

The N-terminal domain of Tob55 has a receptor-like function in the biogenesis of mitochondrial β -barrel proteins

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β -Barrel proteins constitute a distinct class of mitochondrial outer membrane proteins. For import into mitochondria, their precursor forms engage the TOM complex. They are then relayed to the TOB complex, which mediates their insertion into the outer membrane. We studied the structure–function relationships of the core component of the TOB complex, Tob55. Tob55 precursors with deletions in the N-terminal domain were not affected in their targeting to and insertion into the mitochondrial outer membrane. Replacement of wild-type Tob55 by these

deletion variants resulted in reduced growth of cells, and mitochondria isolated from such cells were impaired in their capacity to import β -barrel precursors. The purified N-terminal domain was able to bind β -barrel precursors in a specific manner. Collectively, these results demonstrate that the N-terminal domain of Tob55 recognizes precursors of β -barrel proteins. This recognition may contribute to the coupling of the translocation of β -barrel precursors across the TOM complex to their interaction with the TOB complex.

Introduction

Mitochondria and chloroplasts contain β -barrel proteins in their outer membranes (Gabriel et al., 2001; Rapaport, 2003; Schleiff et al., 2003). The only other biological membrane known to harbor β -barrel proteins is the outer membrane of Gram-negative bacteria (Tamm et al., 2001; Wimley, 2003). This situation is believed to reflect the evolutionary origin of mitochondria and chloroplasts from endosymbionts that belong to the class of Gram-negative bacteria.

Little is known about how newly synthesized β -barrel proteins are sorted in the eukaryotic cell, integrated into lipid bilayers, and assembled into oligomeric structures (Rapaport, 2003; Johnson and Jensen, 2004; Voulhoux and Tommassen, 2004; Paschen et al., 2005). In the case of mitochondria, the precursors are initially recognized by the receptor components of the translocase of the outer mitochondrial membrane (TOM) complex, Tom20 and Tom70. They are then translocated through

the import pore of the TOM complex (Rapaport and Neupert, 1999; Schleiff et al., 1999; Krimmer et al., 2001; Model et al., 2001; Rapaport, 2002). From the TOM complex, β -barrel precursors are relayed to another complex in the outer membrane, the topogenesis of mitochondrial outer membrane β -barrel proteins (TOB) complex, also called the sorting and assembly machinery (SAM) complex (Kozjak et al., 2003; Paschen et al., 2003; Wiedemann et al., 2003). On their way from the TOM to the TOB complex, β -barrel precursors are exposed to the intermembrane space (IMS), where they were reported to interact with small Tim components (Hoppins and Nargang, 2004; Wiedemann et al., 2004; Habib et al., 2005).

The major component of the TOB complex is Tob55 (also named Sam50/Omp85). Tob55 was found to be essential for viability of yeast cells and to promote the insertion of β -barrel proteins into the mitochondrial outer membrane (Kozjak et al., 2003; Paschen et al., 2003; Gentle et al., 2004). Homologues of Tob55 appear to be present in virtually all eukaryotes (Paschen et al., 2003; Gentle et al., 2004; Dolezal et al., 2006). Tob55 belongs to a family of β -barrel-shaped transporters, which includes the bacterial Omp85/YaeT (Voulhoux et al., 2003; Wu et al., 2005), alr2269 (Moslavac et al., 2005), and the plastidic Toc75 (Eckart et al., 2002).

Two further proteins, Tob38 (Tom38/Sam35) and Mas37 (Tom37/Sam37), were identified as subunits of the TOB

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Abbreviations used in this paper: BNGE, blue native gel electrophoresis; DHFR, dihydrofolate reductase; IMS, intermembrane space; MBP, maltose binding protein; PK, proteinase K; POTRA, polypeptide transport associated; SAM, sorting and assembly machinery; TOB, topogenesis of mitochondrial outer membrane β -barrel proteins; TOM, translocase of the outer mitochondrial membrane.

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complex (Wiedemann et al., 2003; Ishikawa et al., 2004; Milenkovic et al., 2004; Waizenegger et al., 2004). The essential protein Tob38 is peripherally associated with Tob55 on the cytosolic surface of the outer membrane and, together with Tob55, forms the TOB core complex. Mas37 plays a, so far, undefined role. Another outer membrane component, Mdm10, a β -barrel protein, was also suggested to be a member of the TOB/SAM complex and to promote the assembly of Tom40 precursor (Meisinger et al., 2004). The role of Mdm10 remains to be further clarified, as it was originally identified as a protein with a role in determining morphology and inheritance of mitochondria (Sogo and Yaffe, 1994).

Despite some progress in our understanding of the structure of the TOB complex, the mechanism by which precursors of β -barrel proteins are transferred from the TOM to the TOB complex is still unknown. Moreover, many questions as to the functions of its subunits and their domains remain to be answered. These answers are crucial for obtaining a comprehensive view on the biogenesis of β -barrel membrane proteins. Here, we report on the contribution of the N-terminal domain of Tob55 to the function of the TOB complex. It has receptor-like function in recognizing precursors of β -barrel proteins in the IMS.

Results

The N-terminal domain of Tob55 is facing the IMS

The N-terminal domain of Tob55, comprising ~ 100 amino acid residues, was suggested not to be part of the β -barrel structure (Fig. 1 A) and to be exposed to the IMS (Paschen et al., 2003). Therefore, precursors of β -barrel membrane proteins that are on their way from the TOM to the TOB complex could interact with this domain. Because the location of the N-terminal domain is an essential element in such a working model, we decided to analyze the topology in detail. We used a Tob55 variant that carried a His₈ tag at the N terminus (Paschen et al., 2003) and an assay that monitors the formation of a characteristic fragment upon treatment of mitochondria with protease. As observed before, Tob55 is cleaved by proteinase K (PK) at a single position, resulting in an N-terminal fragment of ~ 30 kD and a C-terminal fragment of ~ 25 kD (Fig. 1 A; Paschen et al., 2003; Habib et al., 2005). Treatment of mitochondria carrying His₈-tagged Tob55 with PK resulted, as expected, in the formation of a 30-kD fragment that could be immunodecorated with antibodies against the His tag (Fig. 1 B). Formation of this fragment was abolished when the outer membrane was ruptured by either osmotic shock or solubilizing the membrane with detergent (Fig. 1 B). Hence, these results imply that the N terminus of Tob55 is exposed to the IMS.

Tob55 precursor lacking the N-terminal domain is targeted to mitochondria and inserted into the outer membrane

To investigate the function of the N-terminal domain of Tob55, we created constructs in which 50, 80, or 102 of the N-terminal amino acid residues were deleted resulting in Tob55 Δ 50,

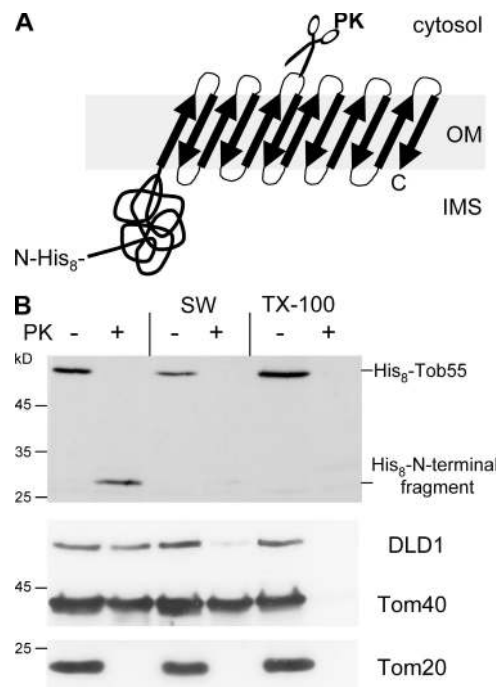


Figure 1. **The N-terminal domain of Tob55 is facing the IMS.** (A) Schematic representation of cleavage of Tob55 by PK, resulting in two proteolytic fragments. (B) Mitochondria from cells expressing His₈-Tob55 were isolated and left intact, subjected to osmotic swelling (SW), or solubilized with Triton X-100 (TX-100). Mitochondria were incubated on ice in the absence or presence of PK. All samples were subjected to SDS-PAGE and immunoblotting with antibodies against His tag (top) or against the marker proteins DLD1 (IMS), Tom40 (embedded in the outer membrane), and Tom20 (an outer membrane protein with a cytosolic domain). Full-length Tob55 and the N-terminal fragment are indicated.

