**Tom7 Regulates Mdm10-mediated Assembly of the Mitochondrial Import Channel Protein Tom40**

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β-Barrel membrane proteins in the mitochondrial outer membrane use the TOM40 complex to enter mitochondria and then the TOB/SAM complex to be assembled into the outer membrane. Tom7, a subunit of the TOM40 complex, regulates association of Mdm10 with the TOB complex. Here, we analyzed the role of Tom7 in assembly of β-barrel proteins, including Tom40, a central channel subunit of the TOM40 complex, and porin. Depletion of Tom7 decreased transient accumulation of Tom40 at the level of the TOB complex and retarded assembly of porin in vitro. On the other hand, overexpression of Tom7 resulted in enhanced accumulation of in vitro imported Tom40 in the TOB complex, yet it did not affect the in vitro assembly of porin. Site-specific photocross-linking in vivo revealed that Tom7 directly interacts with Tom40 through its transmembrane segment and with Mdm10. These results collectively show that Tom7 recruits Mdm10, enhancing its association with the MMM1 complex, to regulate timing of the release of Tom40 from the TOB complex for subsequent assembly into the TOM40 complex.

Eukaryotic cells are divided into many membrane-bound compartments, organelles, the functions of which rely on correct delivery of their resident proteins synthesized in the cytosol. Mitochondria are surrounded by the outer and inner membranes and contain 1,000–1,500 different proteins. Most mitochondrial proteins are synthesized in the cytosol and inserted into the outer and inner membranes and contain 1,000–1,500 different proteins. Most mitochondrial proteins are synthesized in the cytosol and subsequently imported into mitochondria with the aid of translocators, e.g. the TOM40 and TOB/SAM complexes in the outer membrane and the TIM23 and TIM22 complexes in the inner membrane (1–4). The TOM40 complex functions as an entry gate for most mitochondrial proteins by mediating protein translocation across the outer membrane. The TOM40 complex consists of the core complex consisting of Tom40, Tom22, Tom5, Tom6, and Tom7 and peripherally associated receptors Tom20 and Tom70. The TOM40 channel is mainly formed by the β-barrel pore of Tom40, which is likely stabilized by the other subunits, including Tom22. After crossing the outer membrane, mitochondrial proteins are further sorted to different intramitochondrial destinations with the aid of distinct translocators. Notably, a class of outer membrane proteins, β-barrel proteins use a translocator, the TOB complex, in the outer membrane for their membrane insertion (5–8). The TOBcore complex consists of Tob55/Sam50 (9–11), Tom38/Tob38/Sam35 (12–14), and Mas37/Tom37/Sam37 (8, 15). Tob55 is a β-barrel protein itself and a homolog of Omp85/YaeT in the bacterial outer membrane. Tom38 is a receptor of the β-signal, a short C-terminal sequence functioning as a sorting signal of mitochondrial β-barrel proteins (16).

Recently, Mdm10 was found to function with the TOB complex for the assembly of β-barrel membrane proteins (17). Although Mdm10 is partly found in the MMM1 complex with Mmm1, Mmm2, and Mdm12, which tethers the ER and mitochondria (18), a fraction of Mdm10 associates with the TOBcore complex, which is more abundant than the MMM1 complex, to form a larger TOBholo complex (17, 19). Mdm10 occupies a site of the TOBcore complex that can also be used for assembly of other β-barrel proteins (17, 19). Meisinger et al. (17) reported that although the TOBcore complex is required for assembly of all the β-barrel proteins, Mdm10 in the TOBholo complex is only required for the later step of the assembly of Tom40 but not for other β-barrel proteins after dissociation from the TOBcore complex. Because deletion of the MDM10 gene would potentially impair various processes, including lipid metabolism mediated by the MMM1 complex, we lowered the Mdm10 protein level by mutating its β-signal without changing the mitochondrial lipid composition or the protein levels of other TOM40 and TOB components (19). By doing so, we found that depletion of Mdm10 (Mdm10↓) primarily impairs release of Tom40 from the TOB complex, but not the later step of the Tom40 assembly process, suggesting that the dynamic entry of Mdm10 into the TOB complex facilitates dissociation of Tom40 from its transiently associating TOB complex (19).

