Two novel proteins in the mitochondrial outer membrane mediate β-barrel protein assembly

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Mitochondrial outer and inner membranes contain translocators that achieve protein translocation across and/or insertion into the membranes. Recent evidence has shown that mitochondrial β-barrel protein assembly in the outer membrane requires specific translocator proteins in addition to the components of the general translocator complex in the outer membrane, the TOM40 complex. Here we report two novel mitochondrial outer membrane proteins in yeast, Tom13 and Tom38/Sam35, that mediate assembly of mitochondrial β-barrel proteins, Tom40, and/or porin in the outer membrane. Depletion of Tom13 or Tom38/Sam35 affects assembly pathways of the β-barrel proteins differently, suggesting that they mediate different steps of the complex assembly processes of β-barrel proteins in the outer membrane.

Introduction

Mitochondria are essential organelles in eukaryotic cells that are bounded by two biological membranes. Most mitochondrial proteins are synthesized on the cytoplasmic ribosomes and imported into mitochondria. Protein import into mitochondria is mediated by translocators in the mitochondrial membranes, the translocator of the mitochondrial outer membrane (TOM)40 complex in the outer membrane, and the translocator of the mitochondrial inner membrane (TIM)23 and TIM22 complexes in the inner membrane (Herrmann and Neupert, 2000; Endo et al., 2003; Wiedemann et al., 2004a). They function as receptors for mitochondrial proteins and protein-conducting channels through which proteins cross the hydrophobic barrier of the membrane. The protein-conducting channel of the TOM40 complex mainly consists of Tom40, a β-barrel protein (Hill et al., 1998; Künkele et al., 1998). Recently, a new pathway has been identified for assembly of β-barrel proteins in the mitochondrial outer membrane (Wiedemann et al., 2003).

The mitochondrial β-barrel proteins, including Tom40 and porin, are synthesized without a cleavable presequence. They first interact with the TOM40 complex for recognition of internal targeting signals and translocation at least partly across the outer membrane (Wiedemann et al., 2003). Then, their assembly into the outer membrane in a barrel form of β-strands requires outer membrane proteins including Mas37 and Sam50/Tob55 that do not comprise the TOM40 complex. Depletion or functional defects of Mas37 (Wiedemann et al., 2003) or Sam50 (Paschen et al., 2003; Kozjak et al., 2003; Gentle et al., 2004) leads to impairment of the assembly of Tom40 and porin into the outer membrane. Mas37 and Sam50 appear to form a large membrane–protein complex, a second, more specialized TOM complex (the SAM/TOB complex; Kozjak et al., 2003; Paschen et al., 2003). Recently, small Tim proteins in the intermembrane space have also been shown to mediate the assembly of porin and Tom40 in the outer membrane (Hoppins and Nargang, 2004; Wiedemann et al., 2004b).

In the present study, we looked for other components, if any, that mediate assembly of β-barrel proteins in the mitochondrial outer membrane in yeast. For this purpose, we took advantage of the fact that many mitochondrial proteins mediating mitochondrial protein assembly/import are essential or important for the yeast cell viability (Rehling et al., 2003). We systematically analyzed the localization of yeast proteins that are indicated in the database to have essential but unknown functions and identified two novel proteins, Tom13 and Tom38, in the mitochondrial outer

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membrane. We found that these proteins are involved in the assembly of β-barrel proteins, Tom40, and/or porin in the outer membrane.

Results and discussion

Tom13 and Tom38 are mitochondrial outer membrane proteins

Tom13, the gene product of *YOL026C*, and Tom38, that of *YHR083W*, are deposited as essential proteins in the database of the yeast deletion project (Winzeler et al., 1999; http://www-deletion.stanford.edu/YDPM/YDPM_index.html). We analyzed their properties in mitochondrial association by protease treatment and alkaline extraction of isolated yeast mitochondria followed by immunoblotting for the HA or FLAG epitope tags attached to the COOH termini of Tom13 (Fig. 1, Tom13-HA) and Tom38 (Fig. 1, Tom38-FLAG). Both Tom13-HA and Tom38-FLAG were functional in vivo because they restored the growth defects of the null mutants of their original genes (unpublished data). When mitochondria were treated with proteinase K (PK), both Tom13 and Tom38 disappeared, suggesting that their COOH termini are exposed to the cytosol (Fig. 1, lanes 1 and 2). Tom70, a surface-exposed outer membrane protein, was degraded; whereas Tim23, an inner membrane protein exposing a domain to the intermembrane space, and Mdj1p, a soluble matrix protein, remained intact after PK treatment. When the outer membrane of the mitochondria was selectively ruptured to make mitoplasts, treatment of the mitoplasts with PK led to degradation of Tom13, Tom38, Tom70, and Tim23, but not of Mdj1p (Fig. 1, lanes 3 and 4). This finding confirms that Tom13 and Tom38 reside outside the inner membrane. Tom13 was, like integral membrane proteins Tom70 and Tim23, not extracted by alkaline treatment of mitochondria, but was solubilized by treatment of mitochondria with Triton X-100 (Fig. 1, lanes 5–8). In contrast, Tom38 was extracted by both alkaline treatment of mitochondria or treatment of mitochondria with Triton X-100 (Fig. 1, lanes 5–8). These results indicate that Tom13 and Tom38 are an integral membrane protein and a peripheral membrane protein, respectively, of the mitochondrial outer membrane and expose at least their COOH termini to the cytosol.