Tob55 Δ 80, and Tob55 Δ 102, respectively. First, we asked whether the N-terminal domain is required for targeting of Tob55 precursor to mitochondria and its subsequent insertion into the outer membrane. To this end, we cloned these *TOB55* variants into a yeast expression vector and transformed wild-type cells with the resulting plasmids. Upon subcellular fractionation, all Tob55 variants were found in the mitochondrial fraction, like the mitochondrial marker proteins Tom20 (Fig. 2 A). Thus, all Tob55 variants are targeted to mitochondria in vivo.

Mitochondrial targeting and membrane integration of Tob55 variants were further studied using an in vitro import assay with radiolabeled precursor proteins and isolated mitochondria. The read out of the assay was the formation of characteristic proteolytic fragments upon PK treatment (Fig. 1; Habib et al., 2005). Correct membrane insertion in vitro of the N-terminal truncated variants was expected to result in smaller N-terminal fragments, whereas the fragments representing the C-terminal part of the protein should remain unchanged. Indeed, upon incubation of radiolabeled Tob55 truncated variants with isolated mitochondria, the expected proteolytic fragments were formed (Fig. 2 B). Therefore, all variants appear to be targeted to mitochondria and become inserted into the outer membrane to reach the native topology.

The topogenesis of newly inserted Tob55 molecules requires the import receptor Tom20, the translocation pore of

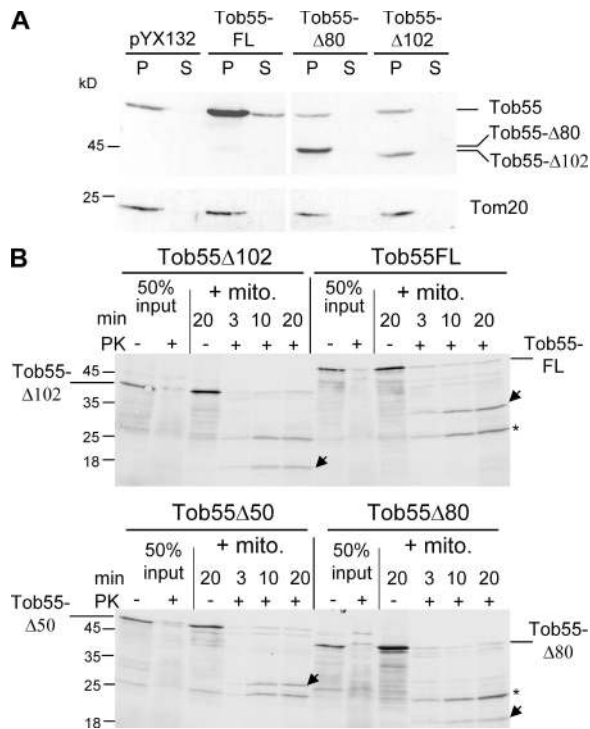


Figure 2. The N-terminal domain of Tob55 precursor molecules is not required for targeting and membrane insertion. (A) Wild-type yeast cells were transformed with either empty vector (pYX132) or vector encoding the indicated variant of Tob55. Cells were ruptured, and crude mitochondria were pelleted by differential centrifugation (P) and analyzed by SDS-PAGE together with the supernatant fraction (S). The membrane was immunodecorated with antibodies against Tob55 and Tom20. The Tob55 variants are indicated. (B) Radiolabeled Tob55 variants were incubated for the indicated time periods in the absence or presence of isolated mitochondria. The samples were divided into aliquots. Two of the aliquots were left untreated (–PK), and the other aliquots were treated with PK (+PK). Samples were subjected to SDS-PAGE and autoradiography. The migration of molecular mass markers is indicated on the left. Untreated radiolabeled precursors, N-terminal proteolytic fragments (arrows), and a C-terminal proteolytic fragment (asterisk) are indicated.

the TOM complex, and the TOB complex (Habib et al., 2005). We analyzed whether the truncated Tob55 variants follow this pathway. First, all variants displayed reduced efficiency of import into mitochondria deficient in Tom20 (Fig. S1, A and B, available at <http://www.jcb.org/cgi/content/full/jcb.200602050/DC1>). Second, blocking the TOM channel before import with a large excess of the recombinant matrix–destined preprotein, pSu9(1–69)–dihydrofolate reductase (DHFR), strongly inhibited the import and membrane insertion of the variant Tob55 proteins (Fig. S1 C). Third, to check for the involvement of the TOB complex, we compared the insertion of the Tob55 variants into wild-type mitochondria to insertion into mitochondria depleted of Tob55. All Tob55 radiolabeled variants were inserted with strongly reduced efficiency into the Tob55-depleted mitochondria (Fig. S1 D). Similar low efficiencies of insertion were observed for all Tob55 variants when import was performed with mitochondria depleted of Tob38 (unpublished data). Thus, preexisting TOB complexes are essential for the membrane insertion of all Tob55 variants. Collectively, Tob55 precursors follow the insertion pathway of β -barrel proteins even when lacking their N-terminal domain.

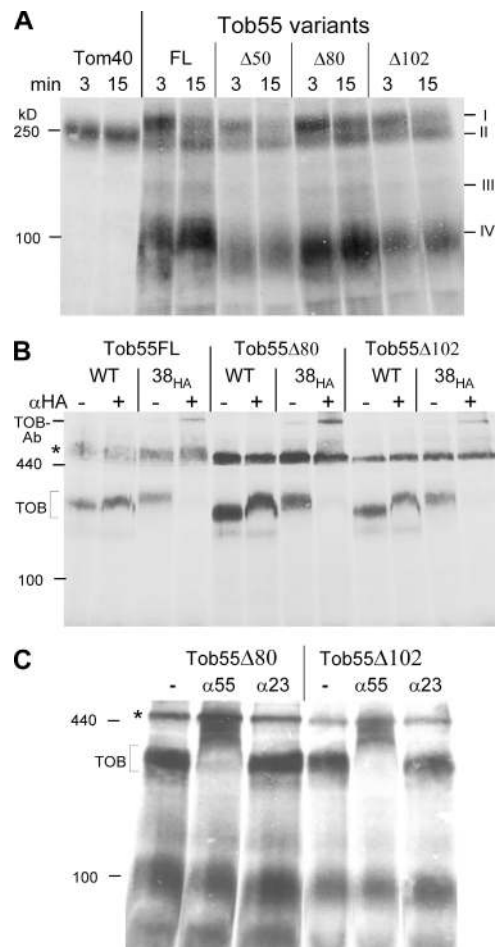


Figure 3. Tob55 variants are assembled into preexisting TOB complexes. (A) Radiolabeled precursors of Tob55 variants and of Tom40 were incubated with wild-type mitochondria for the indicated time periods. Mitochondria were reisolated and analyzed by BNGE followed by autoradiography. Species containing the radiolabeled Tob55 are indicated as in Habib et al. (2005)—I, II, III, and IV. The 250-kD intermediate of Tom40 with the TOB complex is indicated. (B) Tob38 interacts with precursors of Tob55 variants. Wild-type mitochondria (WT) and mitochondria containing an HA-tagged form of Tob38 (38_{HA}) were incubated for 3 min with radiolabeled Tob55 precursors. Mitochondria were reisolated, resuspended in a buffer containing 1% digitonin, and divided into two halves. One aliquot was analyzed directly by BNGE, whereas the other was incubated with antibodies against the HA tag before such an analysis. The gel was further analyzed by autoradiography. The TOB complex and a TOB complex with bound antibody are indicated. An unspecific band is indicated with an asterisk. (C) Tob55 variants interact with preexisting Tob55. Radiolabeled Tob55 variants were incubated for 3 min with isolated mitochondria. Mitochondria were reisolated, resuspended in a buffer containing 1% digitonin, and divided into three aliquots. One aliquot was analyzed directly by BNGE, whereas the other two were incubated before analysis with antibodies against either an N-terminal peptide of Tob55 or Tim23. The TOB complex is indicated. An unspecific band is indicated with an asterisk.