In this study, we altered the ratio of TOBcore complex and TOBholo complex by overexpression or depletion of Tom7, a small subunit of the TOM40 complex, to further analyze the role of Mdm10 in the Tom40 assembly process. Tom7, together with Tom40 and Tom22, is almost ubiquitously present in eukaryotes, indicating that these three subunits of the TOM40 complex represent a minimal and likely ancestral set constituting the functional TOM40 complex (20). Tom7 spans the outer membrane by its central transmembrane (TM) segment and is associated with Tom40 (21). Tom7 was initially suggested to function in an antagonistic manner with Tom6 by destabilizing the dynamic assembly of the TOM40

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2 The abbreviations used are: TM, transmembrane; BN, blue native; BPA, benzylphenylalanine; CL, cardiolipin; PE, phosphatidylethanolamine; ER, endoplasmic reticulum.
complex (22). Then the intermembrane space domain of Tom7 was found to constitute the trans site for presequence binding to facilitate efficient translocation of presequence-containing precursor proteins across the outer membrane (23). Recently, Tom7 was found to affect association of Mdm10 with the TOBcore complex, which results in sequestration of Mdm10 from formation of the TOBholo complex (24). We found that depletion or overexpression of Tom7 prevents Tom40 from association with or dissociation from the TOB complex, respectively, by regulating the association of Mdm10 with the TOB complex. These results support the model that the Mdm10 in cooperation with Tom7 regulates timing of the release of unassembled Tom40 from the TOB core complex to facilitate its coordinated assembly into the TOM40 complex.

EXPERIMENTAL PROCEDURES

**Plasmids**—The amplified DNA fragment encoding the TOM7 gene was inserted between the GAL1 promoter and the CMK1 terminator of the CEN-URA3 plasmid, YEpUG578T, to generate pGAL-TOM7. pGAL1-TOM7 was digested with BamHI to obtain a DNA fragment for the GAL1 promoter containing the 5′-portion of TOM7, which was replaced with a DNA fragment for the TOM7 promoter containing the 5′-portion of the TOM7 gene in pRS314/TOM7-HA (23) to generate pRS314/GAL1pro-TOM7-HA.

For *in vivo* cross-linking, pYO326/GAL1pro-TOM7-HA and its derivative plasmids were constructed as follows. A SacI/XhoI fragment of pRS314/GAL1pro-TOM7-HA harboring the GAL1 promoter and the TOM7-HA gene was introduced into SacI/Xhol sites of the 2μ-URA3 plasmid, pYO326, to generate pYO326/GAL1pro-TOM7-HA. The codon for residue X of Tom7 was replaced with the amber codon by oligonucleotide-directed mutagenesis with an appropriate primer pair. Finally, the amplified DNA fragments were introduced into pYO326 to generate pYO326/GAL1pro-TOM7(X)amb-HA.

**Yeast Strains and Growth Conditions**—The wild-type *Saccharomyces cerevisiae* strain used in this study is W303-1A (MATa ade2-1 his3-11,15 ura3-1 leu2-3,112 trpl-1 can1-100). The tom7Δ strain was constructed as follows. DNA fragments encoding CgHIS3 or kanMX4 were introduced into the chromosomal TOM7 gene of W303-1A, and his + or G418-resistant transformants were selected. FLAG-TOM22, a yeast haploid strain expressing N-terminally FLAG-tagged Tom22 from a plasmid instead of the chromosome, was constructed as follows. MNMS1-C (25), a yeast haploid strain whose chromosomal disruption of TOM22 was complemented with the plasmid harboring the TOM22 gene under the control of the GAL1 promoter, was transformed with pRS314/FLAG-TOM22, and the ura + trp + cells were isolated. The ura + trp + transformants were spread onto SCD (−Trp −Ura) medium with or without 1 mM benzylpenicillin-lalanine (BPA) (Bachem), and cultivated further for 30 h at 30 °C, harvested by centrifugation, resuspended in SCD medium with or without 1 mM benzylpenicillin-lalanine (BPA) (Bachem), and cultivated further for 30 h at 30 °C in the dark until A$_{600}$ reaches 1.0. The culture was then UV-irradiated for 10 min at room temperature. After UV irradiation, cells were harvested and were solubilized with 1% SDS buffer (40 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1% (w/v) SDS, 1 mM PMSF, protease inhibitor mixture (Sigma)) at 95 °C for 5 min and diluted 10-fold in 0.5% TX-100 buffer (0.5% (v/v) Triton X-100, 40 mM Tris-HCl, pH 7.5, 200 mM NaCl). FLAG
tagged Mdm10 and its cross-linked products were affinity-purified by the immobilized anti-FLAG antibody.

