Precise targeting of mitochondrial precursor proteins to mitochondria requires receptor functions of Tom20, Tom22, and Tom70 on the mitochondrial surface. Tom20 is a major import receptor that recognizes preferentially mitochondrial presequences, and Tom70 is a specialized receptor that recognizes presequence-less inner membrane proteins. The cytosolic domain of Tom22 appears to function as a receptor in cooperation with Tom20, but how its substrate specificity differs from that of Tom20 remains unclear. To reveal possible differences in substrate specificities between Tom20 and Tom22, if any, we deleted the receptor domain of Tom20 or Tom22 in mitochondria in vitro by introducing cleavage sites for a tobacco etch virus protease between the receptor domains and transmembrane segments of Tom20 and Tom22. Then mitochondria without the receptor domain of Tom20 or Tom22 were analyzed for their abilities to import various mitochondrial precursor proteins targeted to different mitochondrial subcompartments in vitro. The effects of deletion of the receptor domains on the import of different mitochondrial proteins for different import pathways were quite similar between Tom20 and Tom22. Therefore Tom20 and Tom22 are apparently involved in the same step or sequential steps along the same pathway of targeting signal recognition in import.

Nuclear encoded mitochondrial proteins are synthesized in the cytosol and are imported into mitochondria with the aid of protein translocator complexes in the outer and inner mitochondrial membranes for sorting to one of four compartments: the outer membrane, intermembrane space (IMS),

inner membrane, and matrix (1–4). Most matrix proteins and some inner membrane proteins are synthesized as precursor proteins with an N-terminal presequence, which contains a mitochondrial targeting signal and is cleaved off by the matrix-processing peptidase in the matrix upon import (5–7). Presequences are rich in positively charged residues and have the ability to form an amphiphilic helical structure. On the other hand, polytopic inner membrane proteins, soluble IMS proteins, and outer membrane proteins are mainly synthesized without a presequence, but contain internal targeting signals within mature parts (5–7).

The outer membrane translocator, the TOM40 complex, functions as an entry gate for most mitochondrial proteins. The TOM40 complex contains three receptor subunits, Tom20, Tom22, and Tom70, which recognize mitochondrial targeting signals (5–8). Tom20 is anchored to the outer membrane by its N-terminal transmembrane segment and exposes its C-terminal receptor domain to the cytosol (9–11). Tom20 is the major import receptor that preferentially recognizes presequence-containing proteins. The NMR structure of the receptor domain of rat Tom20 in a complex with a presequence peptide showed that Tom20 has a hydrophobic groove on the surface, to which the hydrophobic side of the amphiphilic helix of the presequence binds (12). Further NMR analyses revealed a weak consensus motif in the presequences for Tom20 recognition as well as a mechanism to accept a wide variety of presequences as substrates (13–15).

Tom70 is anchored to the outer membrane by its N-terminal transmembrane segment and exposes its C-terminal receptor domain to the cytosol as well (16–18). Tom70 preferentially recognizes presequence-less inner membrane proteins and functions as a docking site for cytosolic chaperone Hsp70 (19), although some substrate cross-specificities between Tom20 and Tom70 were reported (17, 18, 20, 21).

Tom22 is anchored to the outer membrane by its hydrophobic segment in the middle of the sequence, exposing the N-terminal and C-terminal domains to the cytosol and IMS, respect-
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tively (22–24). Evidence has been accumulated that the N-terminal and C-terminal domains of Tom22, which are rich in acidic residues, constitute binding sites for positively charged presequences, with Tom20 on the cytosolic side and with Tom40 and Tom7 on the IMS sides of the outer membrane (21, 25–30). Nevertheless, the difference in the substrate specificity of the cytosolic domain of Tom22 from that of Tom20 has remained elusive for the following reasons. First, Tom22, especially the central transmembrane segment, is essential for yeast cell growth under most culturing conditions, rendering it difficult to deplete Tom22 without abrogating functional integrity of the TOM40 complex (31). Second, genetic deletion of Tom20 in turn accompanies a decrease in the amount of Tom22, so the effects of the defective receptor functions of Tom20 and Tom22 on protein import cannot be analyzed separately (22). Third, because the isolated cytosolic domain of Tom22 hardly takes an ordered structure, but still binds to presequences, analyses of presequence binding to the isolated receptor domain of Tom22 may not be reliable.

