Analysis of the Functional Domain of the Rat Liver Mitochondrial Import Receptor Tom20*

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Jun Iwashashi, Soh Yamazaki, Tohru Komiya, Nobuo Nomura‡, Shuh-ichi Nishikawa§, Toshiya Endo¶, and Katsuyoshi Mihara†

From the Department of Molecular Biology, Graduate School of Medical Science, Kyushu University, Fukuoka 812, Japan, the §Kazusa DNA Research Institute, Chiba 292, Japan, the ¶Department of Chemistry, Faculty of Science, Nagoya University, Chikusa-ku, Nagoya 464-01, Japan

Tom20 is an outer mitochondrial membrane protein and functions as a component of the import receptor complex for the cytoplasmically synthesized mitochondrial precursor proteins. It consists of the N-terminal membrane-anchor segment, the tetratricopeptide repeat (TPR) motif, a charged amino acids-rich linker segment between the membrane anchor and the TPR motif, and the C-terminal acidic amino acid cluster. To assess the functional significance of these segments in mammalian Tom20, we cloned rat Tom20 and 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. Tom20N69, a mutant consisting of the membrane anchor and the linker segments, was targeted to mitochondria and complemented the growth and import defects of Δtom20 yeast cells. whereas a mutant lacking the linker segment did not. In vitro protein import into mitochondria isolated from the complemented yeast cells revealed that the precursor targeted to yeast Tom70 was efficiently imported into the mitochondria via rat Tom20N69. Thus the linker segment is essential for the function of rat Tom20, whereas the TPR motif and the C-terminal acidic amino acids are not.

Protein import into mitochondria depends on the import receptors of the outer membrane. These components are Tom70, Tom22, and Tom20 in fungi and yeast, plus an additional component, Tom37, in yeast (1). In yeast, these receptors function as the Tom70-Tom37 and Tom20-Tom22 subcomplexes (1, 2). The precursors are targeted to Tom70/Tom37 through the action of an ATP-requiring cytoplasmic chaperone such as the mitochondrial import stimulation factor (MSF)‡ (2–5), transferred to Tom20/Tom22 ATP dependently, and then translocated across the outer membrane (4). Urea-denatured precursors or those which can assume unfolded conformations by themselves or by the action of hsp70 bypass Tom70-Tom37, bind to Tom20-Tom22, and are then imported into mitochondria independently of the cytoplasmic ATP (4, 5).

Relatively little is known about the import machinery of mammalian mitochondria. The 29-, 42-, and 52-kDa components of the rat liver outer mitochondrial membrane have been reported to be the components of the import machinery (6, 7). However, the function of these proteins remains unknown. The outer mitochondrial membrane proteins, OM37 in rats and metaxin in mice, have been shown to function as the components of the receptor for the precursor-MSF complex (herein referred to as the MSF-receptor; see Refs. 8 and 9). Recently, homologues of Tom20 in humans and an inner membrane component Tim17 in humans and Drosophila melanogaster have been identified and characterized (10–14).

In the present study, we have identified a rat homologue of Tom20 by functional assay, analyzed its role in targeting the precursor to mitochondria, and identified the domain of rat Tom20 responsible for the function of the import receptor. Rat Tom20 complemented both the growth and the mitochondrial import defects of Δtom20 yeast cells on a nonfermentable carbon source, which corresponded well with the correct targeting of the expressed rat Tom20 to yeast mitochondria. Taking advantage of this complementation, we analyzed the functional segment of rat Tom20 as the import receptor since Tom20 exhibits characteristic structural features that are shared by yeast, fungi, and mammals: an N-terminal hydrophobic segment, a putative tetratricopeptide repeat (TPR) motif, a charged amino acids-rich linker region between the hydrophobic segment and the TPR motif, and a cluster of negatively charged amino acid residues at the C terminus. The truncated cDNA coding for the N-terminal 69 amino acid residues (rat Tom20N69) containing the membrane anchor and the linker segments, but lacking the TPR motif and the acidic amino acid cluster, complemented the defects of both growth and mitochondrial import of Δtom20 yeast cells as efficiently as wild-type rat Tom20. Rat Tom20Δ25–69 and Tom20Δ2–18, the mutants lacking the linker domain and most of the transmembrane segment, respectively, did not complement these defects although they were expressed and localized to mitochondria in yeast cells. In vitro import experiments with the mitochondria isolated from rat Tom20N69-complemented yeast cells revealed that the precursor was targeted to yeast Tom70 MSF dependently, then transferred to rat Tom20N69, and finally imported into the mitochondria. These results indicate that the linker segment is essential, whereas the TPR, as well as the C-terminal acidic amino acids, is dispensable for the import receptor function of rat Tom20.