Miscellaneous—For glycerol density gradient centrifugation, mitochondria were solubilized with lysis buffer (20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.1 mM EDTA, 10% (w/v) glycerol, 1 mM PMSF, protease inhibitor mixture (Sigma)) containing 0.5% digitonin on ice for 15 min. After centrifugation at 20,000 \( \times g \) for 15 min at 4 °C, the clarified supernatants were subjected to glycerol density gradient centrifugation (10–40% (w/v) glycerol and 0.2% digitonin) in lysis buffer at 200,000 \( \times g \) for 10 h at 4 °C. Fractions were collected from the top and proteins were precipitated with trichloroacetic acid. Cell labeling with \( { }^{32} \)Pi and subsequent separation of phospholipids by thin layer chromatography were performed as described previously (19, 28).

RESULTS

Tom7 Regulates the Interactions of Mdm10 with the TOB Complex—Tom7, a subunit of the TOM40 complex, was reported to regulate the association of Mdm10 with the TOB complex by direct recruitment of Mdm10 from the TOB complex to Tom7 (24). We thus made the \( \text{tom7}^{\Delta} \) strain with chromosomal disruption of the \( \text{TOM7} \) gene for Tom7 depletion, and the wild-type (WT) strain was transformed with a plasmid where the \( \text{TOM7} \) gene was under the control of galactose-inducible \( \text{GAL1} \) promoter for Tom7 overexpression. Depletion (\( \text{tom7}^{\Delta} \)) or overexpression (Tom7\( ^{1} \)) after cultivation in galactose-containing medium for 6 h did not affect the protein levels of Mdm10 and components of the MMM1 complex (Mdm12 and Mmm1), TOB complex (Tob55, Tom38, and Mas37), TOM40 complex (Tom40, Tom70, Tom20, and Tom22), and TIM23 complex (Tim23) (Fig. 1A, upper panel). Because anti-Tom7 antibodies are not available, we used mitochondria with the HA epitope-tagged version of Tom7 (Tom7-HA), which can replace wild-type Tom7 without any defects in cell growth at 37 °C or on nonfermentable medium (data not shown), to access the protein level of Tom7. In mitochondria with overexpression of Tom7-HA (Tom7-HA\( ^{1} \)),

FIGURE 1. Depletion or overexpression of Tom7 alters the fraction of the holo-form of the TOB complex. A, mitochondria were isolated from W303-1A cells (WT), \( \text{tom7}^{\Delta} \) cells, WT cells with pGAL1-TOM7 (Tom7\( ^{1} \)), \( \text{tom7}^{\Delta} \) cells with pRS314/TOM7-HA (Tom7-HA), and \( \text{tom7}^{\Delta} \) cells with pRS314/TOM7-HA and pGAL1-TOM7-HA (Tom7-HA\( ^{1} \)) after 6 h of cultivation in galactose-containing medium at 23 °C. Mitochondrial proteins were analyzed by SDS-PAGE or Schägger-PAGE followed by immunoblotting with the indicated antibodies. The anti-HA antibody was used for detection of the HA-tagged version of Tom7. B, after solubilization with 1% digitonin, WT, \( \text{tom7}^{\Delta} \), and Tom7\( ^{1} \) mitochondria with FLAG-tagged Tom38 or FLAG-tagged Mdm10 were subjected to BN-PAGE analyses followed by immunoblotting with anti-Tom40 antibodies (\( \alpha \text{Tom40} \)) and the anti-FLAG antibody (\( \alpha \text{FLAG} \)). An asterisk indicates the partially dissociated TOB complex. 10-7 and 10-(7) indicate protein complexes containing Mdm10 with or without Tom7, respectively. C, 100 \( \mu \)g of 1% digitonin-solubilized TOM7-HA and Tom7-HA\( ^{1} \) mitochondria were subjected to 4–14% BN-PAGE for the first dimension. The BN gel was then treated with SDS buffer (100 mM Tris-HCl, pH 6.8, 4%(w/v) SDS, 10 mM DTT) at 60 °C for 30 min. The gel was cut into 16 pieces and subjected to Schägger-PAGE for the second dimension followed by immunoblotting with the anti-HA antibody. 10-7, 10-(7)\( _{n} \), and 7 are the Mdm10-Tom7 complex, the Mdm10-multiple Tom7 complex, and free Tom7, respectively.
the level of Tom7 was over 10-fold higher than that in WT mitochondria (Fig. 1A, lower panel).

