not known. However, Tom20 depletion as well as its overexpression is known to induce altered morphology of mitochondria. Tom20-deficient N. crassa cells contain mitochondria that are highly deficient in cristae (24), suggesting that the appropriate number of Tom20 molecules is important in maintaining the normal morphology of mitochondria.

Recently, Iwahasi et al. (25) investigated the functional domain of rat Tom20. They expressed mutant rat Tom20 proteins in Δtom20 yeast cells and examined their ability to complement the defects of respiration-driven growth and mitochondrial protein import. Based on their results, they concluded that the N-terminal region containing the membrane-anchor and the linker segments is essential for the function of rat Tom20, whereas the TPR motif, glutamine-rich segment and the C-terminal segment are not. The region essential for the Tom20 function in the complementation study is nearly identical to that required for the dominant-negative effect in this study. In the current study, a distinct region (106–125) is required for the stimulation of pOTC-GFP import. In a heterologous system, some subtle interaction between mammalian Tom20 and the yeast translocase complex may be overlooked, as suggested by the fact that when yeast Tom20 was substituted with hTom20 in yeast mitochondria, hTom20 worked somewhat differently from the endogenous yeast Tom20 (26).

From the results of in vitro import and binding assays, both the glutamine-rich segment-containing region (region 106–125) and the C-terminal end of hTom20 (region 126–145), especially the former region, were shown to be involved in the binding to precursors. These results are in close agreement with in vitro experiments showing that the glutamine-rich segment-containing region (region 106–125) and the C-terminal end of hTom20 (region 126–145), especially the former region, were shown to be involved in the binding to precursors. These results are in close agreement...
with those obtained on cultured cells. Schleiff et al. (23) showed recently that a predicted “glutamine face” (region 104–114) and an extreme C-terminal cluster of acidic residues (region 141–145) have effects on binding of some precursor proteins. Stimulation of pOTC-GFP import seems to be attributable to the predicted glutamine face, most of which is included in the region 106–125. The critical necessity of this region for binding to the presequence of pOTC-GFP was shown through the binding assay. In addition, the binding studies suggest that there may be additional interactions between hTom20 and precursor proteins through mature protein domains adjacent to the cleavable presequence. Thus, we have previously shown that the import velocity of pOTCN-GFP was almost the same as that of pOTC but was about 2.5-fold higher than that of pOTC-GFP (19). This is consistent with the ability of both pOTC and pOTCN-GFP to bind to a similar degree to region 25–105 of hTom20, whereas pOTC-GFP does not bind to this region. The interaction between the region 25–105 of hTom20 and the N-terminal portion of mature OTC seems to facilitate the import of pOTC. Previous study on an artificial precursor fused with dihydrofolate reductase has shown that destabilization of the precursor facilitates its import (27). The low import velocity of pOTC-GFP may be attributable to the stability of the GFP domain, whereas the high import velocity of pOTC and pOTCN-GFP may be due to their instability, in which the N-terminal mature portion of OTC may work as an intramolecular chaperone as shown for the F1-ATPase β-subunit (28).

### Fig. 9. Model for the interaction between precursors and hTom20.

This model presents the different domains of hTom20 as in Fig. 1 and shows the likely sites of interaction of pOTC/pOTCN-GFP and pOTC-GFP with hTom20. The observed, more rapid import of pOTC and pOTCN-GFP is explained by there precursors being largely unfolded. This unfolded, import-competent conformation, is prone to aggregation giving an import-incompetent state. The import-competent form interact with hTom20 via the presequence and an adjacent segment of the mature protein. Proteins which are in a more tightly folded conformation such as pOTC-GFP are imported more slowly. The pOTC-GFP interacts with the glutamine-rich segment and C-terminal segment of hTom20 only via its presequence.

### Fig. 7. Binding of precursors to GST-hTom20s.

A, a 35S-labeled reticulocyte lysate translation product was incubated with glutathione agarose prebound with about 0.56 nmol of GST or GST-fused hTom20s for 30 min at 25 °C. After washing, GST derivatives were eluted by adding 15 mM reduced glutathione, 40% of the eluted protein was subjected to 10% SDS-PAGE for fluorography using a FUJIX BAS2000 analyzer to detect labeled precursor. B, the radioactive preproteins eluted were quantitated by image plate analysis (percent binding). Values are represented as means ± S.E. of at least four independent experiments.
Acknowledgments—We thank J. Miyazaki (Osaka University, Japan) for pCAGGS, colleagues of this laboratory (Kumamoto University) for discussions, and M. Imoto for secretarial services.

