Targeting and Assembly of Mitochondrial Tail-anchored Protein Tom5 to the TOM Complex Depend on a Signal Distinct from That of Tail-anchored Proteins Dispersed in the Membrane*

Chika Horie, Hiroyuki Suzuki, Masao Sakaguchi, and Katsuyoshi Mihara‡

From the Department of Molecular Biology, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan

Mitochondrial outer membrane proteins are synthesized without a cleavable presequence but instead contain segments responsible for mitochondrial targeting and membrane integration within the molecule: the transmembrane segment (TMS) and N- or C-terminal flanking segment. We analyzed targeting and integration of Tom5, a C-tail anchor protein associated with the preprotein translocase of the outer membrane, to the yeast mitochondrial outer membrane in vivo using green fluorescent protein as the reporter and compared the signal with other signals for proteins dispersed in the membrane. The functional assembly of Tom5 into the TOM complex was assessed by blue native PAGE and complementation of temperature-sensitive Δtom5 cells. Correct targeting and assembly required (i) an appropriate length TMS rather than hydrophobicity, (ii) a proline residue located at correct position in the TMS and specific residues near the proline, and (iii) that, in contrast to proteins dispersed in the outer membrane, the positive C-terminal segment was dispensable. Based on these findings, we constructed green fluorescent protein fusions with a C-terminal TMS in which the deduced sequences (minimum: Ser-Pro-Met) were inserted at an appropriate position within artificial Leu-Ala repeats. They were targeted to mitochondria and complemented the temperature-sensitive growth phenotype of Δtom5 yeast cells. The membrane-targeting mechanism of Tom5 appears to be distinct from that for proteins that are dispersed in the outer membrane.

Most mitochondrial proteins are synthesized in the cytosol and post-translationally transported to mitochondrial subcompartments. In contrast to proteins destined for the matrix or inner membrane (1–3), the targeting mechanism and the topogenesis of mitochondrial outer membrane proteins remains poorly understood, although targeting signals have been analyzed for several outer membrane proteins (4–11). Mitochondrial outer membrane proteins are synthesized without a cleavable presequence, and the mitochondrial-targeting and topogenic information is contained within the mature protein sequence; usually within α-helical transmembrane segments (TMS) and flanking regions (12, 13). The import receptors of the preprotein translocase of the mitochondrial outer membrane (TOM (translocase of outer membrane) complex) (3, 14), Tom70 (15, 16), and Tom20 (8, 17) are anchored to the membrane through the N-terminal TMS in the Nin-Cout orientation. Tom22, which functions both as the preprotein receptor and organizer of the TOM complex, is anchored to the membrane in the Nout-Cin orientation through the internal TMS (7, 18–20). Tom5, Tom6, and Tom7 are anchored to the outer membrane through the C-terminal TMS (tail-anchored protein). Tom5 functions as the connecting link between import receptors and the translocation channel Tom40 (21). Tom6 (22–24) and Tom7 (25) function as modulators of the TOM channel (26). Tom40, like porin, is predicted to be a β-barrel protein spanning the outer membrane by 12–14 antiparallel β-strands (27–30).

The N-terminal TMS with moderate hydrophobicity and a net positive charge within five residues of the C-terminally flanking region function as the mitochondrial targeting signal of Tom20 (8). As for tail-anchored proteins, we used yeast Tom5 and mammalian OMP25 to demonstrate that the moderate length and helical structure of the TMS and the positive charges in the C-terminal segment (C-segment) following the TMS are critical for targeting to mammalian mitochondria (11). This signal functions only at the C terminus of the reporter protein. In mammalian cells, yeast Tom5 was not integrated into the TOM complex and behaved as a tail-anchored protein that is dispersed in the membranes. Similarly, Isenmann and Wattenberg (4) demonstrated that mitochondrial localization of VAMP-1B is determined by the C-terminal positive charge and the length of the TMS in cultured mammalian cells. Kuroda and Ito (31) and Borgese et al. (9) also demonstrated that basic amino acid residues at the C-terminal tail are critical for correct targeting of mitochondrial outer membrane cytochrome b5.