We then examined the effects of depletion or overexpression of Tom7 on the apparent sizes of the TOM40 complex and TOB complex by BN-PAGE of digitonin-solubilized WT, tom7Δ, and Tom7↑ mitochondria. When we detected the TOM40 complex with anti-Tom40 antibodies, depletion of Tom7 slightly reduced the molecular size of the 450-kDa TOM40 complex, although overexpression of Tom7 did not affect the size of the TOM40 complex (Fig. 1B, left panel). On the other hand, when we detected the TOB complex containing Tom38-FLAG with the anti-FLAG antibody, depletion or overexpression of Tom7 increased or decreased, respectively, the amount of the 350-kDa TOB\textsuperscript{holo} complex on a BN-polyacrylamide gel (Fig. 1B, central panel). The 100-kDa complex containing Mdm10-FLAG detected by the anti-FLAG antibody in WT mitochondria was converted to a slightly smaller band in the fractions that were shifted to the heavier dimension that the bands assigned to the TOM40 complex (Fig. 1C). It is to be noted that Mdm10 in the MMM1 complex is hardly detected by BN-PAGE probably because it falls apart under the conditions for BN-PAGE (data not shown).

**Tom7 Is a Subunit of the MMM1 Complex**—We analyzed complex formation of Tom7-HA by glycerol density gradient centrifugation of solubilized mitochondria containing the FLAG-tagged Tom22 (Fig. 2A). As expected from the fact that Tom7 is a constituent of the TOM40 complex, Tom7-HA was mainly found in the fractions that were shifted to the heavier region when the anti-FLAG antibody was added before fractionation. On the other hand, Tom7-HA of solubilized mitochondria was not efficiently pulled down by the immobilized anti-HA antibody (Fig. 2B, uppermost panel), and the minor pulled down fraction of Tom7 was not associated with the other subunits, Tom40, Tom20, or Tom22, of the TOM40 complex (Fig. 2B, 2nd column panels). Therefore, the HA epitope tag on Tom7 is not fully exposed in the intact TOM40 complex. Instead, the fraction of Tom7-HA pulled down by the anti-HA antibody was associated with Mdm10 (Fig. 2B, 3rd column panels). As a control, Tom7-HA was not pulled down with any of the subunits of the TOB complex, Tob55, Tom38, or Mas37 (Fig. 2B, lowermost panel).

We then solubilized the mitochondria containing Tom7-HA and FLAG-tagged version of Mdm10 (Mdm10-FLAG) or Tom22 (FLAG-Tom22) and subjected them to immunoprecipitation with the anti-FLAG antibody (Fig. 2C). When detected with anti-HA antibodies, Tom7-HA was mainly co-pulled down with FLAG-Tom22, yet a small but distinct fraction was pulled down with Mdm10-FLAG, as well (Fig. 2C, upper right panel). It was rather unexpected that Mdm12 and Mmm1 of the MMM1 complex were also pulled down with Tom7-HA by the anti-HA antibody (Fig. 2B, 3rd column panels). The interactions between Tom7 and the components of the MMM1 complex are not artifacts due to the HA epitope tag attached to Tom7 because Tom7-FLAG was also found to interact with Mdm12-FLAG (Fig. 2D). These results suggest that Tom7 is a subunit of not only the TOM40 complex but also the MMM1 ER-mitochondria tethering complex even under the conditions without overexpression of Tom7. We then asked if Mdm10 associated with the MMM1 complex was affected by the protein level of Tom7 by comparing the results of co-immunoprecipitation of solubilized mitochondria containing Mdm10-FLAG with the anti-FLAG antibody between WT and tom7Δ strains (Fig. 2E). In the absence of Tom7, the components of the TOB complex, Tob55, Tom38, and Mas37, co-immunoprecipitated with Mdm10-FLAG increased by 70–100%, although the co-immunoprecipitated Mdm12 and Mmm1 of the MMM1 complex decreased by 30% (Fig. 2E, lower panel). This suggests that depletion of Tom7 results in dissociation of Mdm10 from the MMM1 complex, thereby promoting association of Mdm10 with the TOB\textsuperscript{core} complex to form the TOB\textsuperscript{holo} complex. On the other hand, when Tom7 was overexpressed, although the TOB subunits co-immunoprecipitated with Mdm10 decreased by 50–60%, the co-immunoprecipitated MMM1 subunits increased by 25–55% (Fig. 2E, lower panel). In WT mitochondria, quantification of Tom7-HA and Mdm10-HA with the anti-HA antibody revealed that the ratio of Tom7 and Mdm10 is 2:1 (data not shown), and 70% of Tom7 was pulled down with FLAG-Tom22 and 7% with Mdm10-FLAG (Fig. 2C, lower panel). Therefore, although a major fraction of Tom7 is present in the TOM40 complex, a small but distinct fraction of Tom7 is associated with Mdm10, which may be at least partly associated with the MMM1 complex.