The N-terminal domain of newly synthesized Tob55 molecules is not required for Tob55 assembly into TOB complexes

Does the N-terminal domain of newly synthesized Tob55 precursors have a function in the integration into TOB complexes? Radiolabeled Tob55 variants were incubated with mitochondria, and the import reactions were analyzed by blue native gel electrophoresis (BNGE). The radiolabeled Tob55 precursor

migrated upon BNGE as several species (Fig. 3 A; Ishikawa et al., 2004; Meisinger et al., 2004; Habib et al., 2005). Initially, the radiolabeled molecule of Tob55 migrated preferentially with the uppermost one (species I). Upon prolonged incubation to allow for assembly into the preexisting TOB complexes, the precursor molecules migrated mostly as species II. This species migrated at an apparent molecular mass smaller than that of an intermediate of Tom40 precursor associated to the TOB complex; thus, it most likely represents an endogenous TOB complex. The three truncated variants assembled into the same complexes as the full-length protein (Fig. 3 A).

Next, ³⁵S-labeled Tob55 variants were incubated with mitochondria isolated from either wild type or a strain containing an HA-tagged version of Tob38 (Tob38_{HA}; Habib et al., 2005). The TOB complex from the latter mitochondria migrates slightly slower upon BNGE than the wild-type complex. The radioactive Tob55 species imported into the Tob38_{HA} mitochondria showed the same reduced electrophoretic mobility, and they were in a complex that was recognized by antibodies against the HA tag (Fig. 3 B). This provides further evidence that all Tob55 variants studied become assembled into preexisting TOB complexes. To obtain additional independent support for this conclusion, further antibody shift experiments were performed (Paschen et al., 2003; Wiedemann et al., 2003). We used an antibody raised against a peptide comprising amino acid residues 1–15 of Tob55, which does not recognize the two truncated variants, Tob55Δ80 and Tob55Δ102. Mitochondria containing imported ³⁵S-labeled Tob55 variants were lysed, incubated with antibodies against the N-terminal peptide of Tob55, and analyzed by BNGE. The addition of this antibody, but not of a control antibody against Tim23, resulted in a shift of the upper

³⁵S-labeled band to higher apparent molecular mass, obviously by formation of a supercomplex with the antibody (Fig. 3 C). Collectively, the absence of the N-terminal domain of Tob55 precursor does not impair its ability to become inserted into the outer membrane and assembled into preexisting TOB complexes.

Deletion of the N-terminal domain of Tob55 results in a growth phenotype of yeast cells

Is the N-terminal domain important for the function of Tob55? Because Tob55 is an essential protein, we had to use the “plasmid shuffling” method to test the ability of the truncated variants to complement the deletion of the wild-type protein (see Materials and methods). Strains that harbored a plasmid encoding full-length or truncated forms of Tob55 were tested for their ability to grow on glycerol- and glucose-containing medium at various temperatures (Fig. 4 A). Growing the cells at 30°C resulted in only minor differences in the growth rates of the various cells. In contrast, incubating the cells at 37 or 24°C resulted in a slower growth in the case of cells expressing Tob55Δ80, and even more so in cells harboring Tob55Δ102. As expected, the growth phenotype was more conspicuous on the nonfermentable carbon source, where yeast cells are dependent on mitochondria for energy production (Fig. 4 A). Thus, already the first 80 amino acid residues of Tob55 are required for optimal function of Tob55 and thus for normal growth of yeast cells.

Deletion of the N-terminal domain results in impaired biogenesis of β-barrel proteins

The growth phenotype of cells harboring deletions in Tob55 led us to investigate whether those cells contain normal levels of

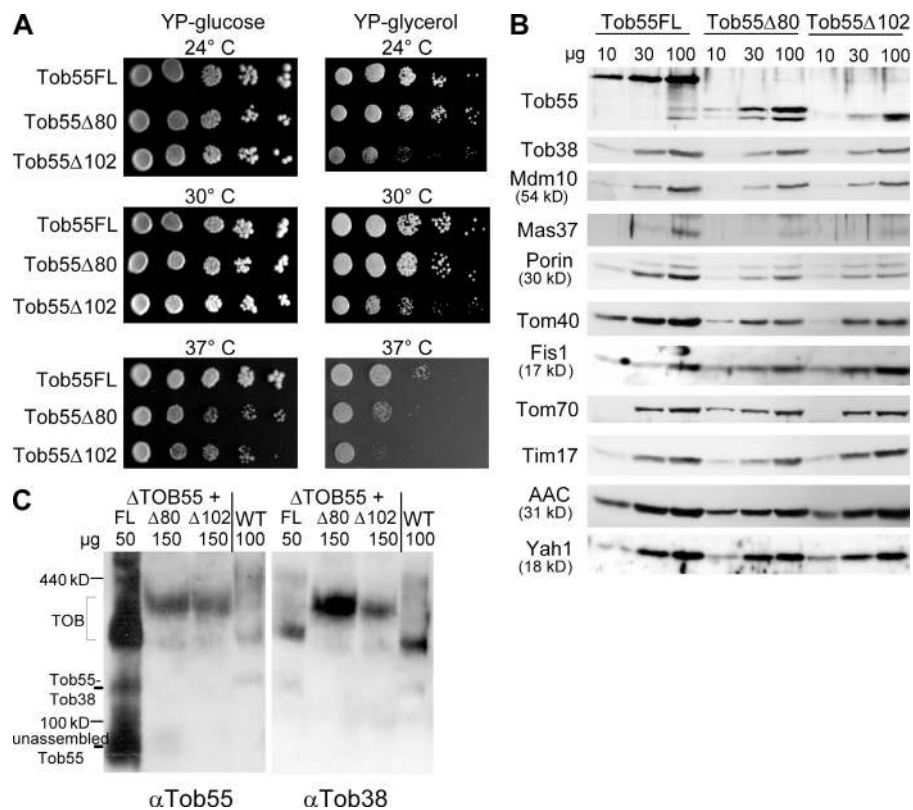


Figure 4. The N-terminal domain of Tob55 is required for optimal growth. (A) Cells harboring plasmid-encoded Tob55 variants were tested by dilution in 10-fold increments for their ability to grow at the indicated temperature on rich medium containing either glycerol or glucose. (B) Mitochondria were isolated from cells harboring plasmid-encoded full-length Tob55 (Tob55FL) or its truncated variants. Mitochondria were analyzed by SDS-PAGE and immunodecoration with antibodies against the indicated mitochondrial proteins. (C) Mitochondria as in B were solubilized in 1% digitonin and analyzed by BNGE and immunodecoration with antibodies against Tob55 and Tob38. The TOB complex, Tob55–Tob38 core complex, and unassembled Tob55 are indicated.

mitochondrial proteins. To that end, we isolated mitochondria from cells harboring plasmid-encoded full-length Tob55 or its truncated variants and controlled the amounts of expressed proteins by immunodecoration. The levels of the β -barrel proteins Tom40, Mdm10, and porin were reduced in mitochondria containing the truncated versions. Similarly, the levels of the truncated variants of Tob55 (β -barrel proteins themselves) and the other two components of the TOB complex, Tob38 and Mas37, were also reduced as compared with mitochondria containing full-length Tob55 (Fig. 4 B). In contrast, other proteins of the various mitochondrial subcompartments were present at roughly control levels (Fig. 4 B). Thus, the N-terminal domain of Tob55 appears to have an important role in the biogenesis of β -barrel proteins.

We further investigated the assembly state of the TOB complex in the various mitochondria by analyzing them with BNGE, a method that usually results in several observed species of TOB complex (Ishikawa et al., 2004; Meisinger et al., 2004; Habib et al., 2005). As we observed that mitochondria harboring the truncated versions of Tob55 contain reduced levels of this protein, we analyzed a larger amount of these mitochondria. The TOB complex from mitochondria harboring the

truncated versions migrated mainly as the higher molecular species of the TOB complex. Of note, all the Tob38 and Mas37 molecules in these mitochondria were assembled with Tob55 (Fig. 4 C and not depicted), excluding the possibility that because of the reduced levels of Tob55, Tob38 and Mas37 build partial complexes, which exert dominant-negative effect. This conclusion is further supported by our previous observations that lower levels of Tob55 result in reduced biogenesis of both Tob38 and Mas37 (Waizenegger et al., 2004; Habib et al., 2005). In contrast to wild-type mitochondria or mitochondria harboring the truncated variants of Tob55, a substantial portion of the plasmid-expressed full-length Tob55 was found as low molecular weight unassembled species (Fig. 4 C). This behavior probably resulted from the fact that, like the other Tob55 variants, it was expressed from an overexpression plasmid, whereas the interacting partners, Tob38 and Mas37, are not overexpressed.