To overcome those experimental difficulties in assessing the substrate specificities of Tom20 and Tom22, we set up a system to delete the receptor domain of Tom20 or Tom22 with minimal effects on the other subunits of the TOM40 complex after isolation of mitochondria. Briefly, we constructed genes for Tom20<sup>TEV</sup> and Tom22<sup>TEV</sup> that contain a tobacco etch virus (TEV) protease cleavage sequence between the cytosolic receptor domains and transmembrane segments (32). Then mitochondria were isolated from the yeast cells expressing Tom20<sup>TEV</sup> or Tom22<sup>TEV</sup> from the plasmid instead of wild-type Tom20 or Tom22 from the genome DNA and were treated with TEV protease to remove the receptor domain of Tom20 or Tom22 <em>in vitro</em>. The obtained mitochondria without the receptor domain of Tom20 or Tom22 were analyzed for their abilities to import various mitochondrial precursor proteins targeted to different mitochondrial subcompartments <em>in vitro</em>. We found that the effects of deletion of the receptor domains on the import of different mitochondrial proteins are quite similar between Tom20 and Tom22. Therefore Tom20 and Tom22 are involved in the same step or sequential steps along the same pathway for targeting signal recognition.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The TOM20 gene containing its own promoter and terminator was amplified by PCR using primers 5'-CAA GAC TCG AGC AGA TCT TGC ATT C-3' and 5'-GCA TGA GCT CAT TTA GGC TAG AGA AAT G-3'. The amplified DNA fragment was digested with XhoI and SacI and introduced into pRS314 to generate pRS314/TOM20<sup>WT</sup>. Construction of pRS314/TOM22<sup>WT</sup> was reported previously (33).

The plasmids containing genes for Tom20<sup>TEV</sup> and Tom22<sup>TEV</sup> were constructed as follows. The TEV protease recognition site (ENLYFQS) was inserted between residues 73 and 74 of Tom20 and between residues 74 and 75 of Tom22 by oligonucleotide-directed mutagenesis using primers 5'-GGT TAC CGA ATT CTT AGA AAA CTT GTA CTT TCA ATC CAT GGA ATT AGC C-3' and 5'-CCC CCC AGG TAA GAG to delete the receptor domain of Tom20 or Tom22 with mini-

<sup>6</sup>T. Shodai and T. Endo, unpublished observations.
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NaCl, and 200 mM imidazole). The eluted fraction was diluted with 6 M urea buffer to 1 mg protein/ml and subjected to refolding by gel filtration with a Sephadex G-25 column equilibrated with refolding buffer (90 mM Tris-HCl, pH 7.5, 1.8 mM EDTA, 9 mM dithiothreitol, and 10% (v/v) glycerol). Refolded TEV protease was collected; concentrated to 1 mg/ml in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM dithiothreitol, and 50% glycerol; and stored at −80 °C.

TEV Protease Treatment—100 μg of protein from isolated mitochondria were incubated with or without 20 μg of purified TEV protease in 200 μl of TEV buffer (250 mM sucrose, 20 mM MOPS-KOH, pH 7.2, and 0.1 mg/ml bovine serum albumin) at 30 °C for 20 min.

RESULTS

Deletion of the Receptor Domains of Tom20 and Tom22 by TEV Protease Treatment in Vitro—To establish a system in which only the cytosolic receptor domain of Tom20 or Tom22 can be deleted in vitro, we introduced a cleavage site for TEV protease between the receptor domain and transmembrane segment of Tom20 or Tom22 at the DNA level (Fig. 1A). The modified proteins, Tom20TEV and Tom22TEV, are fully functional because replacement of endogenous Tom20 or Tom22 did not affect cell growth even at 37 °C (data not shown).