**Tom7 Interacts Directly with Tom40 and Mdm10**—We next asked if Tom7 and Mdm10 directly interact with each other. For this purpose, we took the approach of site-specific photocross-linking in vivo (27, 29). Briefly, we introduced a photoreactive unnatural amino acid, BPA, into various positions in Tom7-HA in yeast cells containing Mdm10-FLAG. The UV irradiation resulted in cross-linking of BPA in Tom7 with nearby proteins. Cross-linked partner proteins can be identified by immunoblotting with antibodies against known components of the mitochondrial outer membrane complexes.

When total cell extracts after UV irradiation were stained with anti-Tom40 antibodies and the anti-HA antibody, ~50-kDa bands were detected for Tom7 containing BPA at positions 25 (only weakly), 29, 32 (most strongly), and 39 (Fig. 3A), indicating that Tom7 directly interacts with Tom40 in the TOM40 complex. After purification of FLAG-tagged proteins (Mdm10-FLAG and its cross-linked products) with a FLAG-agarose column, we efficiently detected a 70-kDa cross-linked product involving BPA at position 25 of Tom7 by anti-Mdm10 antibodies (Fig. 3B). This cross-linked product was observed only when BPA was added to culture media, and cells were UV-irradiated.
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(Fig. 3C). Therefore, Tom7 directly interacts with Mdm10 as well.

Residues 29, 32, and 39 are in the conserved TM segment of Tom7, and residue 25 is located at the cytosolic end of the

TM segment (20). The helical wheel plot shows that those residues are on the same side of the possible α-helix formed by the TM segment (Fig. 3D). Therefore, Tom7 interacts with both Tom40 and Mdm10 through the same side of the TM
antibody) efficiency (% of total) assessed by anti-Mdm10 antibodies.

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**FIGURE 2.** Tom7 is a constituent of both the TOM40 and MMM1 complexes. A, mitochondria isolated from FLAG-TOM22/TOM7-HA cells were solubilized with 0.5% digitonin, incubated with (+FLAG) or without (−FLAG) the anti-FLAG antibody on ice for 30 min, and subjected to 10–40% linear glycerol density gradient centrifugation followed by detection with the indicated antibodies after SDS-PAGE or Schägger-PAGE. B, wild-type mitochondria (WT) and those with HA-tagged Tom7 (TOM7-HA) were solubilized with 0.5% digitonin and subjected to immunoprecipitation with the immobilized anti-HA antibody followed by detection with the indicated antibodies after SDS-PAGE or Schägger-PAGE. C, TOM7(25)amb-HA and one aliquot was UV-radiated. The FLAG-tagged proteins were purified with the immobilized anti-FLAG antibody, and co-immunoprecipitated proteins were detected with the indicated antibodies (upper panel) and the anti-HA antibody (lower panel). For detection of Mdm10 and its cross-linked products, proteins were affinity-purified with the immobilized anti-FLAG antibody and subjected to immunoblotting with anti-Mdm10 antibodies. B, open arrows and asterisk indicate the cross-linked products with Tom7-HA and nonspecific bands, respectively. C, MDM10-FLAG/TOM7 cells with pY0326/GAL1pro-TOM7 amb-HA and p-6 × trNA were grown in galactose medium with or without BPA at 30 °C. The culture was divided into halves, and one aliquot was UV-radiated. The FLAG-tagged proteins were purified with the immobilized anti-FLAG antibody. Black arrowheads indicate cross-linked products involving Tom7-HA. D, helical wheel plot of the TM segment and flanking regions of Tom7 (25–42 amino acids).

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**FIGURE 3.** Tom7 directly interacts with Tom40 and with Mdm10 in vivo. A and B, pRS516 (Vec), pY0326/GAL1 pro-TOM7-HA (WT), or its derivative plasmids (pY0326/GAL1 pro-TOM7(X)amb-HA) containing amber codon for residue X in the TOM7 gene were introduced into MDM10-FLAG/tom7Δ cells containing p-6 × trNA. The transformants were grown in galactose medium containing 1 mM BPA. After UV irradiation, total cell extracts were analyzed by SDS-PAGE and immunoblotting with anti-Tom40 antibodies (A, upper panel) and the anti-HA antibody (A, lower panel). For detection of Mdm10 and its cross-linked products, proteins were affinity-purified with the immobilized anti-FLAG antibody and subjected to immunoblotting with anti-Mdm10 antibodies. B, open arrows and asterisk indicate the cross-linked products with Tom7-HA and nonspecific bands, respectively. C, MDM10-FLAG/tom7Δ cells with pY0326/GAL1 pro-TOM7(p)amb-HA and p-6 × trNA were grown in galactose medium with or without BPA at 30 °C. The culture was divided into halves, and one aliquot was UV-radiated. The FLAG-tagged proteins were purified with the immobilized anti-FLAG antibody. Black arrowheads indicate cross-linked products involving Tom7-HA. D, helical wheel plot of the TM segment and flanking regions of Tom7 (25–42 amino acids).