Systematic analysis of the targeting signal for tail-anchored proteins to mammalian mitochondria using yeast Tom5 and mammalian Omp25 indicated that the basic C-segment of Tom5, which is essential for mitochondrial targeting in mammalian cells, is dispensable in yeast. In the present study, we analyzed the characteristics of the mitochondrial-targeting signal of Tom5 in yeast cells by examining intracellular localization, assembly into the TOM complex, and the ability to com-

* This work was supported by grants from the Ministry of Education, Science, and Culture of Japan (to M. S. and K. M.), from the Takeda Science Foundation, Core Research from Evolutional Science and Technology, and Specially Promoted Research from the Ministry of Education, Science, and Culture of Japan (to K. M.). 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.

† To whom correspondence should be addressed: Dept. of Molecular Biology, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan. Tel.: 81-92-642-6176; Fax: 81-92-642-6183; E-mail: mihara@cell.med.kyushu-u.ac.jp.

The abbreviations used are: TMS, transmembrane segment; BN, blue native; DHFR, dihydrofolate reductase; SU9, a 1–69 segment of precursor for subunit 9 of N. crassa F1-ATPase; GIP, general import pore; RFP, red fluorescent protein; GFP, green fluorescent protein; EGFP, enhanced GFP; ts, temperature-sensitive; C-segment, C-terminal segment; ER, endoplasmic reticulum.

Received for publication, July 2, 2003, and in revised form, July 31, 2003
Published, JBC Papers in Press, August 1, 2003, DOI 10.1074/jbc.M307047200

© 2003 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.
plement temperature-sensitive (ts) Δtom5 cells. These experiments revealed that the C-segment is dispensable, but a TMS with an appropriate length (17–20 residues), rather than hydrophobicity, is critical for the targeting and functional assembly into the TOM complex. In particular, a short segment containing a proline and the adjacent residues in the TMS was critical for the targeting. Unexpectedly, the TMS of Tom5 fused to the C terminus of GFP was assembled into the TOM complex and complemented the ts growth phenotype of Δtom5 cells. Thus, mitochondrial targeting of tail-anchored proteins depends upon the context of the TOM complex depends on a signal distinct from that of proteins that are dispersed in the membrane. The structural characteristics of the targeting signal of Tom5 might be directly recognized by Tom40 in this targeting pathway.

Based on the deduced structural characteristics of the Tom5 signal, model Leu-Ala repeats containing a stretch of proline 39 and the vicinity residues (minimum: Ser-Pro-Met) were synthesized and ligated to the C terminus of GFP. These constructs were targeted to the mitochondria and complemented ts Δtom5 cells, although the integration efficiency into the TOM complex was low.

In vitro import of GFP-Tom5 into mitochondria as assessed by blue native (BN)-PAGE revealed that it did not require the general import pore (GIP) during the assembly process but appeared to bind directly to the exterior of the Tom40 channel.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media—The Saccharomyces cerevisiae strain SEY6210 (MATa leu2-3, 112 ura3-52 his3230 trpl-S901 lys2-801 sac2-3 GAL+) was used throughout the study. Yeast cells were grown at 30 °C on rich medium (YPD: 2% glucose, 2% bacto-trypton (Difco), 1% Bacto-Yeast extract (Difco)). Transformants were grown on synthetic medium (YPD: 2% glucose, 2% bacto-trypton (Difco), 1% Bacto-Yeast extract (Difco)). Transformants were grown on synthetic medium (SD: 2% glucose, 0.6% yeast nitrogen base without amino acids (Difco), and appropriate amino acids) selective for a plasmid-borne marker, e.g. SD-His for His3-harboring plasmids. TOM5 (79753-797704 on chromosome XVI) was replaced with Candida glabrata HIS3 using a PCR-assisted disruption protocol.

