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

Shukry J. Habib, Thomas Waizenegger, Agathe Niewienda, Stefan A. Paschen, Walter Neupert, and Doron Rapaport

Institut für Physiologische Chemie, Universität München, 81377 Munich, Germany

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.

Correspondence to Doron Rapaport: rapaport@med.uni-muenchen.de

S.A. Paschen’s present address is Institut für Medizinische Mikrobiologie, 81675 Munich, Germany.

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.

The online version of this article contains supplemental material.

© The Rockefeller University Press  $15.00
The Journal of Cell Biology, Vol. 176, No. 1, January 1, 2007 77–88
http://www.jcb.org/cgi/doi/10.1083/jcb.200602050

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 complex.
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. 1A) 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. 1A; 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. 1B). Formation of this fragment was abolished when the outer membrane was ruptured by either osmotic shock or solubilizing the membrane with detergent (Fig. 1B). 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 Tob55Δ50, Tob55Δ80, and Tob55Δ102, 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. 2A). 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. 2B). 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
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). Similarly 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.

The N-terminal domain of Tob55 precursor molecules is not required for targeting and membrane insertion.

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 (Tob38HA; 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 Tob38HA 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...
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...
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 μg of mitochondria harboring plasmid-encoded full-length Tob55, 150 μg of mitochondria harboring Tob55Δ80, or 100 μg 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 Tob55Δ80, 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 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 radio-labeled 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. Radio-labeled 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 insertion 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 radio-labeled 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

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 radio-labeled 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 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 dilute 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.
To study the interaction of water-soluble porin with the N-terminal domain of Tob55, we used the seminative 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-To b55 and two control proteins were subjected to seminative SDS-PAGE and transferred onto nitrocellulose membrane. Water-soluble porin was incubated with this membrane, and the membrane was washed and immunodecorated with antibodies against porin. The indicated amounts of MBP-fusion proteins were analyzed by seminative gel and blotted onto nitrocellulose membrane. Water-soluble porin isolated from N. crassa mitochondria (11 μg/ml) was incubated with this membrane, and the membrane was washed and immunodecorated with antibodies against porin.

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 14C-formaldehyde by reductive methylation (Pfaller and Neupert, 1987). To quantify the binding, increasing amounts of 14C-ws-porin were added to 2 μg of either MBP (as control) or MBP-Tob55 bound to amyllose 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 14C-ws-porin was determined. The curves show the binding of 14C-ws-porin to MBP-Tob55 and MBP.

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. 9A; 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. 9A). 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. 9B). 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.

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
the IMS has a crucial role in guiding the precursor of β-barrel of Tob55, which contains the POTRA motif and is exposed in the molecular mechanism of this coupling is not clear. 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-Δ200 leu2-3,112 ura3-52 trp1-Δ63 lys2-801) was used. The tom20null strain YJ7864 and its corresponding parental strain YJ7864 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] bactopeptone, and 2% glucose), YPG (1% [wt/vol] yeast extract, 2% [wt/vol] bactopeptone, 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

Biochemical methods
Mitochondria were prepared by differential centrifugation as described previously (Baumöhl et al., 1982). Water-soluble porin proteins were synthesized in rabbit reticulocyte lysate in the presence of [35S]methionine (MBI Biomedicals) after in vitro transcription by SP6 polymerase from pGEM4 vectors containing the cDNA of interest.

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 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 (Pfeller et al., 1985). Shortly, 5 mg of outer membrane vesicles, which were isolated as described elsewhere (Schön et al., 2003), 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 (Pfeller 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) 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 (Pfeller 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.

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.

For quantification of the binding reactions, increasing amounts of radiolabeled water-soluble porin were analyzed directly by SDS-PAGE and autoradiography.

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 μl reticulocyte lysate, which was not used for in vitro translation. The resin was washed again and incubated for 20 min at 4°C in import buffer with 50 μl 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. Seminative SDS-PAGE and far Western blotting were performed according to published procedures (Youlou et al., 2003).