To provide further support for the involvement of the N-terminal domain in biogenesis of β -barrel proteins, we performed *in vitro* protein import experiments with isolated mitochondria. In accordance with the *in vivo* results, the import efficiencies of newly synthesized β -barrel precursors like Tom40 and porin were substantially reduced in mitochondria

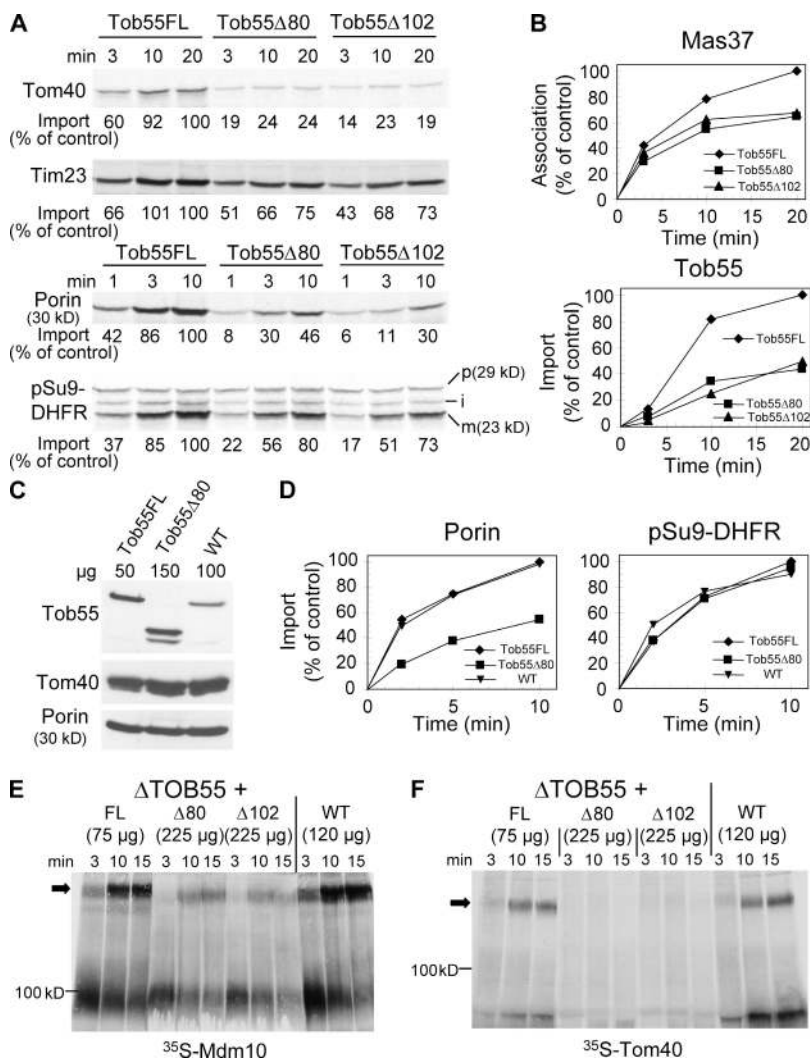


Figure 5. Mitochondria containing Tob55 truncated variants are impaired in import of β -barrel precursors. (A) 50 μ g of mitochondria isolated from cells harboring plasmid-encoded full-length Tob55 (Tob55FL) or its truncated variants were incubated with the indicated radiolabeled precursor proteins for various time periods. Samples were treated with PK, and mitochondria were reisolated. Imported proteins were analyzed by SDS-PAGE and autoradiography. Membrane insertion of porin and Tom40 as well as translocation of Tim23 precursor across the outer membrane were quantified by analyzing the protease-protected precursors, whereas for pSu9-DHFR, the bands corresponding to the mature protein were quantified. The amount of precursor proteins imported into mitochondria harboring full-length Tob55 for the longest time period was set to 100%. The precursor, intermediate, and mature forms of pSu9-DHFR are indicated as p, i, and m, respectively. (B) Radiolabeled precursors of Mas37 and Tob55 were incubated with 50 μ g of mitochondria, as in A. The import (Tob55) and association (Mas37) of the precursor proteins were assayed by the formation of proteolytic fragments (Fig. 2; Habib et al., 2005). The amount of precursor proteins imported into or associated with mitochondria harboring full-length Tob55 for the longest time period was set to 100%. (C) Mitochondria were isolated from wild type (WT) or from cells harboring plasmid-encoded full-length Tob55 or Tob55 Δ 80. The indicated amounts of mitochondria were analyzed by SDS-PAGE and immunodecoration with antibodies against the indicated outer membrane mitochondrial proteins. (D) Mitochondria (50 μ g Tob55FL, 150 μ g Tob55 Δ 80, and 100 μ g wild type) were incubated with the radiolabeled precursor proteins for various time periods. The samples were then treated with PK, and mitochondria were reisolated. Imported proteins were analyzed by SDS-PAGE and autoradiography. Import was analyzed and quantified as in A. (E and F) Radiolabeled precursor of either Mdm10 (E) or Tom40 (F) was incubated with the indicated amounts of mitochondria for various time periods. Imported proteins were analyzed by BNGE and autoradiography. The intermediate of β -barrel precursor with the TOB complex is indicated by an arrow.

containing the truncated versions (Fig. 5 A). Other precursor proteins, such as the inner membrane protein Tim23 and the matrix-destined pSu9-DHFR, were only moderately affected (Fig. 5 A). This latter reduction is probably due to the reduced level of Tom40 in the mitochondria harboring the truncated variants. Next, we investigated the importance of the N-terminal domain in preexisting Tob55 for the insertion of newly synthesized Tob55 precursor molecules and for the association of Mas37 precursors with mitochondria. The topogenesis of both proteins requires functional TOB complex (Habib et al., 2005). A moderate reduction in the association of Mas37 was observed upon incubation with mitochondria harboring the truncated variants of Tob55 (Fig. 5 B). A stronger reduction was observed upon import of Tob55 precursor (Fig. 5 B). We propose that although the association of Mas37 with mitochondria is probably reduced because of the lower levels of Tob55, the insertion of Tob55 precursor (a β -barrel protein itself) is affected by both the reduced levels of Tob55 and the absence of the N-terminal domain.

To exclude the possibility that the effect on the insertion of β -barrel proteins observed for mitochondria harboring truncated variants of Tob55 resulted only from reduced endogenous levels of both Tob55 and Tom40 in those organelles, we performed control *in vitro* import experiments. We incubated the radiolabeled precursor proteins with 50 μ g of mitochondria harboring plasmid-encoded full-length Tob55, 150 μ g of mitochondria harboring Tob55 Δ 80, or 100 μ g of wild-type mitochondria. Under these conditions, comparable amounts of TOB and TOM complexes were present in import reactions with the two former types of mitochondria (Fig. 5 C). The matrix-destined protein, pSu9-DHFR, was imported under these conditions with similar efficiency in all reactions (Fig. 5 D). In contrast, the import of the β -barrel precursor porin into mitochondria carrying the truncated Tob55 variant was still impaired. Of note, although the samples with wild-type mitochondria contain far less Tob55 molecules in comparison with those containing Tob55 Δ 80, the efficiency of porin import into the former mitochondria was substantially higher (Fig. 5 D). Furthermore, the assembly of two other β -barrel proteins, Mdm10 and Tom40, as analyzed by BNGE, was dramatically reduced in mitochondria harboring the truncated versions of Tob55 (Fig. 5, E and F). Thus, the reduced import of β -barrel precursors into mitochondria harboring truncated Tob55 variants is caused mainly by impaired function of the corresponding Tob55 molecules and is not solely due to a reduced level of Tob55. Collectively, these experiments suggest that the N-terminal domain of Tob55 is playing a central role in the biogenesis of β -barrel proteins.