Plasmid Construction—Construction of expression plasmids for enhanced GFP (EGFP) fusion proteins were based on the 2μ plasmid Yplac112 (32). The Psrl/EcoRI sites in Yplac112 were disrupted, and the ADC1 promoter and CYC1 terminator were subcloned into Spkl/EcoRI and EcoRI/HinIII sites, respectively, to create pMID-2. To construct pMID2-EGFP-Tom5, an XbaI/Apal site was created at the EcoRI site of pMID2, into which an EGFP-Tom5 fragment that was excised from pCVM EGFP-Tom5 by XbaI was cloned. To construct pMID2-EGFP-Tom5 derivatives, pCVM plasmids harboring EGFP-Tom5 mutants (11) were digested with XbaI and Apal, and the obtained fragments were inserted into pMID2. To create pMID2-EGFP-Tom6, the 186-bp DNA fragment corresponding to the Tom6 ORF (full-length, 62 residues) was amplified from genomic DNA by PCR using the following primers: 5′-AACGCGCGGATGACGGTATGTTTGCT-3′ (for the N terminus) and 5′-ATAGGGCCCTTATAAATGTTGAGGCAAA-3′ (for the C terminus) and cloned into the SacII/Apal site of pMID2-EGFP. To create pMID2-EGFP-Fis1, the 108-bp DNA fragment of Fis1p (residues 181–216) was amplified from genomic DNA using oligonucleotides: 5′-AACGCGGCGGAGAATAAGCGCAAG-3′ (for the N terminus) and 5′-ATAGGGCCCTTACCTCTTGTCATCT-3′ (for the C terminus) and cloned into pMID-EGFP. To construct pMID2-EGFP-Ure1, the DNA fragment (residues 314–346) was amplified from genomic DNA using the following oligonucleotides: 5′-CAAGCGCGGGCTAGGAGAATAAGCGCAAG-3′ (for the N terminus) and 5′-ATAGGGCCCTTACCTCTTGTCATCT-3′ (for the C terminus) and the fragment was cloned into pMID-EGFP. To create pSD-Ure9 (69-RFP), the N-terminal 66 residues of preSU9 of F0-ATPase subunit 9 were ligated on the DNA level to the N terminus of DeRed (RFP) obtained from pDeRed2-N1 (Clontech), and the fragment was cloned into pMID2.

Subcellular Fractionation—Subcellular fractionation of yeast cells was performed as follows. Yeast cells (SEY6210) were transformed with pMID2-based vectors using a lithium acetate-based method and grown in SD medium without tryptophan at 30 °C to an A590 of 1.0. The cells were harvested and treated with zymolyase 100T (Seikagaku America, Falmouth, MA). The spheroplasts were obtained as homogenized using a polycarbonate filtre (3-μm pores; Nuclepore, Corning, NY). The homogenate was fractionated by differential centrifugation into P1 (6000 × g, 10 min precipitate: mitochondria-rich fraction), P2 (100000 × g, 30 min: microsomal fraction), and the supernatant according to the method of Daum et al. (33). The subcellular fractions were subjected to SDS-PAGE and immunoblotting. The immunoblots were visualized by ECL (Amerham Biosciences), and the images were analyzed by LAS-1000 plus (Fuji Film, Tokyo, Japan).

Blue Native PAGE—BN-PAGE was performed essentially as described previously (34). Mitochondria were isolated from yeast cells expressing the GFP-Tom5 or the GFP-Fis1 constructs by the method described above and subjected to one-dimensional BN-PAGE as described previously (11). The subcellular fractions were subjected to immunoblotting using antibodies against EGFP (35) or rTom40 (36).

Complementation of ts Phenotype of Δtom5 Yeast Cells—Δtom5 yeast cells transformed with various pMID2-based vectors were grown over night at 30 °C in SD (–His, –Trp) liquid culture medium. The cells grown to an A590 of 1.0 were diluted 5-, 25-, or 125-fold, and 5-μl aliquots from each dilution were plated onto two SD (–His, –Trp) plates, which were incubated at either 30 or 37 °C for 2 days.

Fluorescence Microscopy—Yeast cells were co-transformed with pMID-SU9-RFP- and pMID2-based expression plasmids for GFP-Tom5 fusions or other GFP fusions. The cells were grown on YPD medium to log phase at 30 °C and then incubated with 3.7 to 5% (v/v) formaldehyde at 30 °C for 30 min. The cells were isolated by centrifugation and washed twice with phosphate-buffered saline by centrifugation, suspended into VECTASHEILD (Vector Laboratories, Inc., Burlingame, CA), and placed on glass slides. Fluorescent images were taken and analyzed with a confocal laser microscope (Radiance 2000; Bio-Rad) using fluorescence images of SU9-RFP as the mitochondrial reference.