We isolated 20T or 22T mitochondria and incubated them with TEV protease for 20 min at 30 °C. SDS-PAGE followed by immunoblotting with anti-Tom20 or anti-Tom22 antibodies showed that the receptor domain of Tom20TEV or Tom22TEV was completely deleted in 20T or 22T mitochondria, respectively, whereas the amounts of Tom20TEV and Tom22TEV in 22T before TEV protease treatment were nearly the same as those of Tom20 and Tom22 in wild-type mitochondria (20W and 22W) (Fig. 1B). TEV protease treatment did not affect the level of Tom22 in 20T mitochondria or Tom20 in 22T mitochondria, either. We also analyzed the levels of other subunits of the translocator complexes (Tom40 and Tom70 in the TOM40 complex; Tim22 in the TIM22 complex; Tim23 in the TIM23 complex; and Tob55, Mas37, and Tom38 in the TOB/SAM complex) to confirm that they did not change upon TEV protease treatment.

We then solubilized wild-type (20W and 22W) mitochondria and 20T and 22T mitochondria before or after TEV protease treatment with 1% digitonin and analyzed the complex structures of the TOM40 complex by BN-PAGE. The wild-type TOM40 core complex consisting of Tom40, Tom22, Tom5, Tom6, and Tom7 migrates as an ~400-kDa form on BN-polyacrylamide gels (Fig. 1C, 20W and 22W). The intact assembly structure of the TOM40 complex was not significantly affected by deletion of the receptor domain of Tom20 (20T) or Tom22 (22T) by TEV protease treatment, although deletion of the acidic receptor domain of Tom22 by TEV protease treatment reduced the apparent molecular mass to ~300–350 kDa (Fig. 1C, 20T and 22T). Solubilized wild-type (22W) mitochondria and 22T mitochondria before or after TEV protease treatment with 1% digitonin were also analyzed by glycerol density gradient centrifugation, and similar results were obtained (Fig. 1D). Although the TOM40 complex solubilized from 22T mitochondria without TEV protease treatment migrated slightly faster than the one from WT mitochondria on the BN-polyacrylamide gel (Fig. 1C), no significant difference in the apparent sizes of the TOM40 complex between 22T mitochondria without TEV treatment and WT mitochondria was observed by glycerol density gradient centrifugation (Fig. 1D).

Deletion of the Receptor Domains of Tom20 and Tom22 Impairs Mitochondrial Protein Import in a Similar Manner—To analyze the effects of deletion of the receptor domains of Tom20 and Tom22 on mitochondrial protein import, we prepared mitochondria specifically lacking the receptor domain of Tom20 or Tom22 by in vitro TEV protease treatment of 20T or 22T mitochondria, respectively. Then we performed in vitro protein import assays with reduced amounts of those mitochondria so that binding of mitochondrial precursor proteins to the mitochondrial surface became rate-limiting for the overall import process (36).

To reveal the receptor specificity of Tom20 and Tom22, we performed in vitro import of various radiolabeled mitochondrial precursor proteins into 20T and 22T mitochondria. First, we tested presequence-containing matrix-targeted precursor proteins for their import into mitochondria without the receptor domain of Tom20 or Tom22. The presequence of subunit 9 of F$_1$-ATPase fused to mouse dihydrofolate reductase (pSu9-DHFR), the precursor to mitochondrial Hsp60 (pHsp60), and the precursor to the β-subunit of F$_1$-ATPase (pF$_1$β) were synthesized with reticulocyte lysate and subjected to import into 20T and 22T mitochondria. Deletion of the receptor domains of Tom20 and Tom22 significantly impaired the import of pSu9-DHFR, pHsp60, and pF$_1$β (Fig. 2), suggesting that both Tom20 and Tom22 function as receptors for these precursor proteins, as expected. When we compared the effects of removal of the receptor domains of Tom20 and Tom22, the reduction of the import rates (60–90%) appears similar for the receptor domain deletion of Tom20 and Tom22.