The N-terminal domain recognizes β -barrel precursors

To study the function of the N-terminal domain of Tob55, a fusion protein consisting of the N-terminal 120 amino acid residues and maltose binding protein (MBP) was expressed in and purified from *Escherichia coli*. As controls, MBP alone and MBP fused to the cytosolic domain of the mitochondrial outer membrane protein Fis1 (MBP-Fis1) were expressed and

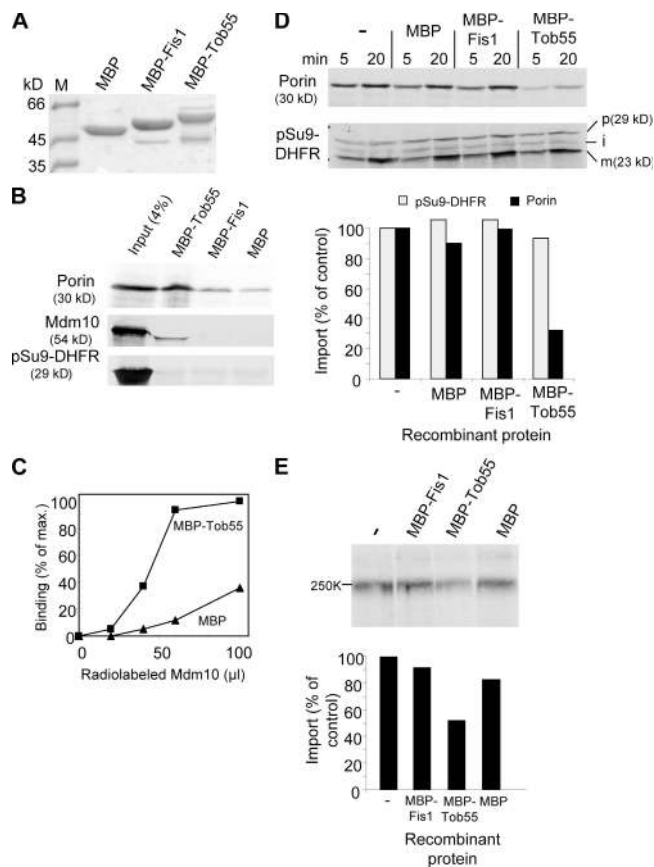


Figure 6. The N-terminal domain of Tob55 can bind β -barrel precursors *in vitro*. (A) MBP alone or fused either to the cytosolic domain of Fis1 (MBP-Fis1) or to residues 1–120 of Tob55 (MBP-Tob55) were expressed in *E. coli* and purified. 5 μ g of each protein were analyzed by SDS-PAGE and Coomassie staining. (B) The indicated recombinant MBP-fusion proteins were bound to amylose resin and incubated with radiolabeled precursor proteins. The resins were washed, and bound proteins were eluted and analyzed by SDS-PAGE and autoradiography. (C) Increasing amounts of radiolabeled Mdm10 in retic. Lysates were incubated with either MBP or MBP-Tob55 bound to amylose resin. The resins were washed, and bound proteins were eluted and analyzed by SDS-PAGE and autoradiography. The highest amount of precursor protein bound to MBP-Tob55 was set to 100%. (D) Purified N-terminal domain of Tob55 added to an import reaction inhibits the import of porin precursor. Radiolabeled precursors of porin or pSu9-DHFR were incubated in import buffer in the presence or absence of the indicated purified MBP-fusion proteins for 10 min at 0°C. Mitochondria were then added, and the mixture was incubated for a further 15 min at 25°C. At the end of the import reaction, PK was added to degrade precursors that were not completely imported. Mitochondria were reisolated, and imported proteins were analyzed by SDS-PAGE and autoradiography. The bands corresponding to protease-protected porin and to the mature form of pSu9-DHFR were quantified. The import without MBP-fusion protein was taken for each precursor as 100%. (E) Radiolabeled precursor of Mdm10 was incubated in import buffer in the presence or absence of the indicated purified MBP-fusion proteins. Mitochondria were then added, and the mixture was incubated for a further 10 min at 25°C. The import reactions were analyzed by BNGE as in Fig. 5 E, and the bands representing Mdm10-TOB intermediate were quantified. The import without MBP-fusion protein was taken as 100%.

purified in parallel (Fig. 6 A). All three proteins were analyzed for their ability to bind precursors of β -barrel proteins, porin and Mdm10. Background binding to the matrix was observed in the case of porin precursor. However, in the case of MBP-Tob55, we observed on top of these background levels specific binding that was severalfold higher than that observed

with the control proteins (Fig. 6 B). Specific binding to MBP-Tob55 was observed also with the β -barrel protein, Mdm10. Only very low unspecific binding of a matrix-destined precursor, pSu9-DHFR, was observed with all proteins (Fig. 6 B). To further verify that the binding to the N-terminal domain is specific and saturable, we added increasing amounts of radiolabeled Mdm10 precursor to equal small amounts of either MBP-Tob55 or MBP alone as control. In each added amount, severalfold more Mdm10 molecules were bound to the MBP-Tob55 in comparison with the control protein. Furthermore, a saturation of the binding was observed when large amounts of precursor were used (Fig. 6 C). Thus, the first 120 amino acid residues of Tob55 appear to be sufficient to support specific interaction with β -barrel precursors.

To obtain further support for this proposal, we investigated whether the N-terminal domain of Tob55 is able to compete out the import of porin and Mdm10. Radiolabeled precursors of porin and Mdm10 were incubated in the presence or absence of competing amounts of the purified MBP-Tob55 or control proteins, and isolated mitochondria were added. The presence of the N-terminal domain of Tob55 substantially impaired the import of both precursors, whereas the control proteins (MBP and MBP-Fis1) did not have a substantial effect (Fig. 6, D and E). Notably, the level of inhibition of porin import depended on the amount of added recombinant MBP-Tob55 (Fig. 7 A). This effect was only observed when the N-terminal domain was in a native state, as preincubation of the latter protein with urea impaired its ability to compete for the import of porin (Fig. 7 B). MBP-Tob55 did not compete out the translocation through the TOM pore of other precursor proteins, such as pSu9-DHFR and Tim23 (Fig. 6 D and Fig. 7, A and B). Hence, it is unlikely that this inhibitory effect is entirely due to an ability of residues 1–120 of Tob55 to cross the TOM pore and thereby to jam the import channel. Moreover, as shown in Figs. 2 and 3, residues 1–102 are not required for import of Tob55 through the TOM complex. Along the same line, when radiolabeled Tob55(1–120) was synthesized in a cell-free system and incubated with isolated mitochondria, it did not become protected from degradation by added proteases (unpublished data). Thus, this domain is not competent for import across the outer membrane. It is also unlikely that the competence of MBP-Tob55 is due to an interaction of the N-terminal domain with the import receptors Tom20 and Tom70; MBP-Tob55 was also able to compete import into mitochondria lacking either Tom20 or Tom70 (unpublished data).

To further substantiate the capacity of the N-terminal domain to bind β -barrel proteins, we investigated the binding of this domain to a water-soluble form of porin (Pfaller et al., 1985). This water-soluble porin, isolated from detergent-purified porin from *Neurospora crassa*, has the properties of the precursor form of porin and can be imported into the mitochondrial outer membrane (Pfaller and Neupert, 1987; Pfaller et al., 1988). We first checked whether the precursor of *N. crassa* porin can be imported into and assembled in the outer membrane of yeast mitochondria. Indeed, the *N. crassa* orthologue was imported into yeast mitochondria in the pathway that involved the general insertion pore. Blocking this pathway with excess