Mitochondrial Import of Preproteins in Vitro—Yeast mitochondria were isolated as described previously (37). Mitochondrial import of reticulocyte lysate-synthesized preproteins (preadrenodoxin, porin, or GFP-Tom5) was performed essentially as described previously (36), except that 5 mM creatine, 5 mM sodium phosphate, and 100 μM creatine kinase were added to the assay mixture. Where indicated, the mitochondria that were incubated with 1 μM SU9-DHF at 0 °C for 15 min in the presence of 5 mM methotrexate and 1 mM NAPDH were used for the import reaction. Membrane insertion of [35S]porin was assessed as follows. After import, the mitochondria were treated with 50 μg/ml proteinase K at 0 °C for 30 min, trichloroacetic acid-precipitated, and subjected to SDS-PAGE. Membrane import of GFP-Tom5 was assessed as follows. After import, the mitochondria were treated with 50 μg/ml proteinase K at 0 °C for 30 min, trichloroacetic acid-precipitated, and subjected to SDS-PAGE. Membrane import of GFP-Tom5 was assessed as follows. After import, the mitochondria were incubated with 0.1 M Na2CO3 (pH 11.5) at 0 °C for 20 min, followed by ultraacentrifugation to recover the membrane precipitates. The mitochondria were treated again with 0.1 M Na2CO3 and subjected to SDS-PAGE. GFP-Tom5 was not recovered to the precipitate fraction in the absence of the membranes (data not shown). Analysis of the in vitro imported [35S]preproteins by BN-PAGE was as follows. After import, the mitochondria were solubilized in 20 μl Triton-1HCl buffer (pH 7.4) containing 1% digitonin, 0.1 mM EDTA, 50 mM NaCl, 10% glycerol, and 1 mM phenylmethylsulfonfluoride. The solubilized supernatant was subjected to one-dimensional BN-PAGE (5–16% gradient gel) followed by fluorography analysis using an FLA2000 (Fuji). Where indicated, mitochondria (10 μg) were pretreated with 100 μg/ml trypsin or proteinase K at 0 °C for 30 min, followed by incubation either with soybean trypsin inhibitor or phenylmethylsulfonyl fluoride to inhibit proteases. The recovered mitochondria were subjected to the import reaction.

RESULTS

Mitochondrial Localization of GFP-Tom5 in Yeast Depends on a Signal Distinct from That for Tail-anchored Proteins Dispersed in the Membrane—We previously analyzed the signal that directs tail-anchored proteins to mammalian mitochondria using yeast Tom5 as a model. These experiments revealed that a TMS with an appropriate length and three basic amino acid residues within the flanking five-residue segment (the C-segment) are critical. Of note, yeast Tom5 was not assembled into the mammalian TOM complex but dispersed in the membrane (11). The signal of Omp25, a tail-anchored protein of the mammalian TOM complex but dispersed in the membrane—
We constructed GFP-Tom5 fusions in which deletions, insertions, or point mutations were introduced to the TMS or the C-segment, expressed in yeast, and examined the intracellular localization by confocal microscopy and subcellular fractionation (Fig. 1). Mitochondria in yeast and COS-7 cells were stained with a RFP fusion protein carrying an N-terminal mitochondrial-targeting signal (SU9-RFP) and MitoTracker, respectively. The expressed GFP-Tom5 localized to mitochondria in both cells (Fig. 1B). This finding was further confirmed by subcellular fractionation (Fig. 1C). We then examined the importance of the C-segment using constructs in which three basic residues were replaced by serine residues (K-S) (Fig. 1A).
GFP-Tom5(K-S) was dispersed throughout the membranes in COS-7 cells (Fig. 1B), confirming our previous finding. In yeast cells, it was unexpectedly correctly localized to mitochondria (Fig. 1B and C), indicating that the basic C-segment is dispensable for correct targeting in yeast. The TMS-deleted construct GFP-Tom5(H9004TM) localized to the cytosol (Figs. 1C and 2B). BN-PAGE indicated that GFP-Tom5(K-S) was efficiently integrated into the ~400-kDa TOM complex (Fig. 1D). Furthermore, GFP-Tom5 constructs (wild type and K-S) but not GFP-Tom5(H9004TM) complemented the ts growth defect of H9004 tom5 yeast cells (Fig. 1E). The C-segment-deleted mutant GFP-Tom5(H9004C) showed the same phenotype as GFP-Tom5(K-S) (data not shown). Thus, correct targeting and functional integration of Tom5 into the yeast TOM complex does not require the basic C-segment.