Tom13 and Tom38 comprise 113 and 329 amino-acid residues, respectively, and their calculated molecular masses are 12.8 and 37.4 kD, respectively. The BLAST searches allowed us to find only a few homologues of Tom13 and Tom38 in other organisms (see online supplemental material, available at hppt://www.jcb.org/cgi/content/full/jcb.200405138/DC1). The amino acid sequence of Tom13 has a hydrophobic segment (residues 39–68) in the middle of the molecule, a part of which could span the membrane once or twice, leaving the COOH terminus exposed to the cytosol. In contrast, the amino acid sequence of Tom38 does not exhibit a hydrophobic segment that is long enough to span the membrane. During preparation of the manuscript on this study, identification of the gene product of *YHR083W* was reported, and it was termed Sam35 (Milenkovic et al., 2004). Because the name of Sam35 does not reflect its molecular
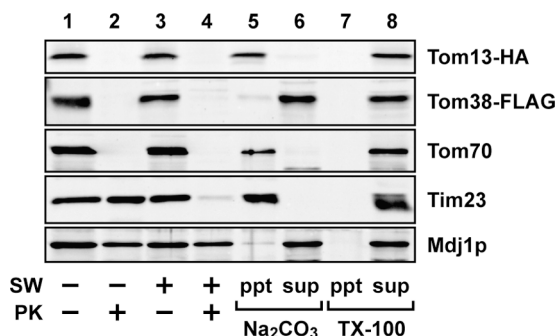

Figure 1. **Tom13 and Tom38 are mitochondrial outer membrane proteins.** Mitochondria were prepared from yeast strains expressing Tom13-HA or Tom38-FLAG. Mitochondria and mitoplasts generated by osmotic swelling (SW) were treated with 500 μg/ml PK for 30 min on ice. Mitochondria were treated with either 0.1 M Na_2CO_3 or 1% Triton X-100 with 500 mM NaCl (TX-100), and then pellets (ppt) and supernatants (sup) were separated by centrifugation. Proteins were detected by immunoblotting with the antibodies against the HA epitope (for Tom13-HA mitochondria), the FLAG epitope (for Tom38-FLAG mitochondria), or indicated proteins (for Tom38-FLAG mitochondria).

mass (37.4 kD), we tentatively call it Tom38 instead of Sam35 according to the original rule of the agreed nomenclature of the proteins involved in mitochondrial protein import/assembly (Pfanner et al., 1996).

Tom13 and Tom38 are involved in protein import into mitochondria in vivo

To assess the functions of Tom13 and Tom38, we constructed yeast strains GAL-TOM13 and GAL-TOM38 in which the galactose-inducible *GAL7* promoter was integrated into the chromosome in front of *YOL026C* and *YHR083W*, respectively, to achieve regulated expression of Tom13 and Tom38 by galactose. Tom38 is essential for viability of yeast cells as confirmed by tetrad analysis (Fig. 2 B), and the GAL-TOM38 cell growth slowed down significantly 20 h after shift to galactose-free medium (Fig. 2 A).

Although the growth of GAL-TOM13 cells also slowed down 30 h after shift to galactose-free medium, they did not reach complete growth arrest 60 h after the shift (Fig. 2 A). This finding prompted us to reexamine the essentiality of the *TOM13* gene in yeast cell viability. When we deleted the *TOM13* gene in diploid cells and subjected them to tetrad analysis, two out of the four spores grew normally while the other two yielded significantly slow-growing colonies (Fig. 2 B). The latter colonies indeed had the disrupted *TOM13* gene and did not grow on nonfermentable medium (unpublished data), suggesting that depletion of the *TOM13* gene is not lethal but renders cells respiration deficient. The apparent discrepancy between our observation and the results of the yeast deletion project (Winzeler et al., 1999) is not clear.