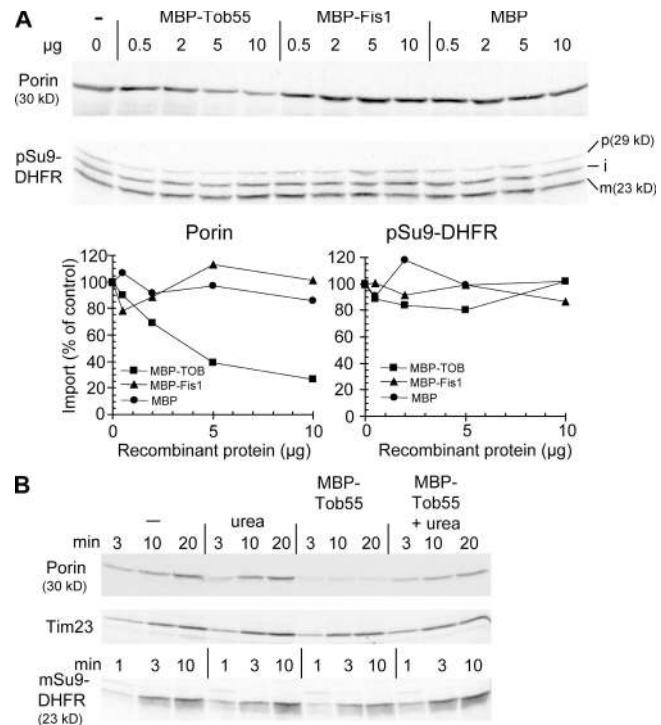
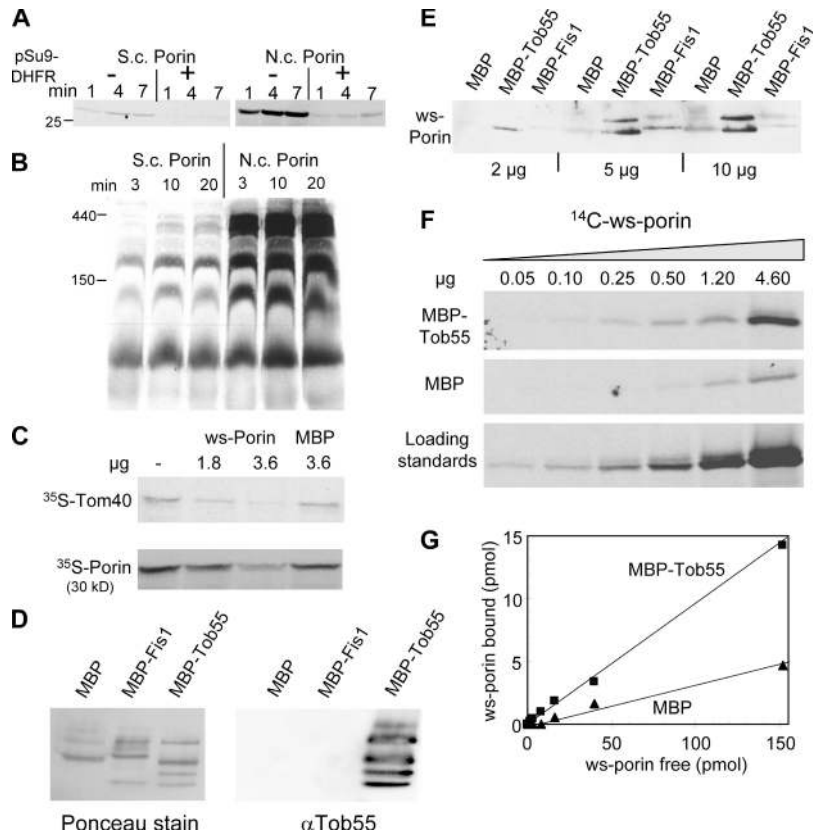


Figure 7. The inhibition by MBP-Tob55 is concentration dependent and requires folded structure. (A) Inhibition of porin import depends on the concentration of added recombinant MBP-Tob55. Radiolabeled precursors of porin or pSu9-DHFR were incubated in import buffer in the presence or absence of the indicated amounts of purified MBP-fusion proteins. Mitochondria were then added, and the mixture was incubated for a further 15 min at 25°C. At the end of the import reaction, PK was added, and further treatment was as described in the legend to Fig. 6 D. (B) Unfolded N-terminal domain of Tob55 cannot inhibit the import of porin. Mitochondria were incubated for 2 min on ice in import buffer with either native MBP-Tob55 or MBP-Tob55 pretreated with urea or with diluted urea solution as a control. Radiolabeled precursors of porin, Tim23, or pSu9-DHFR were then added, and the mixture was incubated further for the indicated time periods. At the end of the import reaction, PK was added and further treatment was done as described in the legend to Fig. 6 D.

recombinant preprotein, pSu9-DHFR, inhibited membrane integration (Fig. 8 A; Krimmer et al., 2001; Paschen et al., 2003; Wiedemann et al., 2003). Furthermore, as was observed for yeast β -barrel precursors, the import of *N. crassa* porin into yeast mitochondria was impaired in mitochondria lacking Tom20 or harboring reduced levels of Tob38 (unpublished data; Krimmer et al., 2001; Ishikawa et al., 2004; Milenkovic et al., 2004; Waizenegger et al., 2004). Porin is known to form several oligomeric structures that can be observed by BNGE (Krimmer et al., 2001; Gentle et al., 2004; Waizenegger et al., 2004). The radiolabeled *N. crassa* porin was assembled upon its import into yeast mitochondria into the same oligomeric structures as the yeast protein (Fig. 8 B). We further verified that the water-soluble porin is import competent as was published before (Pfaller and Neupert, 1987; Pfaller et al., 1988). Indeed, water-soluble porin but not the control protein, MBP, was able to compete out the import of two other β -barrel precursors, Tom40 and Porin (Fig. 8 C). Collectively, we conclude that *N. crassa* porin and its water-soluble form can use the yeast machinery for biogenesis of β -barrel proteins.

Figure 8. The N-terminal domain of Tob55 can recognize water-soluble porin.

(A) Precursor of *N. crassa* porin is correctly inserted in vitro into the outer membrane of yeast mitochondria. Radiolabeled precursors of porin from either yeast (S.c. porin) or *N. crassa* (N.c. porin) were incubated with yeast mitochondria for the indicated time periods in the presence or absence of excess recombinant matrix–destined precursor, pSu9-DHFR. At the end of the import reactions, PK was added and inserted porin was analyzed by SDS-PAGE and autoradiography. (B) Precursor of *N. crassa* porin assembles into oligomeric structures in yeast mitochondria. Radiolabeled precursors of porin from either yeast or *N. crassa* were incubated with yeast mitochondria for the indicated time periods. At the end of the import reactions, mitochondria were lysed with digitonin (1%), and assembled porin was analyzed by BNGE and autoradiography. (C) Water-soluble porin (ws-porin) can compete out the import of β -barrel precursors. Radiolabeled precursors of porin or Tom40 were incubated in import buffer in the presence or absence of the indicated proteins. Mitochondria were then added, and the mixture was incubated for a further 15 min at 25°C. At the end of the import reaction, PK was added to degrade precursors that were not completely imported. Mitochondria were reisolated, and inserted proteins were analyzed by SDS-PAGE and autoradiography. (D) MBP-Tob55 migrates on seminitative gel as multiple bands. 10 μ g each of MBP-fusion proteins were analyzed by seminitative SDS-PAGE and further stained with Ponceau (left) or immunodecorated with antibody against Tob55 (right). (E) The N-terminal domain of Tob55 recognizes water-soluble porin. The indicated amounts of MBP-fusion proteins were analyzed by seminitative gel and blotted onto nitrocellulose membrane. Water-soluble porin isolated from *N. crassa* mitochondria (11 μ g/ml) was incubated with this membrane, and the membrane was washed and immunodecorated with antibodies against porin. (F) Increasing amounts of 14 C-ws-porin were added to 2 μ g of either MBP-Tob55 (top) or MBP (middle) bound to amylose beads or were loaded directly on a gel (bottom). After incubation, the resins were washed as described in Materials and methods, and bound proteins were eluted and analyzed by SDS-PAGE and autoradiography. (G) The bands in F were quantified, and a calibration curve was created according to the loading standards. For each binding reaction, the amount of bound 14 C-ws-porin was determined. The curves show the binding of 14 C-ws-porin to MBP-Tob55 and MBP.



To study the interaction of water-soluble porin with the N-terminal domain of Tob55, we used the seminitative gel electrophoresis, which was used successfully to study the interaction of Omp85 with bacterial β -barrel proteins (Voulhoux et al., 2003). Under these conditions, MBP-Tob55 migrated as two dominant bands (Fig. 8 D). Various amounts of MBP-Tob55 and two control proteins were subjected to seminitative SDS-PAGE and transferred onto nitrocellulose membrane. Water-soluble porin was incubated with this membrane, and the bound porin was detected by immunodecoration. The N-terminal domain of Tob55 could bind specifically water-soluble porin in a concentration-dependent manner (Fig. 8 E).