EXPERIMENTAL PROCEDURES

Materials—MSF and hsp70 were purified from rat liver cytosol according to Hachiya et al. (3) and Deshaies et al. (15), respectively.
Recombinant pAd was purified in 7 M urea as described (16). Antibodies against rat Tom20 were prepared by immunizing a rabbit with the recombinant protein that had been separated by SDS-PAGE. Antibodies against rat monoamine oxidase (MAO), yeast Bmh1p, yeast Kar2p, and yeast Tom70 were generous gifts from Akio Ito (Kyushu University), Norio Sakaguchi (Kyushu University), Masao Tokunaga (Keio University), and Gotfried Schatz (Basel Biocenter), respectively. Antibodies against rat liver microsomal cytochrome P450(M-1), rat liver cytoplasmic hemoprotein H450, and yeast mitochondrial porin were as described (17–19). Monoclonal antibody against hsp60 (SPA-807) was purchased from Stressgen Biotechnologies Corp.

Shedding of putative carbohydrate from the lipoprotein by Strep tag I—Saccharomyces cerevisiae is known to produce a 50-kDa protein with a molecular weight of 70,000 daltons. This protein is N-glycosylated, and the carbohydrate chain is cleaved by treatment with endo H (20). We also used this method for the detection of the carbohydrate chain of the recombinant rat Tom20.

Succinate-cytochrome c reductase was assayed as described (21). Yeast cell fractionation was carried out according to the method of Daum et al. (22), except that Zymolyase 100T was used to prepare the spheroplasts. Yeast lysates were prepared according to Yamazaki et al. (23).

RESULTS

Isolation of Rat Tom20 cDNA—A search of the EBI Data Bank revealed that a human cDNA with an open reading frame encoding a protein of 145 amino acid residues (DDBJ, accession number D13641) exhibited a significant homology to Tom20 encoding a protein of 145 amino acid residues (DDBJ, accession number D13641) as the substrate. pAd import into rat liver mitochondria was performed as follows. Rat liver mitochondria (100 µg) were treated with 100 µg of pre-immune IgGs or 50 µg of anti-rat Tom20 IgGs at 0 °C for 30 min in 50 µl of the import buffer, washed once with the import buffer, and subjected to the import using pAd-hsp70 or pAd-MSF-hsp70 as the substrate in which all of the components were labeled with 125I (20).

Other Methods—Succinate-cytochrome c reductase was assayed as described (21). Yeast cell fractionation was carried out according to the method of Daum et al. (22), except that Zymolyase 100T was used to prepare the spheroplasts. Yeast lysates were prepared according to Yamazaki et al. (23).
FIG. 2. Localization of rat Tom20 to the outer mitochondrial membrane. A, 100 μg each of mitochondrial, microsomal, and cytosolic proteins from rat liver were subjected to SDS-PAGE and immunoblotting using IgGs against rat Tom20, MAO, cytochrome P450(M-1), or hemoprotein H450. B, rat liver mitochondria were subjected to hypotonic treatment and then fractionated by sucrose density gradient centrifugation. Each fraction was assayed for the amounts of total protein, succinate-cytochrome c reductase, MAO, and rat Tom20.