Next, we analyzed the in vitro import of presequence-less inner membrane proteins ADP/ATP carrier (AAC), Tim17, and Tim23. These presequence-less polytopic inner membrane proteins are known to use the TIM22 complex to be inserted into the inner membrane. AAC and Tim23 were also shown to use another outer membrane receptor, Tom70, for targeting to mitochondria (16–18, 37), although in the absence of Tom70, AAC import becomes Tom20-dependent as well (18). Deletion of the receptor domains of Tom20 and Tom22 moderately decreased the import rates of these proteins (by 20–30%) (Fig. 3). Therefore even in the presence of Tom70, Tom20 and Tom22, in addition to Tom70, are involved in recognition of internal targeting signals of these presequence-less inner membrane proteins.

Then we tested soluble IMS proteins Tim9, Tim13, and cytochrome c for their import into mitochondria without the receptor domain of Tom20 or Tom22. It was reported that the import of Tim13 (38) and cytochrome c (39) was not affected by trypsin treatment of mitochondria, although deletion of the entire Tom22 molecule moderately impaired the import of cytochrome c in vitro (40). Here we found that even after specific removal of the receptor domain of Tom20 or Tom22 by TEV protease treatment, import of these proteins into mito-
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chondria was not affected at all (Fig. 4). Therefore the receptor domain of Tom20 or Tom22 does not recognize mitochondrial targeting signals of those presequence-less soluble IMS proteins.

We also tested outer membrane proteins porin, Tom40, and rat peripheral benzodiazepine receptor (PBR) for their import into mitochondria without the receptor domain of Tom20 or

FIGURE 1. Introduction of the cleavage site for TEV protease in Tom20 and Tom22. A, the strategy is shown for deletion of the receptor domain of Tom20 or Tom22. Chromosomal deletion of the TOM20 or TOM22 gene is complemented by the plasmid-borne gene for Tom20 (20W), Tom22 (22W), Tom20$_{TEV}$ (20T), or Tom22$_{TEV}$ (22T). B, 20W, 20T, 22W, and 22T mitochondria were isolated from the corresponding yeast cells, and with (+) or without (−) TEV protease treatment (20 min, 30 °C) followed by re-isolation, proteins were analyzed by SDS-PAGE followed by immunoblotting with the indicated antibodies. C, 20W, 20T, 22W, and 22T mitochondria were, with or without TEV protease treatment, solubilized with 1% digitonin, 25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, and 50 mM 6-aminohexanoic acid and subjected to BN-PAGE followed by immunoblotting with anti-Tom40 antibodies. D, 22W and 22T mitochondria were treated with or without TEV protease, subsequently solubilized with 1% digitonin as in C, and subjected to glycerol density gradient centrifugation (10–40% glycerol and 0.3% digitonin in the solubilization buffer) at 200,000 × g for 12 h at 4 °C. After centrifugation, fractions were collected from the top, and proteins were analyzed by SDS-PAGE and immunoblotting with antibodies against Tom40, Tom22, and Tim23. N.D., not detected.
Tom22. Porin and Tom40 are β-barrel proteins in the outer membrane, whereas PBR is a polytopic outer membrane protein with five transmembrane segments. Previous studies showed that integration of porin into the outer membrane (41) and assembly of Tom40 into the TOB/SAM complex (42) require both Tom20 and Tom22. On the other hand, import of PBR into mammalian mitochondria was found to depend on Tom70, but not on Tom20, Tom22, or even Tom40 (43). In vitro import of porin into mitochondria without the receptor domain of Tom20 or Tom22 was significantly retarded as compared with control mitochondria with intact Tom20 and Tom22 (Fig. 5A). The assembly process of imported Tom40 into the endogenous TOM40 complex can be followed by BN-PAGE after solubilization of mitochondria. Tom40 imported into wild-type control mitochondria (20T or 22T mitochondria without TEV protease treatment) was first associated with the TOB/SAM complex to exhibit the 250-kDa assembly II intermediate (Fig. 5B, upper panels). Prolonged incubation allowed imported Tom40 to shift to the 450-kDa final TOM40 complex (data not shown). Removal of the receptor domain of Tom20 or Tom22 by TEV protease treat-
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Deletion of the Tom22 Receptor Domain Impairs Assembly of Tom40 into the TOM40 Complex, but Not Its Insertion into the Outer Membrane—Next, we analyzed the effects of deletion of the receptor domain of Tom20 or Tom22 on the assembly of imported protein, a proteinase K-resistant fragment (f) was quantified as an assembled species. The amounts of species assembled into the outer membrane (assemblies I and II for Tom40) without TEV protease treatment after a 15-, 16-, and 30-min incubation were set to 100% for Tom40, PBR, and TEV protease, respectively. Open and filled circles indicate import into TEV protease-untreated and -treated mitochondria, respectively.