Next, we aimed to obtain more quantitative information on the interaction of water-soluble porin with the N-terminal domain of Tob55. To that end, water-soluble porin was radiolabeled with 14 C-formaldehyde by reductive methylation (Pfaller and Neupert, 1987). To quantify the binding, increasing amounts of 14 C-ws-porin were added to 2 μ g of either MBP (as control) or MBP-Tob55 bound to amylose beads or were loaded directly on a gel to serve as loading standards. The beads were washed, and bound water-soluble porin was eluted and analyzed. Much higher levels of binding were observed with MBP-Tob55 as compared with the control protein (Fig. 8 G). Unfortunately, we could not demonstrate saturable binding for 14 C-ws-porin

because, under our experimental conditions, it tends to aggregate at higher concentrations. As shown in Fig. 8 F, the binding was concentration dependent at concentrations ranging from 1 to 152 nM. When we subtracted the unspecific binding to MBP from the specific binding and analyzed the resulting data as a Scatchard plot, we obtained a binding parameter of $K_d = 12$ nM. Of note, this value is in the same range as the dissociation constant of the binding of presequence-containing precursor protein and the TOM complex (Stan et al., 2000). Collectively, these results demonstrate that the N-terminal domain of Tob55 is able to interact with precursors of β -barrel proteins.

Translocation of porin precursor across the TOM complex is required for its efficient insertion into the outer membrane
Although β -barrel precursors were suggested to be exposed to the IMS on their transit from the TOM to the TOB complex (Kozjak et al., 2003; Paschen et al., 2003; Wiedemann et al., 2003, 2004; Hoppins and Nargang, 2004; Habib et al., 2005), an open question is whether the TOB complex is required for the transfer of β -barrel precursors across the outer membrane. We observed a reduced import of β -barrel precursors into mitochondria containing truncated Tob55 variants (Fig. 5). Because we analyzed the import as protection against externally added

protease, our observations suggest that functional Tob55 is required for translocation of β -barrel precursor across the outer membrane.

Next, we wanted to investigate whether translocation of β -barrel precursors across the import pore of the TOM complex is required for efficient insertion of these precursor proteins into the outer membrane. To that end, we blocked the TOM channel with an excess of matrix-destined precursor to reduce both membrane insertion of β -barrel proteins and the import of matrix-destined precursor proteins (Fig. S1 and Fig. 9 A; Hwang et al., 1989; Rapaport and Neupert, 1999; Krimmer et al., 2001; Stan et al., 2003). As was observed before, rupturing the outer membrane resulted in substantial reduction in the insertion efficiency of porin precursor (Smith et al., 1994). This reduction is probably caused by the loss of the small Tim proteins, which were shown to be involved in the biogenesis of β -barrel proteins (Hoppins and Nargang, 2004; Wiedemann et al., 2004; Habib et al., 2005). Surprisingly, blocking the TOM complex in mitochondria with ruptured outer membrane strongly impaired the insertion of β -barrel precursors (Fig. 9 A). This behavior is different from that of matrix-destined precursors, where rupturing the outer membrane can overcome such blockage of the TOM channel (Hwang et al., 1989). The residual insertion of porin precursor into the outer membrane of ruptured mitochondria did not result from the insertion capacity of subpopulation of intact mitochondria. We could not detect any DLD1 (a marker IMS protein) upon treatment of the ruptured mitochondria with external protease, suggesting that all mitochondria were ruptured (Fig. 9 B). Thus, we propose that the efficient recognition of β -barrel precursors by the TOB complex requires a preceding translocation across the import channel of the TOM complex.

Discussion

We present here evidence for the involvement of the N-terminal domain of Tob55 in recognition of β -barrel precursors and thus in the transfer of precursors from the IMS to the TOB complex. Such an involvement is in agreement with the location of this domain in the IMS. It is also in line with the suggestion that the N-terminal region of the bacterial Omp85/YaeT recognizes β -barrel precursors in the periplasmic space (Bos and Tommassen, 2004). This region was named POTRA (polypeptide transport-associated domain; Sanchez-Pulido et al., 2003). According to prediction programs, the POTRA domain of yeast Tob55 covers amino acid residues 29–108. Notably, chloroplast Toc75, another protein belonging to the β -barrel-type pores, also has a POTRA-like region at its N terminus. In contrast to Tob55, Toc75 is involved in translocation of precursor proteins with chloroplast targeting signals across the outer membrane. It is currently unclear whether this protein is also involved in the insertion of β -barrel precursors. The N-terminal domain of Toc75 was reported to play a role in the recognition of stroma-destined precursor proteins (Ertel et al., 2005). Collectively, a receptor-like function of a hydrophilic N-terminal domain might be a common feature of the β -barrel translocases of the extended Tob55/Toc75/Omp85 family.

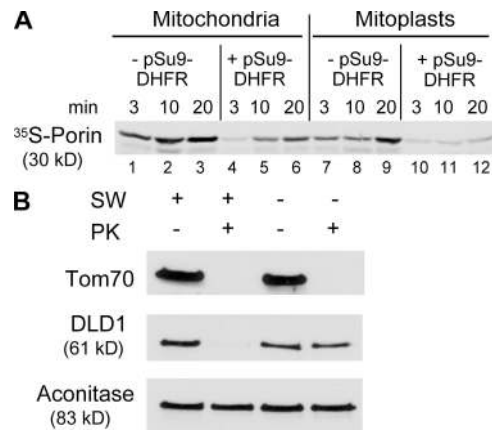


Figure 9. Translocation of porin precursor across the TOM complex is required for its efficient insertion into the outer membrane. (A) Radiolabeled precursor of porin was incubated for the indicated time periods with isolated intact mitochondria or with mitochondria that had been subjected to osmotic swelling (mitoplasts). The incubation was in the presence or absence of excess recombinant matrix-destined precursor, pSu9-DHFR. At the end of the import reaction, mitochondria were treated with PK and proteins were analyzed by SDS-PAGE and autoradiography. (B) To control the efficiency of both rupturing the mitochondrial outer membrane and the protease treatment, samples from treated and untreated mitochondria were analyzed by SDS-PAGE and immunodecoration with antibodies against Tom70 (outer membrane protein), DLD1 (IMS protein), and aconitase (matrix protein).

The growth behavior of cells with a Tob55 that lacks the N-terminal domain underscores the functional importance of this part of the protein. This domain appears to be required neither for targeting of Tob55 to mitochondria nor for its assembly into the TOB complex. There may be, however, a role of the N-terminal domain in the structural organization of the TOB complex, as we observed that the TOB complex containing the deletion variants of Tob55 has altered migration behavior in native gel system. We cannot exclude the possibility that part of the reduction in the biogenesis of β -barrel proteins in the strains containing the deletion variants is due to altered conformation of the TOB complex. However, our data strongly support our suggestion that this reduction results from the absence of the binding capacity of the N-terminal domain. Tob55 interacts with the other two components of the TOB complex, Tob38 and Mas37. The location of the N-terminal domain in the IMS makes it an unlikely candidate for such an interaction, as the two other subunits are attached to the cytosolic surface of the outer membrane (Wiedemann et al., 2003; Ishikawa et al., 2004; Milenkovic et al., 2004; Waizenegger et al., 2004).

What could be the signal in the β -barrel precursors that is being recognized by the N-terminal domain? Our current observations with Tob55 and previous results with the precursors of Tom40 and porin suggest that this signal does not reside in the N-terminal domain of the precursor proteins (Court et al., 1996; Rapaport et al., 2001). Currently, six putative β -barrel proteins in yeast mitochondria are known: two isoforms of porin, Tom40, Tob55 itself, and two proteins that seem to be involved in maintenance of mitochondrial morphology, Mdm10 and Mmm2. Despite their similar overall structure, these proteins show extensive divergence of their primary sequences. A linear

consensus sequence as recognition and/or sorting signal is therefore unlikely. In the bacterial system, there are some hints as to signals in the precursors that are transported through the periplasm to the outer membrane (de Cock et al., 1997; Hennecke et al., 2005). It is currently unclear, however, whether these signals play a role in the insertion pathway mediated by *Omp85/YaeT*.