Intracellular and Submitochondrial Localizations of Rat Tom20—Subfractionation of the rat liver indicated that rat Tom20 was cofractionated with a marker enzyme of the outer mitochondrial membrane, MAO, but not with cytochrome P450(M-1) or hemoprotein H450, the marker proteins of microsomes (27) and cytosol (18), respectively (Fig. 2A). When the mitochondria were subjected to hypotonic treatment followed by sucrose density gradient centrifugation, rat Tom20 co-sedimented with MAO at around fraction 5 but not with succinate-cytochrome c reductase, the marker enzyme of the inner membrane (Fig. 2B). Thus rat Tom20 is the protein of the outer mitochondrial membrane.

Effect of Anti-Tom20 IgGs on the Protein Import into Rat Liver Mitochondria—It has been reported in yeast mitochondria that the precursors complexed with MSF are docked onto Tom70-Tom37 first, then transferred to Tom20-Tom22, and finally translocated across the outer membrane (4). Urea-denatured precursors or precursors that are able to maintain the unfolded conformations by themselves or through the action of hsp70 bypass Tom70-Tom37 and are directly targeted to Tom20-Tom22. We examined this with rat liver mitochondria and assessed the function of rat Tom20 during the initial step of the precursor import. pAd, MSF, and hsp70 were125I-labeled and mixed to preform the pAd-hsp70 and pAd-MSF-hsp70 complexes, and then the import of pAd in these complexes into the antibody-treated mitochondria was examined (Fig. 3). Anti-rat Tom20 IgGs did not inhibit MSF-dependent binding of pAd but inhibited its import into the matrix (Fig. 3, lanes 5 and 6), whereas they inhibited both the binding and the import of pAd in the hsp70-dependent pathway (lanes 7 and 8). These results indicate that Tom20 functions at the junction of both import pathways. The pAd-MSF complex first docks onto the MSF receptor located upstream of Tom20, and pAd is then translocated into the mitochondria via Tom20, whereas pAd in the hsp70 complex binds directly to Tom20 and is then imported into the matrix. As reported previously, MSF was released to the supernatant in an ATP-dependent manner, whereas hsp70 was spontaneously released to the supernatant during this import reaction (lanes 2, 4, 6, and 8) (20). A significant amount of pAd remained bound to the antibody-treated mitochondrial fraction (lane 7). This is probably because a fraction of pAd bypassed Tom20 and was targeted to the unidentified component, such as the mammalian homologue of Tom22 since Tom22 has been reported to cooperate with Tom20 to function as an import receptor in N. crassa (28).

The Functional Domain of Rat Tom20 as Analyzed by Complementation of the Growth Defect of Δtom20 Yeast Cells—
Introduction of the vector carrying rat Tom20 cDNA (pD2R20) into the mutant cells complemented the growth defect of the cells on a nonfermentable carbon source although the growth rate was slightly slower than that of wild-type cells (Figs. 4A–C). Western blotting of the lysate of the complemented cells indicated that rat Tom20 was expressed in \( \text{D}_{\text{tom20}} \) yeast cells (Fig. 5) and that the expressed protein was co-fractionated with mitochondrial porin, but not with the proteins of microsomal or cytosolic fractions, indicating that it was correctly targeted to the mitochondria in yeast cells (Fig. 6). It should be noted that rat Tom20 recovered to yeast mitochondria was easily proteolysed to form a 15-kDa fragment. Since this fragment was resistant to alkali extraction, the processing seemed to occur at the C-terminal region of rat Tom20 in the cytoplasmic side.

We took advantage of this complementation to analyze the functional domain of rat Tom20 in \( \text{D}_{\text{tom20}} \) yeast cells. It is noted in this context that the importance of the TPR motif of yeast Tom20 in the physical interaction with Tom70 carrying seven TPR motifs has been reported (29). We constructed yeast expression vectors harboring cDNAs coding for 1–140 (pD2R20N140), 1–103 (pD2R20N103), and 1–69 (pD2R20N69) of rat Tom20 and the cDNA (pD2R20\( \Delta 25-69 \) or pD2R20\( \Delta 2-18 \)) in which residues 25–69 (the linker region) or 2–18 (~70% of the membrane-anchor segment) of Tom20 had been deleted, and we examined their ability to complement the defect of the respiration-dependent growth of \( \text{D}_{\text{tom20}} \) yeast cells.