REFERENCES
1. Pfanner, N., Craig, E. A., and Meijer, M. (1994) Trends Biochem. Sci. 19, 368–372
2. Lithgow, T., Glick, B. S., and Schatz, G. (1995) Trends Biochem. Sci. 20, 98–101
3. Lill, R., and Neupert, W. (1996) Trends Cell Biol. 6, 56–61
4. Schatz, G. (1996) J. Biol. Chem. 271, 31763–31766
5. Ramage, L., Junne, T., Hahne, K., Lithgow, T., and Schatz, G. (1993) EMBO J. 12, 4115–4123
6. Moczko, M., Ehmann, B., Gartner, F., Honlinger, A., Schafer, E., and Pfanner, N. (1994) J. Biol. Chem. 269, 9045–9051
7. Kiesler, M., Becker, K., Pfanner, N., and Neupert, W. (1993) J. Membr. Biol. 135, 191–207
8. Lithgow, T., Junne, T., Suda, K., Gratzner, S., and Schatz, G. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 11973–11977
9. Gratzner, S., Lithgow, T., Bauer, R. E., Lamping, E., Patlaufl, F., Kohlwein, S., D., Haucke, V., Junne, T., Schatz, G., and Horst, M. (1995) J. Cell Biol. 129, 25–34
10. Hines, V., Brandt, A., Griffiths, G., Horstmann, H., Brutsch, H., and Schatz, G. (1990) EMBO J. 9, 3191–3200
11. Sellner, T., Pfaller, R., Griffiths, G., Pfanner, N., and Neupert, W. (1990) Cell 62, 107–115
12. Haucke, V., Lithgow, T., Rospert, S., Hahne, K., and Schatz, G. (1995) J. Biol. Chem. 270, 5565–5570
13. Lithgow, T., and Schatz, G. (1995) J. Biol. Chem. 270, 14267–14269
14. Haucke, V., Horst, M., Schatz, G., and Lithgow, T. (1996) EMBO J. 15, 1231–1237
15. Seki, N., Moczko, M., Nagase, T., and Zufall, N. (1995) FEBS Lett. 375, 307–310
16. Goping, I. S., Millar, D. G., and Shore, G. C. (1995) FEBS Lett. 373, 45–50
17. Hanson, B., Nuttai, S., and Hoogenraad, N. (1996) Eur. J. Biochem. 235, 750–753
18. Terada, K., Kanazawa, M., Yano, M., Hanson, B., Hoogenraad, N., and Mori, M. (1997) FEBS Lett. 403, 309–312
19. Yano, M., Kanazawa, M., Terada, K., Namekari, C., Yamaizumi, M., Hanson, B., Hoogenraad, N., and Mori, M. (1997) J. Biol. Chem. 272, 8459–8465
20. Niwa, H., Yamamura, K., and Miyazaki, J. (1991) Gene (Amst.) 108, 193–199
21. Kanazawa, M., Terada, K., Kato, S., and Mori, M. (1997) J. Biochem. (Tokyo) 112, 890–895
22. Takeya, M., and Takahashi, K. (1988) Ultrastruct. Pathol. 12, 651–658
23. Schleiff, E., Shore, G. C., and Goping, I. S. (1997) J. Biol. Chem. 272, 17784–17789
24. Harkness, T. A., Nargang, F. E., Klei, I., Neupert, W., and Lill, R. (1994) J. Cell Biol. 124, 637–648
25. Iwahashi, J., Yamazaki, S., Komiya, T., Nomura, N., Nishikawa, S., Endo, T., and Mibaru, K. (1997) J. Biol. Chem. 272, 18467–18472
26. McFride, H. M., Goping, I. S., and Shore, G. C. (1996) J. Cell Biol. 134, 307–313
27. Vestweber, D., and Schatz, G. (1988) EMBO J. 7, 1147–1151
28. Hajek, P., Koh, J. Y., Jones, L., and Bedwell, D. M. (1997) Mol. Cell. Biol. 17, 7169–7177