We then examined the targeting properties of yeast Fis1, a tail-anchored outer membrane protein that is dispersed in the membrane. Fis1p is anchored to the mitochondrial outer membrane through a 20-amino acid C-terminal TMS followed by a 5-residue C-segment with four basic charges (Fig. 1A). Fis1p functions in conjunction with a peripheral protein, Mdv1p, and a cytosolic dynamin-like protein, Dnm1p, to regulate mitochondrial fission reactions (38). Expression of GFP-Fis1 in yeast cells localized to the mitochondria, whereas the C-segment mutant (GFP-Fis1(K-R-S)) was mistargeted to the ER and co-fractionated with Kar2p (Fig. 1, B and C; during cell fractionation the ER-targeted construct was somehow cleaved, and the fragment was released to the cytosol (asterisk)), thus confirming, also in yeast, the importance of the basic C-segment for mitochondrial targeting of the membrane-dispersed tail-anchored proteins. GFP-Fis1 failed to assemble into the TOM complex (Fig. 1D) and to complement the ts growth defect of the Δtom5 yeast cells (Fig. 1E). These results indicated that the mitochondrial-targeting signal of Tom5 is clearly distinct from the signal of tail-anchored proteins dispersed in the outer membrane.

**Length of TMS Rather Than Hydrophobicity Is Critical for Targeting and Integration of GFP-Tom5 Fusions to the TOM Complex**—We then examined the importance of TMS for the functional targeting of GFP-Tom5. TM(+1V), in which the TMS was elongated by a valine residue, localized to the mitochondria, whereas TM(+3V) and TM(+5V) were targeted mainly to the ER as judged by co-localization with Kar2p and cell fractionation (Fig. 2, A–C). TM(+1V) complemented the ts growth defect of Δtom5 cells (Fig. 2D). TM(+2V) localized to the mitochondria (Fig. 2, B and C), although it failed to comple-
ment the ts growth defect of Δtom5 yeast cells (Fig. 2D), indicating that the length of TMS is critical for association with the TOM complex. BN-PAGE was used to confirm that TM(+2V) was not assembled into the TOM complex (Fig. 2E). Furthermore, the C-segment mutant TM(+2V)/K-S, in which positive charges were depleted, localized to the ER (Fig. 2, B and C), consistent with previous results. Together, these results indicate the presence of two targeting pathways for mitochondrial tail-anchored proteins: the C-segment-dependent and -independent pathways.

We then examined the effect of hydrophobicity in the TMS on mitochondrial targeting. The N- and C-terminal segments of the TMS of GFP-Tom5 were replaced by valine and leucine residues to increase hydrophobicity without changing the length (TM(VL)) (Fig. 2A). This manipulation increased the average hydrophobicity from 1.28 to 2.44. It did not affect mitochondrial-targeting of the fusion construct (Fig. 2, B and C), and the construct assembled into the TOM complex and complemented the ts growth defect of the Δtom5 cells (Table I). Similar results were obtained with the mutant in which proline 39 was replaced by leucine (GFP-Tom5(P39L)); it mislocalized to dot-like structures that probably represent secretory organelles and, therefore, failed to complement the ts growth defect of the Δtom5 cells (data not shown). The other GFP-Tom5 constructs with a single-residue deletion in residues 40–45 complemented the ts growth defect of the Δtom5 cells (Table I), of which Δ44–45 and Δ44L were confirmed to be integrated into the TOM complex (data not shown). The constructs carrying a single residue-deletion within residues 30–38 were targeted to the mitochondria. Deletions within residues 35–38 compromised the complementation activity of GFP-Tom5, whereas those within the N-terminal periphery of the TMS (residues 30–34) did not (Table I). Neither the complementation activity nor the mitochondria-targeting function was compromised by the replacement of any of residues 35–38 by alanine (data not shown), suggesting that the 35–38 stretch did not require specific amino acid residues but that the length is critical.

To summarize, proline 39 is most critical for functional assembly into the TOM complex, and the mutation or deletion of proline induced mislocalization to the ER pathway. The residues localizing in the C-terminal vicinity of proline 39 mainly affected the targeting efficiency, whereas those in the N-terminal vicinity (residues 35–38) affected functional complementation. The defect of assembly into the TOM complex was confirmed for several of the complementation-defective constructs.
The ability of complementation of Δtom5 cells was as follows: ++, growth rate equivalent to wild-type cells; +, growth rate lower than that of wild-type cells; −, no complementation.