Total lysates were prepared from wild-type, GAL-TOM13, or GAL-TOM38 cells at various times after shift from galactose-containing medium to galactose-free medium and were analyzed by immunoblotting for various mitochondrial proteins (Fig. 2 C). The amounts of nonmitochondrial Ssa1p and Sec63p or Tom70, a mitochondrial outer membrane
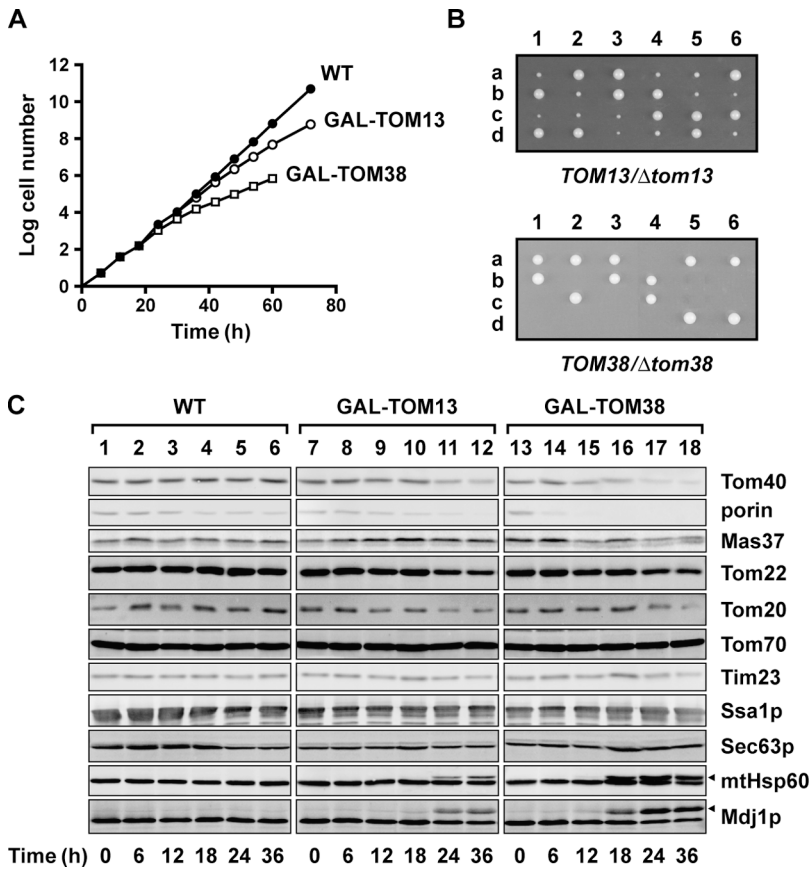
A

B

C

Figure 2.   **Tom13 and Tom38 are involved in mitochondrial protein import in vivo.** (A) Yeast strains in which Tom13 or Tom38 is down-regulated slowed their growth on galactose-free medium. Wild-type cells (WT) and those carrying the *TOM13* (GAL-TOM13) or *TOM38* gene (GAL-TOM38) under control of the *GAL7* promoter were first grown on galactose-containing medium and then transferred to galactose-free medium. (B) One of the two chromosomal *TOM13* (top) or *TOM38* (bottom) genes in a yeast diploid strain, W303-AB, was disrupted, the diploid cells were sporulated, and six different asci were dissected. The four spores recovered from each asci were allowed to germinate and to grow on YPD for 45 h at 30°C. (C) Total lysates were prepared from WT, GAL-TOM13, and GAL-TOM38 cells, which were grown at 23°C for12 h in lactate medium (+0.1% galactose), diluted, and then grown at 23°C for the indicated hours in lactate medium (+0.1% glucose). Total proteins were isolated and analyzed by SDS-PAGE and immunoblotting with antibodies against the indicated proteins. The arrowheads indicate the accumulated precursor forms of mtHsp60 and Mdj1p.