**FIG. 4.** Complementation of the defect of respiration-dependent growth of \( \text{D}_{\text{tom20}} \) yeast cells by rat Tom20 proteins. A, wild-type yeast cells transformed with pD2 or \( \Delta \text{tom20} \) yeast cells transformed with pD2, pD2R20, pD2R20N140, pD2R20N103, or pD2R20N69 were streaked onto synthetic medium plates containing 2% glucose or 3% glycerol, and the plates were incubated at 30 °C for 2 or 4 days, respectively. B, \( \Delta \text{tom20} \) yeast cells harboring pD2R20, pD2R20\( \Delta 25-69 \), or pD2R20\( \Delta 2-18 \) were grown on a glucose-containing or glycerol-containing plate as described in panel A. C, yeast cells grown overnight at 30 °C in glucose-containing medium were diluted with 20–50 volumes of glycerol-containing medium and cultured at 30 °C. Cell growth was monitored by measuring the absorbance at 600 nm.

**FIG. 5.** Expression of full-length and mutant rat Tom20 proteins in yeast cells as detected by Western blotting. Yeast cell lysates were subjected to SDS-PAGE, and full-length and truncated rat Tom20 proteins were detected by immunoblotting as described in Fig. 2A. Left and right panels show the results of different experiments. The amounts of the lysates used were from 0.5 and 0.8 \( A_{600} \) units of cells, respectively, and the time of exposure was 5 and 60 min, respectively. The position of rat Tom20N69 is indicated by an arrow in the left panel.

**FIG. 6.** Subcellular localization of rat Tom20 proteins expressed in \( \text{D}_{\text{tom20}} \) yeast cells. A, \( \Delta \text{tom20} \) yeast cells harboring pD2R20 were fractionated into mitochondria, microsomes, and cytosol. 100-μg fractions were subjected to SDS-PAGE and immunoblot analysis with the IgGs against porin, Kar2p, or Bmh1p to assess mitochondria, microsomes, and cytosol, respectively. Note that anti-Bmh1p IgGs cross-react with Bmh2p in the cytosol. B, \( \Delta \text{tom20} \) cells harboring pD2R20 or pD2R20\( \Delta 25-69 \) were fractionated into mitochondria, microsomes, and the cytosol fractions, and each fraction was subjected to immunoblot analysis with anti-Tom20 IgGs.
Summary of experiments on Δtom20 yeast cells expressing rat Tom20 constructs

The N-terminal transmembrane segment (TM), the putative tetratricopeptide repeat (TPR) motif, and the C-terminal acidic amino acid cluster in rat Tom20 are indicated. The amount of the expressed proteins was determined by scanning the X-ray films in Western blotting analysis using a dual-wavelength scanner (Shimadzu CS-930).

| Rat Tom20 constructs expressed in Δtom20 yeast cells | Respiration-dependent growth | Expression of Tom20s | Localization of Tom20s | Recovery of mitochondrial import of hsp60 | In vitro import of pAd |
|------------------------------------------------------|-----------------------------|---------------------|------------------------|----------------------------------------|-----------------------|
| Wild type (145 amino acids)                          | +                           | 100                 | Mitochondria           | +                                      | +                     |
| N140                                                 | +                           | 97                  | ND*                    | +                                      | +                     |
| N103                                                 | +                           | 74                  | ND                     | +                                      | +                     |
| N69                                                  | +                           | 1.4                 | Mitochondria           | +                                      | +                     |
| Δ25–69                                               | –                           | 4.7                 | Mitochondria           | ND                                     |                       |
| Δ2–18                                                | –                           | 31                  | Mitochondria           | ND                                     | ND                    |

* ND, not determined.