BNGE
Mitochondria were lysed in 50 μl 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, 13 min, 2°C), 5 μl sample buffer (5% [wt/vol] Coomassie brilliant blue G-250, 10% glycerol, and 500 mM 6-aminohexanoic acid, pH 7.0) was 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 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.
import receptor Tom20, the translocation pore of the TOM complex, and the TOB55 complex. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200602050/DC1.

We thank P. Heckmeyer for technical assistance and K. Hell for helpful discussions.

This work was supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 594 (B12, D. Rapaport), the Fonds der Chemischen Industrie (W. Neupert), and predoctoral fellowships from the Minerva Stiftung (S.J. Habib) and the Boehringer Ingelheim Fonds (T. Waizenegger).

Submitted: 9 February 2006
Accepted: 29 November 2006

References

Bos, M.P., and J. Tommassen. 2004. Biogenesis of the Gram-negative bacterial outer membrane. Curr. Opin. Microbiol. 7:610–616.

Court, D.A., R. Kleene, W. Neupert, and R. Lill. 1996. Role of the N- and C-termini of porin in import into the outer membrane of Neospora mitochondria. FEBS Lett. 390:73–77.

Duun, G., S. Gasser, and G. Schatz. 1982. Import of proteins into mitochondria: energy-dependent, two-step processing of the intermembrane space enzyme cytchrome b$_{1}$ by isolated yeast mitochondria. J. Biol. Chem. 257:13075–13080.

de Cock, H., M. Struve, M. Kleerebezem, T. van der Krit, and J. Tommassen. 1997. Role of the carboxy-terminal phenylalanine in the biogenesis of outer membrane protein PhoD of Escherichia coli K-12. J. Mol. Biol. 269:473–478.

Dolezel, P., V. Likic, J. Tacezy, and T. Lithgow. 2006. Evolution of the molecular machines for protein import into mitochondria. Science. 313:314–318.

Eckart, K., L. Eichacker, K. Sohrt, E. Schleiff, L. Heins, and J. Soll. 2002. A Toc75-like protein import channel is abundant in chloroplasts. EMBO Rep. 3:557–562.

Ertel, F., O. Mirus, R. Bredemeier, S. Moslavac, T. Becker, and E. Schleiff. 2005. The evolutionarily related b-barrel polypeptide transporters from Pismum sativum and Nostoc PCC7120 contain two distinct functional domains. J. Biol. Chem. 280:28281–28289.

Gabriel, K., S.K. Buchanan, and T. Lithgow. 2001. The alpha and beta: protein translocation across mitochondrial and plastid outer membranes. Trends Biochem. Sci. 26:36–40.

Gentle, I., K. Gabriel, P. Beech, R. Waller, and T. Lithgow. 2004. The Omp85 family of proteins is essential for outer membrane biogenesis in mitochondria and bacteria. J. Cell Biol. 164:19–24.

Habib, S.J., T. Waizenegger, M. Lech, W. Neupert, and D. Rapaport. 2005. Assembly of the TOM complex of mitochondria. J. Biol. Chem. 280:6434–6440.

Hennecke, G., J. Nolte, R. Volkmer-Engert, J. Schneider-Mergener, and S. Behrens. 2005. The periplasmic chaperone SurA exploits two features characteristic of integral outer membrane proteins for selective substrate recognition. J. Biol. Chem. 280:23540–23548.

Hoppins, S.C., and F.E. Nargang. 2004. The Tim8-Tim13 complex of Neurospora crassa. J. Biol. Chem. 269:8188–8193.

Hoppins, R., H.F. Steger, J. Rassow, N. Pfanner, and W. Neupert. 1988. Import pathways of precursor proteins into mitochondria: multiple receptor sites are followed by a common insertion site. J. Cell Biol. 107:2483–2490.

Parrilla, R., and W. Neupert. 1987. High-affinity binding sites involved in the import of porin into mitochondria. EMBO J. 6:2635–2642.

Parrilla, R., H. Freitag, M. Harremy, R. Benz, and W. Neupert. 1985. A water-soluble form of porin from the mitochondrial outer membrane of Neospora crassa. J. Biol. Chem. 260:3709–3713.