protein with the NH$_2$-terminal transmembrane anchor, were not affected in GAL-TOM13 or GAL-TOM38 cells as well as in wild-type cells by the shift to galactose-free medium. However, the amounts of β-barrel Tom40 in GAL-TOM13 and GAL-TOM38 cells decreased 24 and 12 h after the shift to galactose-free medium, respectively. Depletion of Tom13 also led to dissociation of the intact TOM40 complex 18 h after the shift to galactose-free medium (see online supplemental material). The level of another β-barrel protein, porin, was significantly lowered in GAL-TOM38 cells as compared with that in wild-type cells. The amount of Tom22 (the subunit of the TOM40 complex) also decreased, and precursor forms of the matrix proteins mtHsp60 and Mdj1p accumulated in parallel with the decrease in the amount of Tom40 in both GAL-TOM13 and GAL-TOM38 cells. Similar observation that the precursor forms of mitochondrial proteins accumulated in cells was previously made upon depletion of Sam50 (unpublished data). These results suggest the roles of Tom13 and Tom38 in mitochondrial protein import in vivo.

Tom13 and Tom38 are involved in different steps of the assembly of β-barrel outer membrane proteins

We tested the in vitro import abilities of mitochondria isolated from Tom13-depleted (Tom13↓) cells and from Tom38-depleted (Tom38↓) cells after 14 and 10 h cultivation, respectively, in the absence of galactose for various radiolabeled precursor proteins. Mitochondria isolated from Tom13↓ and Tom38↓ cells did not exhibit decrease in ΔΨ,

which is essential for protein import via the TIM23 or TIM22 complexes (unpublished data). We analyzed the import of radiolabeled matrix and inner-membrane proteins: the precursor of mtHsp60, a presequence-containing precursor protein that uses the TOM40 complex and the TIM23 complex to move across the outer and inner membranes, respectively, and ADP/ATP carrier (AAC), a presequence-less polytopic inner membrane protein, which uses the TOM40 complex to move across the outer membrane and the TIM22 complex to be inserted into the inner membrane. The import rates of the precursor of mtHsp60 (Fig. 3 A) and of AAC (Fig. 3 B) were not affected by depletion of Tom13 or Tom38 at all.

Next, we analyzed the assembly of Tom40 in wild-type, Tom13↓, and Tom38↓ mitochondria. Model et al. (2001) reported that Tom40 was assembled into the 450-kD TOM40 complex via two successive intermediates of the 250-kD complex (the assembly intermediate I) and the 100-kD complex (the assembly intermediate II). Indeed, when we incubated radiolabeled Tom40 with isolated mitochondria from wild-type cells (14 or 10 h after shift to galactose-free medium) and analyzed the proteins by blue-native PAGE (BN-PAGE), we observed its accumulation in both the 250-kD double bands and the 100-kD band, and subsequently in the 450-kD band (Fig. 4 A, lanes 1–5). The 250-kD band represents the assembly intermediates I involving the SAM complex and the 450-kD band represents the final TOM40 complex because addition of the anti-Mas37 antibodies and the anti-Tom22 antibodies, respectively, shifted
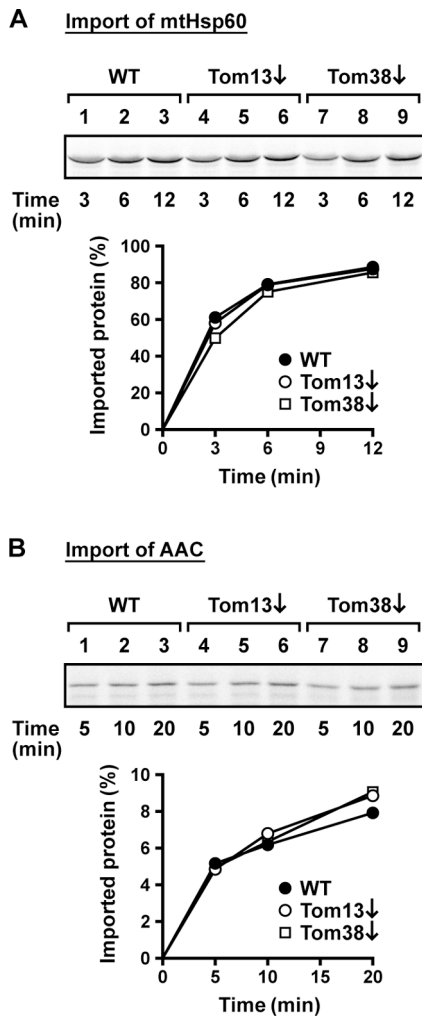
A   Import of mtHsp60