FIG. 7. Effect of the expression of wild type and mutant forms of rat Tom20 upon mitochondrial import of pre-hsp60 in Δtom20 yeast cells. Δtom20 cells expressing wild type and mutant rat Tom20 proteins were grown at 30 °C overnight in glucose-containing medium. The yeast cell lysates were prepared and were subjected to SDS-PAGE followed by Western blotting with the monoclonal antibodies against hsp60. The positions of pre-hsp60 (p) and hsp60 (m) are indicated in the figure.

Δtom20 cells harboring plasmids for wild type or C-terminal-truncated rat Tom20 proteins were able to grow on the glycerol-containing plate although their growth was slightly slower than that of wild-type cells (Fig. 4A). In contrast, Δtom20 cells transformed with pD2R20Δ25–69 or pD2R20Δ2–18 could not grow on the glycerol-containing medium (Fig. 4, B and C).

Δtom20 cells harboring these plasmids expressed Tom20 proteins with the expected molecular sizes although the extent of expression differed between them (Fig. 5). It is worth noting that the expression of Tom20N69 and Tom20Δ25–69 was significantly lower than that of wild type or other mutant Tom20 proteins and was probably due to their instability in yeast cells, although the expression of Tom20Δ25–69 was ~3-fold higher than that of Tom20N69 (Table I). Cell fractionation indicated that Tom20N69 (not shown) and Tom20Δ25–69 were both targeted to mitochondria (Fig. 6B). Nevertheless, they were distinct in their complementation of the growth defect of Δtom20 cells: Tom20N69 complemented the defect, whereas Tom20Δ25–69 did not. The membrane-anchor mutant Tom20Δ2–18 was expressed at about 30% of the level of wild-type Tom20 but was unable to complement the growth defect of Δtom20 yeast cells (Table I). Taken together, these results suggest that the linker domain and the membrane anchor-segment are essential for the function of rat Tom20, whereas the TPR motif as well as the C-terminal acidic amino acid cluster are not.

Tom20Δ25–69 Is Unable to Complement the Defect of Mitochondrial Protein Import in Δtom20 Yeast Cells—Western bloting with monoclonal anti-hsp60 antibody revealed a significant accumulation of pre-hsp60 in Δtom20 cells harboring pD2 (Fig. 7). This import deficiency was complemented by the expression of wild-type rat Tom20 or rat Tom20N69. No accumulation of pre-hsp60 was observed in Δtom20 cells expressing rat Tom20N140 or Tom20N103 (data not shown). In marked contrast, pD2R20Δ25–69 was unable to complement the defect of mitochondrial import of pre-hsp60 in Δtom20 cells (Fig. 7). Thus the TPR motif and the C-terminal acidic amino acid cluster are dispensable for the complementation of the defect of mitochondrial protein import in Δtom20 yeast cells.

FIG. 8. Effect of IgGs against rat Tom20 or yeast Tom70 on the in vitro import of pAd into mitochondria isolated from Δtom20 yeast cells expressing rat Tom20N69. Mitochondria isolated from Δtom20 yeast cells expressing rat Tom20N69 were incubated with or without preimmune IgGs or IgGs against yeast Tom70 or rat Tom20, was incubated with or subjected to the import reaction using 125I-pAd-MSF complex or 125I-pAd-hsp70 complex as the substrate. The reaction mixtures were treated with 100 μg/ml proteinase K at 0 °C for 30 min and analyzed by SDS-PAGE and autoradiography. Other conditions are described under “Experimental Procedures.” Percent inhibitions of MSF-dependent import were as follows: α-rat Tom70, 72% and α-rat Tom20, 91%. Percent inhibitions of hsp70-dependent import were as follows: α-yeast Tom70, 0% and α-rat Tom20, 92%.