<table>
<thead>
<tr>
<th>Localization</th>
<th>Complementation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mt</td>
<td>++</td>
</tr>
<tr>
<td>Mt</td>
<td>+</td>
</tr>
<tr>
<td>Mt</td>
<td>+</td>
</tr>
<tr>
<td>Mt</td>
<td>+</td>
</tr>
<tr>
<td>Mt</td>
<td>+</td>
</tr>
<tr>
<td>Mt</td>
<td>+</td>
</tr>
<tr>
<td>Mt</td>
<td>+</td>
</tr>
<tr>
<td>Mt/Cyt</td>
<td>+</td>
</tr>
<tr>
<td>Mt</td>
<td>+</td>
</tr>
<tr>
<td>Mt</td>
<td>+</td>
</tr>
<tr>
<td>Mt/Cyt</td>
<td>+</td>
</tr>
<tr>
<td>Mt/Cyt</td>
<td>+</td>
</tr>
<tr>
<td>Mt/Cyt</td>
<td>+</td>
</tr>
<tr>
<td>Mt</td>
<td>+</td>
</tr>
<tr>
<td>Mt/Cyt</td>
<td>+</td>
</tr>
<tr>
<td>Mt/Cyt</td>
<td>+</td>
</tr>
</tbody>
</table>

* Yeast cells expressing GFP-Tom5Δ35L exhibit growth defect even at nonpermissive temperature.

(for Δ37V and Δ38S; data not shown). Δ35L was targeted to mitochondria and exhibited strong growth inhibition of Δtom5 cells at a nonpermissive temperature for unknown reasons (Table I, footnote a).

We then addressed the importance of the proline residue position in the TMS (summarized in Table II). When proline 39 was shifted toward the C terminus by one or two residues, the mutants Δ39P/+40P and Δ39P/+41P were more or less targeted to the mitochondria and complemented the ts growth defect of Δtom5 cells, whereas Δ39P/+42P and Δ39P/+43P mislocalized mainly to the cytosol and did not, or only weakly, complemented the growth defect. On the other hand, when proline 39 was shifted toward the N terminus in the TMS, the mutants (Δ39P/+38P, Δ39P/+37P, Δ39P/+36P, and Δ39P/+35P) were mainly targeted to the ER pathways. We further confirmed that these constructs could not, or could only weakly, complement the ts growth defect of the Δtom5 cells. Similarly, GFP-Tom5Δ5/+33P, in which a proline residue was inserted at position 33 to keep proline 39 intact, localized to the mitochondria and cytosol but failed to complement the ts growth defect of the Δtom5 cells (data not shown). These results suggest that the region upstream of proline 39 in the TMS should be in a helical structure for correct targeting to the TOM complex (Chaw-Fassmann’s algorithm predicts a helical structure for the region upstream of proline 39, and the residues that are considered to function during preprotein-transfer from Tom22 to Tom40 (21). Unexpectedly, GFP-Tom5Δ1–22, which lacks the acidic N-terminal 22 residues of Tom5, was targeted to mitochondria and complemented the ts growth defect of Δtom5 cells (Fig. 3, B and C). The basic charges in the C-segment were not required for mitochondrial localization and growth complementation (Tom5Δ1–22(K-S) in Fig. 3, B and C). As a control, the TMS of Fis1 or the tail-anchored tSNARE of the ER, Ufe1 (40), was not functional, although they were targeted to mitochondria (Fig. 3, B and C, and data not shown; the C-segment of Ufe1 was replaced by that of Tom5 to produce mitochondrial localization), thus indicating that this complementation was not due to nonspecific interactions of the hydrophobic TMS of tail-anchored proteins. BN-PAGE revealed that GFP-Tom5Δ1–22 was integrated into the TOM complex (Fig. 3D). These results suggested that the N-terminal cytoplasmic domain of Tom5 is dispensable for membrane targeting and functional assembly into the TOM.

GFP Fusions with the Tom5 Model TMS Complement the ts Growth Defect of Δtom5 Cells—Thus, the minimum requirement for mitochondrial targeting and complementation of the ts growth defect of Δtom5 cells was determined. The structural requirements were (i) a TMS of 17–20 residues, (ii) a proline residue positioned 5–7 residues from the C-terminal border of the TMS, (iii) specific amino acid residues in the N-terminal vicinity of proline 39, and (iv) a presumed helical structure of the region upstream of proline 39 in the TMS. Based on these requirements, we constructed artificial 19-residue TMS in which a stretch of proline 39 and the vicinity residues in the Tom5 TMS was inserted into Leu-Ala repeats so that the proline residue could be positioned 7 residues from the C-terminal border of the membrane and ligated them to the C terminus of GFP (Fig. 4A). The constructs (LA7/VSPMI and (LA8/SPM were targeted to the mitochondria and complemented the ts growth defect of the Δtom5 yeast cells (Fig. 4, B and C), although they had a targeting fidelity lower than constructs with authentic Tom5 TMS and some fraction localized to the ER (Fig. 4B and data not shown). In contrast, (LA6/FLWVSPM efficiently targeted to mitochondria and complemented the ts growth defect of the Δtom5 yeast cells (Fig. 4, B and C). On the other hand, the GFP constructs fused to the synthetic Leu-Ala repeats with a proline insertion, (LA7/P and (LA9/P, were targeted to the mitochondria and ER, respectively, and failed to complement the ts growth defect of Δtom5 cells (Fig. 4, B and C, and data not shown).