B   Import of AAC

Figure 3.   **Depletion of Tom13 or Tom38 does not affect import of mtHsp60 or AAC into mitochondria in vitro.** Mitochondria were isolated from yeast strains W303-1A (WT), GAL-TOM13 (Tom13↓), and GAL-TOM38 (Tom38↓) after cultivation in lactate medium (+0.1% glucose) for 14, 14, and 10 h, respectively, at 23°C. Radiolabeled mtHsp60 precursor and AAC were incubated with Tom13↓ (lanes 4–6 and open circles), Tom38↓ (lanes 7–9 and open squares), and WT mitochondria (lanes 1–3 and closed circles) at 23°C for the indicated times. The mitochondria were treated with PK, and the imported proteins were analyzed by SDS-PAGE and radioimaging. The amounts of radiolabeled proteins added to each reaction are set to 100%.

them to a higher molecular mass range on the BN-PAGE gel (Fig. 4 B, lane 3 and 4).

When we incubated Tom40 with Tom13↓ mitochondria, its assembly into the final TOM40 complex was markedly retarded (Fig. 4 A, lanes 6–10), whereas incorporation into the assembly intermediate I or II was not affected significantly. Although Tom40 accumulated more in the lower molecular mass band of the double bands for the assembly intermediate I, both bands represent the assembly intermediate I because they shifted to a higher molecular mass range upon addition of the anti-Mas37 antibodies (Fig. 4 B, lane 6). These results suggest that Tom13 is involved in the late step of the Tom40 assembly between the assembly intermediate II and the final TOM40 complex.

In contrast, when we incubated Tom40 with Tom38↓ mitochondria, Tom40 associated with the 450-kD TOM40 complex without incorporation into the assembly intermediate I or II. The resulting 450-kD complex involves Tom22 because addition of the anti-Tom22 antibodies caused a shift of the band to a higher molecular mass range (Fig. 4 B, lane 8). We reasoned that the observed association of Tom40 with the TOM40 complex did not reflect the normal assembly of Tom40, but instead nonproductive association of the Tom40 precursor with the preexisting TOM40 complex. To test this possibility, we assessed the membrane topology of radiolabeled Tom40 from the Tom38↓ mitochondria by alkaline extraction and trypsin digestion of both mitochondria and mitoplasts. Endogenous Tom40 is resistant to alkaline extraction and produces a slightly smaller form by the cleavage of a 2.5-kD peptide by trypsin (Fig. 4 C, lanes 10 and 11; Hill et al., 1998). Indeed, radiolabeled Tom40 imported into Tom38↓ mitochondria (in the 450-kD complex) was more sensitive to trypsin digestion and alkaline extraction than that into wild-type mitochondria (Fig. 4 C, lanes 3–8 and their quantification), suggesting that acquisition of the final assembly state of radiolabeled Tom40 was somehow defective in Tom38↓ mitochondria. Interestingly, mitochondria with the temperature-sensitive mutant of Tom38/Sam35 (Milenkovic et al., 2004) showed the block of in vitro synthesized Tom40 at the level of the assembly intermediate I, which is consistent with our observation, but showed no association with the 450-kD TOM40 complex, which exhibits a contrast to our observation with Tom38↓ mitochondria. Perhaps the temperature-sensitive Tom38 mutant can still allow the SAM complex to receive Tom40 from the TOM40 complex, yet the resulting Tom40-containing complex is unstable.

We also tested in vitro import of another β-barrel outer membrane protein, porin, into wild-type, Tom13↓, and Tom38↓ mitochondria (Fig. 4 D). Insertion of porin into the outer membrane is reflected in its resistance against protease digestion in vitro (Krimmer et al., 2001), as observed for wild-type mitochondria (Fig. 4 D). Interestingly, import of porin into mitochondria was substantially impaired in Tom38↓ mitochondria, but not in Tom13↓ mitochondria. A possible explanation for this observation is that, although Tom40 and porin use Tom38 for their assembly, Tom40, but not porin, requires additional components including Tom13 in the late step of its assembly. This means that the assembly pathways of porin and Tom40 branch out past the step of association with the SAM complex.