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Tom20 into the TOM40 complex. Tom20 is a peripheral subunit of the TOM40 complex and is associated with the TOM40 holocomplex only after a mild solubilization process (e.g. with 0.4% digitonin). Radiolabeled Tom20 was incubated with 20T or 22T mitochondria with or without TEV protease treatment. Subsequent BN-PAGE analyses of 20T mitochondria solubilized with 0.4% digitonin showed that assembly of Tom20 into the TOM40 complex was not affected by deletion of the receptor domain of Tom20, which is consistent with a previous observation (44). On the other hand, assembly of Tom20 into the TOM40 complex in vitro significantly depended on the receptor domain of Tom22. First, even without treatment with TEV protease, replacement of wild-type Tom22 with Tom22\textsuperscript{TEV} decreased the assembly efficiency of Tom20 into the TOM40 complex in vitro (Fig. 6A). However, this effect does not appear serious in vivo because 22T mitochondria without TEV protease treatment could import mitochondrial precursor proteins as efficiently as 22W mitochondria did (data not shown). After TEV protease treatment of 22T mitochondria, the assembly efficiency of Tom20 into the TOM40 complex was further decreased significantly as compared with the 22T mitochondria before TEV protease treatment (Fig. 6A).

To determine whether the receptor domain of Tom22 catalyzes the assembly of Tom20 into the TOM40 complex or stabilizes the association of Tom20 with the TOM40 complex, we performed in vitro import of Tom20 into 22T mitochondria without cleavage by TEV protease and subsequently subjected the mitochondria to incubation with TEV protease. Now Tom20 assembled in the TOM40 complex was completely gone after digestion with TEV protease (Fig. 6B). Therefore the receptor domain of Tom22 is essential for Tom20 to stay bound to the TOM40 core complex. In contrast, membrane insertion as monitored by alkaline extraction of Tom20 after incubation with 20T and 22T mitochondria was not affected by removal of the receptor domain of Tom20 or Tom22 by TEV protease (Fig. 6C). Therefore, whereas insertion of Tom20 into the outer membrane does not require Tom20 or Tom22, the Tom22 receptor domain is essential to stabilize the assembly of Tom20 into the TOM40 complex.

DISCUSSION

Because trypsin treatment of mitochondria, which removes the surface-exposed parts of the receptor subunits of the TOM40 complex, significantly impairs protein import, the outer membrane receptor subunits are collectively important for mitochondrial protein entry, although trypsin-treated mitochondria still retain a residual import ability via the “bypass import” pathway (8). However, the differences or similarities in substrate specificities between Tom20 and Tom22 have not been systematically analyzed. In the present study, we developed a system to delete the cytosolic receptor domain of Tom20 or Tom22 with minimal effects on the other components of the TOM40 complex after isolation of mitochondria, and we analyzed the specific effects of deletion of the Tom20 or Tom22 receptor domain on mitochondrial protein import. The results were quantified and summarized in Fig. 7. Evidently, many sequence-less proteins, including substrates for another receptor, Tom70, such as polytopic inner membrane proteins, are