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helix, which in turn indicates that Tom40 and Mdm10 are mutually exclusive for binding to Tom7.

Tom7 Regulates Mdm10-mediated Dissociation of Tom40 from the TOB Complex—Then we analyzed in vitro import of β-barrel proteins into WT mitochondria and those without Tom7 (tom7Δ) or with overexpressed Tom7 (Tom7 †). As a control, matrix-targeted pSu9(69)-DHFR was imported into WT and Tom7 † mitochondria with similar efficiencies, and into tom7Δ mitochondria with slightly decreased efficiency (Fig. 4A). The radiolabeled Tom40 and porin were then syn-
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FIGURE 4. Effects of depletion or overexpression of Tom7 on the β-barrel membrane protein assembly in vitro. A, mitochondria were isolated from W303-1A (WT) cells, tom7Δ cells, and WT cells with pGAL1-TOM7 (Tom7 †) after 6 h of cultivation in galactose-containing medium at 23 °C and were incubated with radiolabeled pSu9(69)-DHFR at 23 °C for indicated times. After PK treatment, imported proteins were analyzed by SDS-PAGE and radioimaging. B, WT, tom7Δ, and Tom7 † mitochondria were incubated with radiolabeled Tom40 or porin at 23 °C for the indicated times. The mitochondria were solubilized with 1% digitonin and analyzed by BN-PAGE and radioimaging. Asterisks indicate nonspecific bands. C, efficiency of formation of the assembly (Assemb.) I and assembly II intermediates (10 and 90 min of incubation, respectively) and porin complexes (30 min of incubation) in tom7Δ and Tom7 † mitochondria were quantified. Error bars represent S.D. from three independent experiments. The amounts of the assembled complexes in corresponding WT mitochondria were set to 100%. D and E, WT, tom7Δ, and Tom7 † mitochondria were incubated with radiolabeled Mdm10 or porin at 23 °C for indicated times. Then mitochondria were treated with 10 μg/ml PK on ice for 20 min and were extracted with 0.1 M Na2CO3. Supernatants (sup) and pellets (ppt) were separated by ultracentrifugation (100,000 g for 30 min) and analyzed by SDS-PAGE and radioimaging (D, upper panels). The PK-resistant pellets were quantified (D, lower panel). The mitochondria were solubilized with 1% digitonin and subjected to BN-PAGE and radioimaging (E, upper panel). Mdm10 assembled in the TOB complex was quantified (E, lower panel). The single and double asterisks indicate nonspecific and uncharacterized bands, respectively.

The mitochondria were reisolated, solubilized with digitonin, and subjected to BN-PAGE analyses to follow assembly of Tom40 and porin in the outer membrane. Assembly of Tom40 in WT mitochondria proceeds via two assembly intermediates of 250 kDa (assembly I) at the level of the TOB complex and 100 kDa (assembly II) before the final 450-kDa complex is formed (Fig. 4B) (30). On the other hand, porin and other β-barrel proteins form their final assembly structures without accumulating intermediates at the level of the TOB complex (Fig. 4B).

When imported into tom7Δ mitochondria, the assembly I intermediate of Tom40 was less accumulated than WT mitochondria (Fig. 4B, upper left panel). On the other hand, when imported into Tom7 † mitochondria, the assembly I intermediate accumulated at longer incubation time than WT mitochondria (Fig. 4, B, lower left panel, and C). Interestingly, assembly of porin was impaired in tom7Δ mitochondria but was somehow enhanced in Tom7 † mitochondria (Fig. 4, B, right panel, and C).
panels, and C). These results with tom7Δ and Tom7 → mitochondria are consistent with those previously observed for Mdm10 → and Mdm10 ← (by the mutation in the b-signal) mitochondria, respectively (19), as will be discussed under “Discussion.”