Tom38 forms a complex with Mas37 and Sam50

Because Tom38 plays a role in the Tom40 assembly similar to those by Mas37 and Sam50, Tom38 could be a subunit of the SAM complex. This is indeed the case (Fig. 5). When solubilized mitochondria with Tom38-FLAG were analyzed by BN-PAGE, the anti-FLAG antibody detected four bands with apparent molecular masses of 360, 290 (Fig. 5 A, lane 1, band a), 230 (Fig. 5 A, lane 1, band b), and 170 kD (Fig. 5 A, lane 1, band c). Bands a and b shifted to a higher molecular mass region upon addition of the anti-Mas37 antibodies (Fig. 5 A, lane 2). When solubilized mitochondria
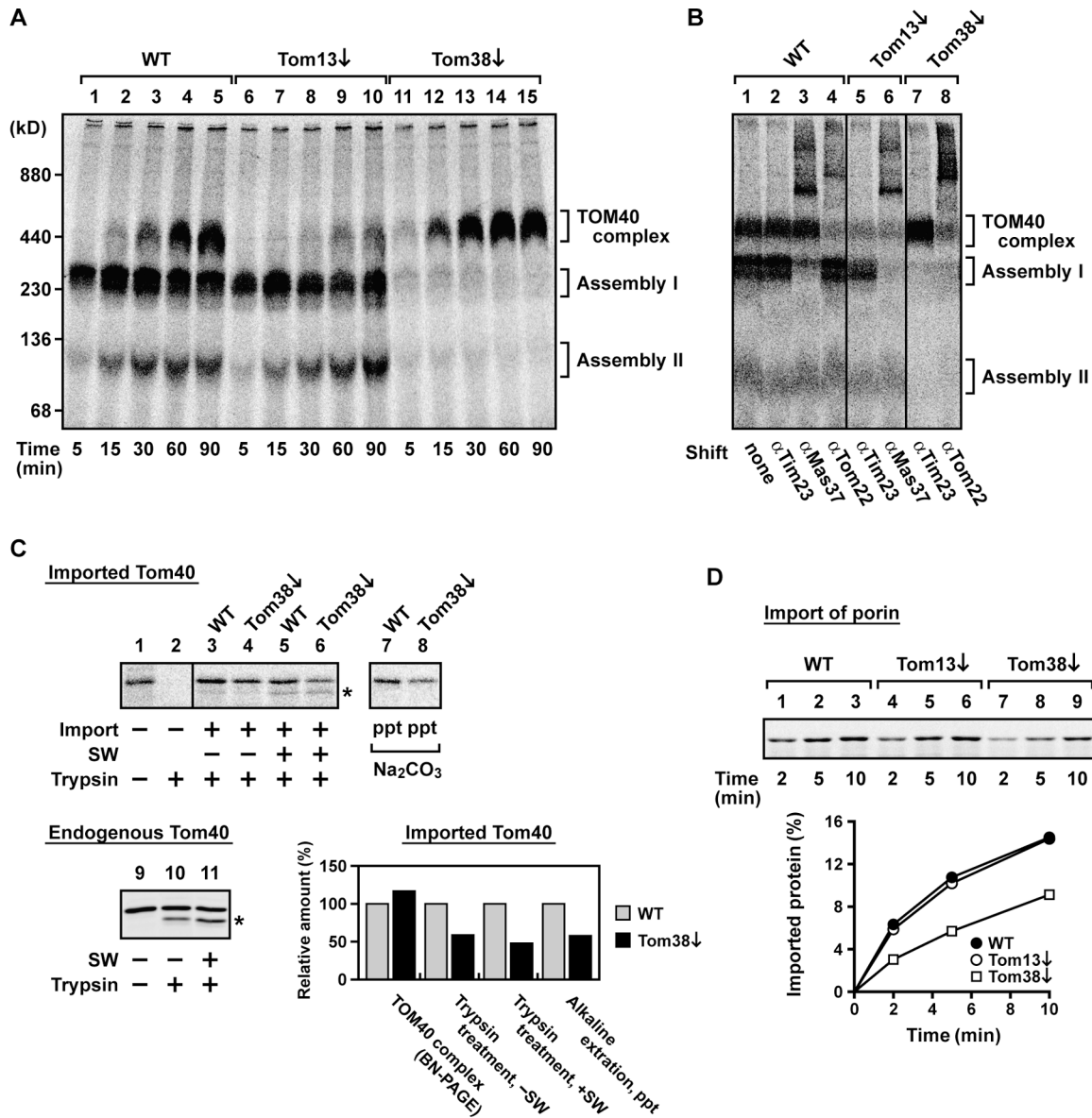
Figure 4.   **Depletion of Tom13 or Tom38 affects assembly of Tom40 and porin in the mitochondrial outer membrane in vitro.** (A) Mitochondria were isolated from yeast strains W303-1A (WT), GAL-TOM13 (Tom13↓), and GAL-TOM38 (Tom38↓) after cultivation in lactate medium (+0.1% glucose) for 14, 14, and 10 h, respectively, at 23°C. Radiolabeled yeast Tom40 was incubated with WT, Tom13↓, and Tom38↓ mitochondria at 23°C for the indicated times. The mitochondria were reisolated by centrifugation, solubilized with 1% digitonin (20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.5 mM EDTA, and 10% glycerol) for 10 min on ice, and subjected to BN-PAGE analyses followed by radioimaging. Assembly I, the assembly intermediate I; Assembly II, the assembly intermediate II. (B) Radiolabeled Tom40 was imported into WT, Tom13↓, and Tom38↓ mitochondria for 60 min at 23°C. After reisolation, the mitochondria were solubilized with 1% digitonin; incubated with the antibodies against Tim23 (αTim23), Mas37 (αMas37), and Tom22 (αTom22) or without antibodies (none); and analyzed by BN-PAGE followed by radioimaging. (C) Radiolabeled Tom40 was imported into WT and Tom38↓ mitochondria for 90 min at 23°C and subjected to osmotic swelling (SW), trypsin treatment (100 μg/ml for 30 min on ice), or alkaline extraction (Na_2CO_3, as Fig. 1). As a control, translated Tom40 was treated with (lane 2) or without (lane 1) trypsin. Relative amounts of radiolabeled Tom40 imported into Tom38↓ mitochondria were compared with those into wild-type mitochondria (set to 100%): TOM40 complex (BN-PAGE), lane 15/lane 5 in A; Trypsin treatment, −SW, lane 4/lane 3 in C; Trypsin treatment, +SW, lane 6/lane 5 in C; Alkaline extraction, ppt, lane 8/lane 7 in C. Endogenous Tom40 was also subjected to similar trypsin treatment followed by immunoblotting with anti-Tom40 antibodies. Asterisks indicate a clipped form of Tom40. (D) In vitro import of porin into WT (closed circles), Tom13↓ (open circles), and Tom38↓ (open squares) mitochondria were performed at 23°C as described in Fig. 3. The amounts of imported, PK-protected protein were plotted.