Rat Tom20N69 in Δtom20 Yeast Mitochondria Functions Normally as the Precursor Receptor in Vitro—To further confirm the results obtained above, we performed an in vitro import assay using mitochondria isolated from Δtom20 cells expressing rat Tom20N69. 125I-labeled pAd was incubated with MSF or hsp70 to preform the complexes, and the import of pAd in the complexes into mitochondria was examined. As shown in Fig. 8, pAd was actively imported into the mitochondria MSF dependently, and this import was inhibited by IgGs against...
yeast Tom70 or rat Tom20 (Fig. 8, top panel). In marked contrast, the hsp70-dependent import was inhibited only by IgGs against rat Tom20 but not by those against yeast Tom70 (Fig. 8, bottom panel). These results clearly indicate that rat Tom20N69 in the complemented yeast mitochondria functions normally as the import receptor and that pAD docked onto yeast Tom70 is transferred to rat Tom20N69 and then imported into the mitochondria.

**DISCUSSION**

In this paper, we have characterized the function of rat Tom20 in precursor targeting to and import into the mitochondria in vitro and identified the region responsible for its function as the import receptor both in vivo and in vitro (summarized in Table I).

The antibodies against rat Tom20 strongly inhibited hsp70-dependent binding as well as the import of the precursor into rat liver mitochondria. In contrast, they did not interfere with the MSF-dependent binding of the precursor to the mitochondria but did inhibit its import into mitochondria. These results are consistent with our previous results showing that the precursors that can maintain the unfolded conformations by themselves or by complexing with hsp70 are directly targeted to Tom20, that the precursors that are complexed with MSF first dock at the MSF receptor located upstream of Tom20, and that the ATP-hydrolysis induced transport of the precursors via Tom20 (20). Similar results have been reported with yeast mitochondria (4). Thus, the essential part of the import apparatus seems to be conserved among species.

Taking advantage of rat Tom20-induced suppression of the growth defect of Δtom20 cells on a nonfermentable carbon source, we analyzed the functional segment of rat Tom20. The respiration defect of Δtom20 cells and their adaptation within days to the loss of Tom20 have been reported to be correlated with the loss of Tom22 and with its restoration, respectively (30, 31). However, Δtom20 cells used in the present study grew normally in the glucose-containing medium and maintained their respiratory deficiency throughout the experiments. Furthermore, Western blotting revealed that yeast Tom20 was absent, and the amount of Tom22 did not alter to any appreciable extent in Δtom20 cells expressing rat Tom20 mutants (data not shown). Thus, Tom22 is not the limiting factor for the defects of Δtom20 yeast cells under the present experimental conditions. Unexpectedly, Tom20N69, which contains both the membrane anchor and the linker segments but lacks the TPR domain for this function, whereas the linker domain is dispensable for this function, whereas the linker domain is not. In support of this notion, only the membrane anchor and the downstream linker segment of Tom20 exhibit pronounced sequence conservation among species. Since Tom20 has been reported to bind precursor proteins through electrostatic interactions with the positively charged precursors in _S. cerevisiae_ and _N. crassa_ (28, 32), we speculate that the charged amino acid-rich linker segment (K/R = 17 residues and D/E = 8 residues in a segment of 45 residues) is important for the recognition of the presence of the precursors.

Haucke et al. (29) have shown that the TPR motif of yeast Tom20 increases interaction of Tom20 with the Tom70Tom37 complex, but its mutation does not inactivate the receptor function of Tom20. However, we could not detect the requirement of the corresponding segment of rat Tom20 in the precursor import process either in vivo or in vitro. A possible explanation for this difference could be that the decreased interaction between Tom70 and Tom20 caused by the TPR-deletion of Tom20 was suppressed by the overexpression of the Tom20 mutants. Another possibility is that different regions of rat and yeast Tom20s are responsible for the interaction with Tom70 since human Tom20 has been reported to lack homology to the A-domain of the typical TPR motif and shows only weak homology to the core motif of the TPR B-domain (10). Analyses of precursor-receptor interactions or precursor transfer between the receptors using the cytoplasmic domains of Tom20, Tom70, or their mutants are required to clarify these results as well as to clarify the mechanisms by which the mitochondria-targeting signals are correctly recognized and translocated across the outer mitochondrial membrane.

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