Irrespective of which signals are being recognized by the N-terminal domain of *Tob55*, we can propose a working model for the biogenesis of mitochondrial β -barrel membrane proteins. Precursors of these proteins are translocated across the outer membrane to the IMS by the TOM complex. This way of delivery is required for the subsequent productive recognition of precursor molecules by the TOB complex and cannot be bypassed by opening the outer membrane. The N-terminal domain of *Tob55* is playing an important role in the initial interaction of the TOB complex with the precursor proteins, most likely as soon as they emerge from the TOM complex. In the absence of this domain, the translocation across the TOM complex and subsequent membrane integration of β -barrel precursors are impaired. Thus, it appears that the translocation of β -barrel precursor proteins across the outer membrane and their recognition by the TOB complex are coupled processes. Currently, the molecular mechanism of this coupling is not clear.

In conclusion, our data show that the N-terminal domain of *Tob55*, which contains the POTRA motif and is exposed in the IMS has a crucial role in guiding the precursor of β -barrel proteins from the IMS into the outer membrane. It will be very important to understand at the molecular level all the events that lead to the membrane integration of β -barrel precursors.

Materials and methods

Yeast strains and growth conditions

Standard genetic techniques were used for the growth and manipulation of yeast strains (Sherman et al., 1986). The wild-type strain YPH499 (MATA *ade2-101 his3- Δ 200 leu2- Δ 1 ura3-52 trp1- Δ 63 lys2-801*) was used. The *tom20*-null strain YTJB64 and its corresponding parental strain YTJB4 were used (a gift from G. Schatz, Biozentrum der Universität Basel, Basel, Switzerland). Transformation of yeast was performed using the lithium-acetate method. Yeast cells were grown under aerobic conditions on YPD (1% [wt/vol] yeast extract, 2% [wt/vol] bacto-peptone, and 2% glucose), YPG (1% [wt/vol] yeast extract, 2% [wt/vol] bacto-peptone, and 3% glycerol), or synthetic medium.

Construction of *TOB55* genomic disruption strain

The *TOB55* gene was cloned by PCR from yeast genomic DNA using primers based on the published sequence. The PCR product was inserted into the yeast expression vector pVTU-102, which contains the selectable marker *URA3*, and the resulting plasmid was transformed into the wild-type strain YPH499. The genomic *TOB55* open reading frame in this strain was replaced with the *HIS3* marker gene by homologous recombination. In the resulting *His⁺Ura⁺* strain, the complete coding sequence of the *TOB55* gene was deleted and a wild-type *TOB55* gene was present on a 2 μ plasmid; this strain was termed YSH1. For growth tests on plates, cells were grown to log phase and diluted to an OD₆₀₀ of 0.3. Cells were then diluted in 10-fold increments, and 5 μ l of each was spotted onto the indicated solid media.

Complementation assay with variants of *Tob55*

The PCR products encoding full-length *Tob55* or *Tob55* variants with various deletions in the N-terminal domain were inserted into a centromeric yeast expression vector, pYX132xTIp-*TRP1* (Invitrogen). To test for the ability of the *Tob55* variants to complement the function of *Tob55*, these plasmids were transformed into the YSH1 strain, and *Ura⁺Trp⁺* transformants were streaked onto 5-fluoro-orotic acid plates. This treatment resulted in the

elimination of the wild-type copy of *Tob55*, which is encoded on the *URA3*-containing plasmid and thus allowed to investigate whether the variant on the *TRP1*-containing vector could support growth.

Binding assay with water-soluble porin

Native porin from *N. crassa* was isolated by modification of a published procedure (Pfaller et al., 1985). Shortly, 5 mg of outer membrane vesicles, which were isolated as described elsewhere (Schmitt et al., 2006), were solubilized in 1 ml of buffer containing 50 mM Hepes-KOH, 1 mM PMSF, 10% glycerol, and 2% Triton X-100. After a clarifying spin (36,670 *g*, 10 min, 2°C), the supernatant was loaded onto an anion-exchange column (ResQ; GE Healthcare). The flow-through that contains porin was collected. Further treatment to obtain water-soluble porin was done as described previously (Pfaller et al., 1985).

The labeling of water-soluble porin with ¹⁴C-formaldehyde was accomplished by reductive methylation in the presence of NaBH₃CN, as described previously (Pfaller et al., 1985). For binding experiments, various amounts of radiolabeled water-soluble porin were added in binding buffer (100 mM KCl, 0.025% BSA, 10% glycerol, and 100 mM sodium phosphate, pH 6.8) to MBP or MBP-*Tob55* prebound to amylose beads. We performed our experiments in low temperature (4°C) and in the presence of BSA and salt because it was reported that these conditions can reduce the tendency of water-soluble porin to adhere to surfaces and thus to cause unspecific binding (Pfaller and Neupert, 1987). After incubation at 4°C for 35 min, the beads were washed once with binding buffer, with binding buffer without BSA, and finally with buffer containing 100 mM NaCl and 90 mM Tris-base. Bound proteins were eluted with sample buffer and analyzed by SDS-PAGE and autoradiography. For quantification of the binding reactions, increasing amounts of radiolabeled water-soluble porin were analyzed directly by SDS-PAGE and autoradiography.

Biochemical methods

Mitochondria were prepared by differential centrifugation as described previously (Daum et al., 1982). Radiolabeled precursor proteins were synthesized in rabbit reticulocyte lysate in the presence of [³⁵S]methionine (MP Biomedicals) after *in vitro* transcription by SP6 polymerase from pGEM4 vectors containing the cDNA of interest.

Import experiments were performed at 25°C in an import buffer containing 250 mM sucrose, 0.25 mg/ml BSA, 80 mM KCl, 5 mM MgCl₂, 10 mM MOPS-KOH, 2 mM NADH, and 2 mM ATP, pH 7.2. Mitochondria containing plasmid-encoded *Tob55* were preincubated at 37°C for 10 min before the addition of the radiolabeled precursor proteins.

The DNAs encoding either the N-terminal domain of *Tob55* (amino acid residues 1–120) or the cytosolic domain of *Fis1* (amino acid residues 1–98) were cloned into the pMalCRI plasmid (New England Biolabs, Inc.) and expressed in *E. coli* BL21 cells as soluble fusion proteins with MBP. Purification of the protein was performed according to the manufacturer's instructions. For *in vitro* binding assays, *E. coli* cells were lysed and proteins were applied to amylose resin. Unbound proteins were washed out with MBP-column buffer (20 mM Hepes, pH 7.4, 100 mM NaCl, 2 mM EDTA, and 1 mM PMSF). To minimize unspecific binding, the resin was further washed with import buffer containing 3% BSA and then incubated for 20 min with 50 μ l reticulocyte lysate, which was not used for *in vitro* translocation. The resin was washed again and incubated for 20 min at 4°C in import buffer with 50 μ l reticulocyte lysate containing radiolabeled proteins. In the case of Mdm10, the binding was performed in the presence of 0.3% digitonin. The resin was then washed twice with import buffer, and bound proteins were eluted with 1 M NaCl. Seminitative SDS-PAGE and far Western blotting were performed according to published procedures (Voulhoux et al., 2003).

BNGE

Mitochondria were lysed in 50 μ l digitonin buffer (1% digitonin, 20 mM Tris-HCl, 0.1 mM EDTA, 50 mM NaCl, 10% glycerol, and 1 mM PMSF, pH 7.4). After incubation for 15 min at 4°C and a clarifying spin (36,670 *g*, 15 min, 2°C), 5 μ l sample buffer (5% [wt/vol] Coomassie brilliant blue G-250, 100 mM Bis-Tris, and 500 mM δ -aminocaproic acid, pH 7.0) were added, and the mixture was analyzed by electrophoresis in a 6–13% gradient blue native gel (Schägger et al., 1994). Antibody shift experiments were performed as described previously (Paschen et al., 2003).

Online supplemental material

Fig. S1 includes results of experiments demonstrating that *Tob55* precursors follow the insertion pathway of β -barrel proteins even when lacking their N-terminal domain. Specifically, their correct topogenesis requires the

import receptor Tom20, the translocation pore of the TOM complex, and the TOB complex. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200602050/DC1>.

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