with Sam50 bearing the COOH-terminal FLAG epitope tag (Sam50-FLAG) were analyzed by BN-PAGE, the anti-FLAG antibody detected bands a, b, and c (Fig. 5 A, lane 3), and addition of the anti-Mas37 antibodies caused shifts of bands a and b, but not band c (Fig. 5 A, lane 4). These re-

sults suggest that bands a and b represent a complex consisting of Mas37, Tom38, and Sam50. Band c may represent a complex containing Tom38 and Sam50, but not Mas37. Evidently, Tom38 forms a complex with Mas37 and Sam50, although interactions among these subunits may be some-

A
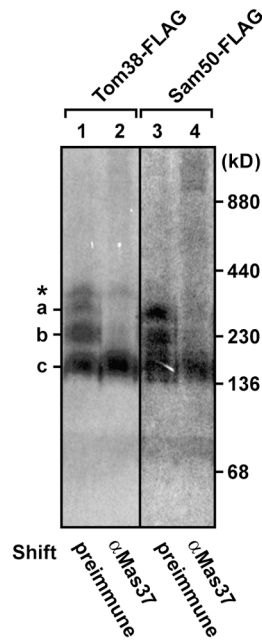
B
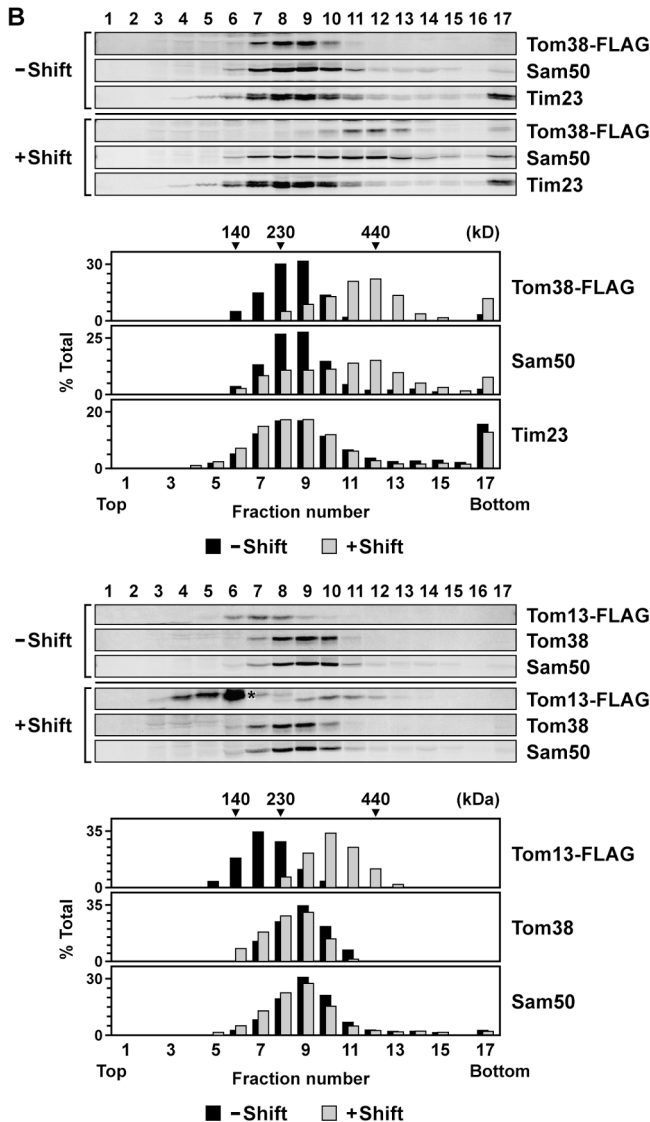
how dynamic. The interactions between Tom38 and Sam50 were also confirmed by coimmunoprecipitation (see online supplemental material).