BN-PAGE revealed that the constructs with artificial TMS that complemented ts Δtom5 cells were integrated into the TOM complex but with lower integration efficiency; (LA7/VSPMI and (LA8/SPM might have dissociated from the TOM complex during electrophoresis because of low affinity (Fig. 4D). We concluded that the model sequence containing Ser-Pro-Met is the minimum signal for membrane targeting and assembly into the TOM complex.

GFP-Tom5 Does Not Share the General Import Pore with Matrix-targeted Preproteins—We then probed the targeting and insertion pathway of GFP-Tom5 to the mitochondrial outer membrane...
membrane. The in vitro insertion of GFP-Tom5 revealed that neither the surface import receptors nor GIP that are normally used by presequence-containing preproteins were involved (Fig. 5). When the GIP of the outer membrane was blocked by accumulating chemical amounts of a translocation intermediate of the fusion protein SU9-dihydrofolate reductase (SU9-DHFR) in the presence of methotrexate and NADPH, the import of matrix-targeted preprotein preadrenodoxin was strongly blocked (Fig. 5A, left panel). Under the same condition, insertion of the outer membrane protein porin, which also requires GIP for the membrane insertion (41) was inhibited by ~30% (Fig. 5A, middle panel). The import of porin into Δtom5 mitochondria was reduced relative to wild type mitochondria, confirming the previous report (21), and the import was significantly blocked by the addition of SU9-DHFR (Fig. 5A, middle panel). These results verified that the GIP was satisfactorily

Table II

<table>
<thead>
<tr>
<th>Localization</th>
<th>Complementation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mt/ER</td>
<td>–</td>
</tr>
<tr>
<td>Mt</td>
<td>++</td>
</tr>
<tr>
<td>Mt/Cyt</td>
<td>+</td>
</tr>
<tr>
<td>Mt</td>
<td>–</td>
</tr>
</tbody>
</table>

Fig. 4. Complementation of ts Δtom5 phenotype by GFP constructs carrying model TMS. The GFP constructs with the indicated synthetic TMS (A) were expressed in yeast cells. The fluorescent images (B), ability of complementation of ts growth defect of Δtom5 cells (C), and assembly into the TOM complex as revealed by BN-PAGE (D) were analyzed. Mt, mitochondria; WT, wild type.
Mitochondria-targeting Signal of Yeast Tom5

occupied by SU9-DHFR. In contrast, however, neither of the above manipulations inhibited alkaline-resistant integration of GFP-Tom5 into mitochondria (Fig. 5A, right panel). Similar results were obtained for porin and GFP-Tom5 when their assembly into the oligomeric structures was monitored by BN-PAGE. The assembly of porin (41) was inhibited by either GIP blockade or combination of the Tom5 depletion and GIP blockade, whereas the assembly of GFP-Tom5 into the TOM complex was not (Fig. 5B). Furthermore, trypsin treatment of mitochondria to remove import receptors or proteinase K treatment to remove Tom40 significantly compromised the membrane integration of porin (Fig. 5C), confirming a previous report that the insertion of porin occurs via the import receptor Tom20 and GIP (41). In contrast, assembly of GFP-Tom5 into the TOM complex was not affected by trypsin treatment, whereas proteinase K treatment to decrease the Tom40 level significantly compromised its assembly (Fig. 5C), suggesting that integration of GFP-Tom5 required Tom40 but not the import receptors. Together, these data suggested that the Tom5 precursor binds directly to the exterior part of the Tom40 channel.