Because Mdm10 itself is a b-barrel protein, we compared the effects of the protein level of Tom7 on association with the TOB complex between imported Mdm10 and endogenous Mdm10. Radiolabeled Mdm10 was imported into isolated WT, tom7Δ, or Tom7 → mitochondria in vitro. Then import efficiency or outer membrane insertion efficiency was analyzed by PK treatment followed by carbonate extraction and the association yield with the TOB complex by BN-PAGE. Although outer membrane insertion efficiency of Mdm10 was the largest for WT mitochondria (Fig. 4D), the association yield of imported Mdm10 with the TOB complex was the largest for tom7Δ mitochondria (Fig. 4E). Different effects of the Tom7 depletion between the outer membrane insertion and association with the TOB complex suggest that import via the TOB complex and association with the TOB complex are separable and distinct processes for Mdm10. In other words, radiolabeled Mdm10 is inserted into the outer membrane via the TOB complex first and then enters the TOB complex again, and the latter step is promoted by the absence of Tom7, although the former step is retarded by Tom7 depletion. This indicates that the TOB complex-bound Mdm10 that is enhanced by the absence of Tom7 most likely represents a structural constituent of the TOB complex but not an assembly intermediate.

Overexpression of Tom7 and Tom40 Causes Synthetic Growth Defects in Yeast Cells—Recently, Kornmann et al. (18) found that MMM1 complex is localized in the juxtaposition between the ER and mitochondria, which may facilitate phospholipid transfer and/or calcium signaling. Indeed, depletion of any of the MMM1 subunits, Mmm1, Mmm2, Mdm10, and Mdm12, was found to decrease the amounts of cardiolipin (CL) and/or phosphatidylethanolamine (PE), the synthesis of which involves cooperation of the enzymes localized in mitochondria or in the ER (18, 19, 31). We thus asked whether the protein level of Tom7 affects the composition of phospholipids such as CL and PE. Total phospholipids were extracted from WT, tom7Δ, and Tom7 → cells grown in the presence of 32P, and were separated by thin layer chromatography. As shown in Fig. 5A, the lipid levels of CL or PE in tom7Δ and Tom7 → cells were similar to those in WT cells. This suggests that Tom7, which is partly accommodated in the MMM1 complex, controls segregation of Mdm10 between the TOB and MMM1 complexes, yet it is not involved in the phospholipid transfer by the MMM1 complex.

To further assess the roles of Tom7 in the Tom40 assembly in vivo, we tested the effects of deletion or overexpression of Tom7 together with overexpression of Tom40 on cell growth. Deletion of the TOM7 gene did not affect the cell growth irrespective of overexpression of Tom40 (Fig. 5B). However importantly, simultaneous overexpression of Tom7 and Tom40 caused synthetic growth defects (Fig. 5C, Galactose). Decrease in the level of Mdm10 by the KY mutation (K484A,Y491A) or KFY mutation (K484A,F489A,Y491A) (19) led, like the Tom7 overexpression, to synthetic growth defects with the Tom40 overexpression (Fig. 5D, Galactose). These results suggest that sequestration of Mdm10 by overexpressed Tom7 cannot manage proper assembly of overexpressed Tom40, confirming that Tom7 contributes to proper assembly of Tom40 in vivo.

**DISCUSSION**

Yeast mitochondria contain at least four b-barrel proteins in the outer membrane as follows: Tom40, porin, Mdm10,
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and Tob55. These β-barrel membrane proteins take the common pathway for their assembly into the outer membrane; they use the TOM40 complex to cross the outer membrane and subsequently the TOB complex to be inserted into the outer membrane from the intermembrane space side. However, it is also evident that there is a clear difference between the assembly process of Tom40 and those of other β-barrel membrane proteins at least in vitro. First, when imported in vitro, Tom40 accumulates transiently at the level of the TOB complex as the assembly I form during its assembly, although other β-barrel proteins do not. Second, although assembly of all the β-barrel proteins requires the TOBcore complex without Mdm10, assembly of only Tom40 requires the TOBholo complex containing Mdm10 (17, 19). We recently proposed the following model that is consistent with the above observations (19). After crossing the outer membrane through the TOM40 channel, Tom40 and other β-barrel proteins associate with the TOBcore complex. Then association of Mdm10 with the TOBcore complex to form the TOBholo complex promotes simultaneous dissociation of Tom40 from the TOBcore complex, although other β-barrel proteins can leave the TOBcore complex without requiring assistance of Mdm10. The results in this study further support this model.