When solubilized mitochondria containing Tom38-FLAG or Tom13-FLAG were subjected to glycerol density gradient centrifugation, the apparent molecular masses of Tom38-FLAG and Sam50 were 280 kD, and both Tom38-FLAG and Sam50 were shifted to the fractions of 440 kD by prior incubation with the anti-FLAG antibody (Fig. 5 B, top). In contrast, the apparent molecular mass of Tom13-FLAG was 180 kD, and the anti-FLAG antibody caused shift of Tom13-FLAG, but not of Tom38 or Sam50, to the fractions of 330 kD (Fig. 5 B, bottom). Therefore, Tom13 constitutes a complex of 180 kD, which is distinct from the SAM complex, but mediates the Tom40 assembly. Tom13 may be perhaps a component specific for assembly of only Tom40. Evidently, the pathway of the β-barrel protein assembly in the outer membrane is more complex than previously envisaged and may well consist of substrate protein–specific sub-pathways that depend on TOM (SAM) components differently.

Materials and methods

Import assays

Radiolabeled precursor proteins were synthesized in rabbit reticulocyte lysates by coupled transcription/translation in the presence of [^{35}S]methionine. Mitochondria were isolated from yeast strains W303-1A, GAL-TOM13, and GAL-TOM38. Import reactions and following treatment with PK and Na_2CO_3 were preformed as described previously (Yamamoto et al., 2002). BN-PAGE analyses were performed according to Dekker et al. (1997).

Online supplemental material

Online supplemental material describes construction of plasmids and yeast strains, growth conditions for yeast strains, possible homologues of Tom13 and Tom38, effects of Tom13 depletion on the 450-kD TOM40 complex, and interactions between Tom38 and Sam50 as detected by coimmunoprecipitation. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200405138/DC1.

Figure 5. **Tom38 forms a complex with Mas37 and Sam50.** (A) Mitochondria containing Tom38-FLAG or Sam50-FLAG were solubilized as in Fig. 4 A and incubated with preimmune serum (Shift, preimmune) or anti-Mas37 antibodies (Shift, αMas37) for 1 h on ice. Solubilized protein complexes were analyzed by BN-PAGE and immunoblotting with the anti-FLAG antibody. The band marked with an asterisk is variable in different experiments. (B) Mitochondria containing Tom38-FLAG (top) or Tom13-FLAG (bottom) were solubilized with 0.8% digitonin (25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 50 mM 6-aminohexanoic acid, and 10% glycerol) in the presence (+Shift) or absence (−Shift) of the anti-FLAG antibody for 1 h on ice. Solubilized protein complexes were analyzed by glycerol density gradient centrifugation (10–30% glycerol and 0.2% digitonin in the same buffer for solubilization) at 200,000 g for 6 h at 4°C. After centrifugation, fractions were collected from the top and analyzed by immunoblotting using antibodies against the FLAG epitope, Tom38, Sam50, and Tim23. Numbers indicate fractions (from top to bottom). The asterisk indicates IgG.

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