FIGURE 6. In vitro import and assembly of Tom20 into mitochondria without the receptor domain of Tom20 or Tom22. A, radiolabeled Tom20 was incubated with TEV protease-untreated (open circles) or TEV protease-treated (filled circles) 20T and 22T mitochondria (0.25 mg of protein/ml) at 25 °C for the indicated times. The mitochondria were re-isolated, solubilized with 0.4% digitonin, and subjected to BN-PAGE analyses. Imported Tom20 was assembled into the TOM40 complex, and the amounts of assembled Tom20 were quantified. The amounts of the assembled species in TEV-untreated mitochondria after a 12-min incubation were set to 100%. B, radiolabeled Tom20 was incubated with TEV protease-untreated 22T mitochondria at 25 °C for 12 min. The mitochondria were re-isolated by centrifugation and subjected to treatment with or without TEV protease. The mitochondria were solubilized with 0.4% digitonin and subjected to BN-PAGE followed by radioimaging. C, radiolabeled Tom20 was incubated with the indicated concentrations of TEV protease-untreated (white bars) or TEV protease-treated (black bars) 20T or 22T mitochondria (Mito.) at 25 °C for 3 min. The mitochondria were re-isolated and subjected to alkaline treatment with 0.1 M Na2CO3, pH 11.2, and pellets and supernatants were separated by centrifugation (100,000 × g for 20 min at 4 °C). Proteins in the pellet fractions were analyzed by SDS-PAGE and radioimaging. Total amounts of added Tom20 were set to 100%.
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FIGURE 7. Efficiencies of in vitro import of various mitochondrial precursor proteins into 20W, 20T, 22W, and 22T mitochondria after TEV protease treatment. Import efficiencies are the amounts of imported proteins after the longest incubation times for each import reaction assay. Import efficiencies for mitochondria without TEV protease treatment were set to 100%. Cyt. c, apocytochrome c.

FIGURE 8. Models of receptor functions in mitochondrial protein import. Hexagons with short arrows represent the steps of recognition by the receptor domains of Tom20, Tom22, and Tom70. Gray arrows indicate protein flux into mitochondria. 5, 6, 7, 22, and 40 for the TOM40 channel represent Tom5, Tom6, Tom7, Tom22, and Tom40, respectively, in the TOM40 core complex constituting a protein conducting channel.

recognized by Tom20 and Tom22. This suggests that Tom20, together with Tom22, is not a mere receptor for a class of mitochondrial proteins, but a master receptor for most mitochondrial proteins.

More interestingly, reduction levels in import efficiencies by deletion of the receptor domains of Tom20 and Tom22 are quite similar. This suggests that the receptor domains of Tom20 and Tom22 are involved in the same pathway for mitochondrial targeting signal recognition (Fig. 8). In addition, because this observation holds for the substrates for Tom70 as well, the currently proposed model (45) that substrates for Tom70 recognition are transferred to Tom22, but not Tom20, to receive recognition by only Tom22 (Fig. 8B) is questionable. Rather, the present results suggest that recognition substrates for Tom70 may well be passed on to Tom20 for recognition by both Tom20 and Tom22 (Fig. 8A).

Are the receptor domains of Tom20 and Tom22 involved in the same step or sequential steps along the same recognition pathway? A previous study showed that the recognition step involving Tom22 is not earlier than that involving Tom20 (21). Because the cytosolic domain of Tom22 is rich in acidic residues, it may well recognize the basic side of the amphiphilic helix formed by presequences through ionic interactions. Therefore Tom20 and Tom22 may recognize opposite sides of the same presequence in the same step, with Tom20 recognizing the hydrophobic surface and Tom22 the hydrophilic surface (25, 46). This interpretation is supported by the recent finding that in Saccharomyces castellii Tom22 has lost an acidic region, whereas Tom20 gained one during evolution, so Tom20 and Tom22 apparently contribute their domains to a single, composite receptor (47). Alternatively, presequences bound to Tom20 through hydrophobic interactions may be cleared from Tom20 by stronger ionic interactions with the acidic receptor domain of Tom22. It is also possible that Tom22 is not directly involved in the recognition of mitochondrial targeting signals, but instead optimizes the receptor function of Tom20 by, for example, physically linking Tom20 to the TOM40 core complex. This interpretation is consistent with the observation that imported Tom20 was not assembled into the TOM40 complex in the absence of the Tom22 cytosolic domain (Fig. 6B). Further information on the high-resolution structure of the cytosolic domain of Tom22 and its interactions with the receptor domains of Tom20 will be important to discriminate those possibilities.

Acknowledgments—We thank Dr. Katsuyoshi Mihara (Kyushu University) for the gene for PBR, Dr. Steve Gould for the expression plasmid for TEV protease, and members of the Endo laboratory for discussion and comments.

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