DISCUSSION

In the present study, we characterized the Tom5 signal required for targeting and functional assembly into the TOM complex in yeast cells and compared it with the signal that directs membrane-dispersed tail-anchored proteins to the outer membrane. The structural characteristics of the signal are in marked contrast to those of proteins not restricted to the TOM complex that are dispersed in the outer membrane. The signal of the dispersed proteins required an appropriate length α-helical TMS and the following basic C-terminal segment (11). The proline residue was not critical for targeting (see Fis1 in Fig. 1,
A and B). These structural characteristics are conserved in yeast and mammals, although a slightly longer or a slightly more hydrophobic TMS is required in mammals (11).

Allen et al. (39) demonstrated that a conserved proline residue in the TMS of Tom7 and other tail-anchored subunits of the TOM complex, Tom6, Tom5, and intermembrane anchor Tom22 is required for their efficient targeting to the outer membrane. Our detailed study with yeast Tom5 has validated this and further revealed that proline and the adjacent residues are critical and should be located within a narrow range from the C-terminal border of the membrane. These characteristic features of Tom5 are not conserved in other tail-anchored components of the TOM complex. In fact, the Tom6 TMS could not replace the function of Tom5 in our study (data not shown). Dembowksi et al. (10) demonstrated with Neurospora crassa Tom6 that the C-terminal domain consisting of the 16-residue TMS and the 6-residue C-segment with a net negative charge contains the information required for the initial recognition of Tom6 that the C-terminal domain consisting of the 16-residue TMS and the 6-residue C-segment with a net negative charge.

The import of Tom22, a bitopic outer membrane protein, into the TOM complex depends on the presence of a 26-residue region (within 26 residues) of the TMS and the 6-residue C-segment. This result has been confirmed in yeast and mammals, although a slightly longer or a slightly more hydrophobic TMS is required in mammals (11).

What component initially recognizes these structural features of Tom5 TMS? In the in vitro import assay, small Tom proteins Tom5 and Tom7 are inserted directly into the mature ~400-kDa TOM complex, whereas Tom6 is inserted into the ~100-kDa intermediate complex before maturation to the 400-kDa complex (42). On the other hand, when radiolabeled Tom40 precursor is imported into mitochondria, it is first targeted to the membrane via the receptor proteins Tom20 and Tom22 and then assembles with endogenous Tom5 to form the intermediate 250-kDa complex exposed to the intermembrane space. The 250-kDa complex matures to a 400-kDa complex via a 100-kDa intermediate. These results also suggest that newly synthesized Tom5 directly binds to Tom40 in the maturation pathway of the TOM complex. In both cases, therefore, Tom40 functions as the receptor for Tom5. In addition, we demonstrated here that GFP-Tom5 was inserted irrespective of the blockade of the GIP, in marked contrast to N. crassa Tom6 and Tom7, whose assembly into the TOM complex occurs via the GIP (10). Together, these results suggest that Tom5 binds directly to the exterior site of the Tom40 channel in the TOM complex. If this is the case, then the structural characteristics of the determined signal might be required to fit with the surface of the predicted β-barrel structure of the Tom40 channel. When this configuration is disrupted, the protein is no longer recognized by Tom40 and is mistargeted to the ER via a post-translational targeting pathway and probably assisted by cytoplasmic chaperones.

The insertion pathway of the basic C-segment-dependent tail anchor protein into the mitochondrial outer membrane remains unclear. Our preliminary results indicated that insertion of GFP-Fis1 is insensitive to blockade of the GIP by the addition of SU9-DHFR. Motz et al. (43) recently demonstrated in yeast that mitochondrial targeting of Bcl2 requires two basic residues in the C-segment, and its insertion depends on the import receptor Tom20, bypassing the GIP or the TOM core complex. It should be clarified, therefore, whether this is the general import pathway for the tail-anchored mitochondrial outer membrane proteins that are targeted in the basic C-segment dependent fashion.

Acknowledgments—We thank M. Tokunaga (Kagoshima University) and T. Endo (Nagoya University) for providing yeast Kar2p and Tom40 antibodies, respectively.

REFERENCES
Mitochondria-targeting Signal of Yeast Tom5

Targeting and Assembly of Mitochondrial Tail-anchored Protein Tom5 to the TOM Complex Depend on a Signal Distinct from That of Tail-anchored Proteins Dispersed in the Membrane

Chika Horie, Hiroyuki Suzuki, Masao Sakaguchi and Katsuyoshi Mihara

doi: 10.1074/jbc.M307047200 originally published online August 1, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M307047200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 43 references, 20 of which can be accessed free at http://www.jbc.org/content/278/42/41462.full.html#ref-list-1