When Tom7 is depleted (this study) or Mdm10 is overexpressed (19), Mdm10 available for association with the TOB complex increases, resulting in an increase in the TOBholo complex containing Mdm10 (Fig. 1B) (19). Because Mdm10 and assembling β-barrel proteins are mutually exclusive on the TOB complex, the increase in the TOBholo complex over the TOBcore complex leads to inefficient association of β-barrel proteins with the TOB complex, which leads to retarded assembly of the porin in the outer membrane, for example (Fig. 4B) (19). However, effects of the increase in the TOBholo complex on the assembly of Tom40 into the TOM40 complex is not that simple. Because Mdm10 free of Tom7 accelerates dissociation of Tom40 from the TOB complex, a moderate increase in Mdm10 available for association with the TOB complex in tom7Δ mitochondria could enhance the entire assembly process of Tom40 despite the decreased efficiency (50% of WT, Fig. 4C) of the formation of the assembly I intermediate (Fig. 4B). However, when Mdm10 that can form the TOBholo complex was increased significantly in Mdm10 Δ mitochondria, the assembly process of Tom40 is hampered to 10% of WT mitochondria, which blocks entry of most Tom40 molecules into the TOBcore complex, so that formation of the final TOM40 complex is strongly impaired (19).

In Neurospora crassa mitochondria lacking Tom7, in vitro imported Tom40 was rapidly accumulated at the final 40-kDa TOM40 complex without obvious formation of any transient intermediate (32), which is compatible with our observation. However, Meisinger et al. (24) reported that the formation of the assembly I intermediate occurred with approximate WT efficiency in yeast tom7Δ mitochondria, although the subsequent assembly of Tom40 into the final TOM40 complex was enhanced in tom7Δ mitochondria. Unaffected formation of the assembly I intermediate is inconsistent with our present observation (Fig. 4B), but the reason for the discrepancy is not clear. Meisinger et al. (24) also observed that yeast mitochondria lacking Tom7 were impaired in porin assembly, which fits our observations (Fig. 4B). In N. crassa mitochondria lacking Tom7, assembly of porin was retarded in vitro as well (32).

When Tom7 was in turn overexpressed, Mdm10 was recruited to the Tom7 that overflowed from the TOM40 complex, resulting in conversion of the TOB complex from its holo-form to a core form. The increase in the TOBcore complex without Mdm10 enhances association of Tom40 and other β-barrel proteins with the TOB complex and accelerates subsequent assembly of β-barrel proteins except for Tom40 (Fig. 4B). Because Tom40 requires Mdm10 for its dissociation from Tom40 from the TOB complex, a decrease in Mdm10 available for association with the TOB complex impairs the Tom40 assembly in the early step as observed previously for mitochondria with decreased Mdm10 with a mutated β-signal (19).

By using in vivo site-specific photocross-linking, we found that Tom7 directly interacts with Tom40 and Mdm10 and that the binding sites for Tom40 and for Mdm10 are close to each other but are not the same (Fig. 3D). The physical contact of the TM segment of Tom7 with Tom40 probably provides the first experimental evidence for the direct interaction between the membrane-embedded parts of the α-helical integral membrane protein (Tom7) and β-barrel membrane protein (Tom40) in the same complex. Because binding sites for Tom40 and Mdm10 are on the same side of the α-helical TM segment of Tom7, it is unlikely that Tom40 and Mdm10 interact with Tom7 simultaneously. Rather, the Tom7 that overflowed from the TOM40 complex may well recruit Mdm10, and this Mdm10–Tom7 pair at least partly associates with the MMM1 tethering complex. Although the presence of Tom7 is not essential for possible lipid transfer functions of the MMM1 complex, Tom7 appears to increase efficiency of the assembly of Mdm10 into the MMM1 complex (Fig. 2E). Involvement of Tom7 in other functions of the MMM1 complex remains to be studied in the future.

Because the dynamic equilibrium of Mdm10 between the TOB complex-bound form and Tom7-bound form may be important for regulation of the timing of the Tom40 assembly with its partner, the protein level of Mdm10 needs to be precisely regulated. In connection to this, depletion of Tom7, which does not impair the in vitro assembly of Tom40 (Fig. 4B), does not cause serious cell growth defects (Fig. 5B). On the other hand, overexpression of Tom7, which retards the assembly process of Tom40 in vitro (Fig. 4B), resulted in cell growth defects when Tom40 was also overexpressed (Fig. 5C). This is consistent with our model that Mdm10 in cooperation with Tom7 might contribute to adjusting the timing of Tom40 release from the TOB complex for subsequent assembly with the other subunits of the TOM40 complex.

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