Waizenegger, T., S.J. Habib, M. Lech, W. Neupert, and F.E. Nargang. 2001. Structural requirements of Tom40 for assembly into preexisting TOM complexes of mitochondria. Mol. Biol. Cell. 12:1189–1198.

Sanchez-Pulido, L., D. Devos, S. Geneveois, M. Vicente, and A. Valencia. 2003. POTRA: a conserved domain in the FtsQ family and a class of beta-barrel outer membrane proteins. Trends Biochem. Sci. 28:523–526.

Schägger, H., W.A. Cramer, and G. von Jagow. 1994. Analysis of molecular masses and oligomeric states of protein complexes by blue native electrophoresis and isolation of membrane protein complexes by two-dimensional native electrophoresis. Anal. Biochem. 217:220–230.

Schleiff, E., J.R. Silvius, and G.C. Shore. 1999. Direct membrane insertion of voltage-dependent anion-selective channel protein catalyzed by mitochondrial Tom20. J. Cell Biol. 145:973–978.

Schleiff, E., L.A. Eichacker, K. Eckart, T. Becker, O. Mirus, T. Stahl, and J. Soll. 2003. Prediction of the plant beta-barrel proteome: a case study of the chloroplast outer envelope. Protein Sci. 12:748–759.

Schmitt, S., H. Prokisch, T. Schlunk, D.G. Camp II, U. Ahting, T. Waizenegger, C. Scharfe, T. Meitinger, A. Immhof, W. Neupert, et al. 2006. Proteome analysis of mitochondrial outer membrane from Neurospora crassa. Proteomics. 6:72–80.

Sherman, F., G.R. Fink, and J. Hicks. 1986. Methods in Yeast Genetics: A Laboratory Course. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 186 pp.

Smith, M., S. Hicks, K. Baker, and R. MacCauley. 1994. Rupture of the mitochondrial outer membrane impairs porin assembly. J. Biol. Chem. 269:2460–2464.

Sogo, L.F., and M.P. Yaffe. 1994. Regulation of mitochondrial morphology and inheritance by Mdm10p, a protein of the mitochondrial outer membrane. J. Cell Biol. 126:1361–1373.

Stan, T., U. Ahling, M. Demberowski, K.-P. Künkele, S. Nussberger, S. Neupert, and D. Rapaport. 2000. Recognition of preproteins by the isolated TOM complex of mitochondria. EMBO J. 19:4895–4902.

Stan, T., J. Brix, J. Schneider-Mergener, N. Pfanner, W. Neupert, and D. Rapaport. 2003. Mitochondrial protein import: recognition of internal import signals of BCS1 by the TOM complex. Mol. Cell. Biol. 23:2239–2250.

Tamm, L.K., A. Arora, and J.H. Kleinschmidt. 2001. Structure and assembly of b-barrel membrane proteins. J. Biol. Chem. 276:32439–32402.

Voulhoux, R., and J. Tommassen. 2004. Omp85, an evolutionarily conserved bacterial protein involved in outer-membrane-protein assembly. Res. Microbiol. 155:129–135.

Voulhoux, R., M.P. Bos, J. Gears, M. Mols, and J. Tommassen. 2003. Role of a highly conserved bacterial b-barrel in outer membrane protein assembly. Science. 299:262–265.

Waizenegger, T., S.J. Habib, M. Lech, D. Mokranjac, S.A. Paschen, K. Hell, W. Neupert, and D. Rapaport. 2004. Tob38, a novel essential component in the biogenesis of beta-barrel proteins of mitochondria. EMBO Rep. 5:704–709.
Wiedemann, N., V. Kozjak, A. Chacinska, B. Schönfish, S. Rospert, M.T. Ryan, N. Pfanner, and C. Meisinger. 2003. Machinery for protein sorting and assembly in the mitochondrial outer membrane. *Nature*. 424:565–571.

Wiedemann, N., K.N. Truscott, S. Pfannschmidt, B. Guiard, C. Meisinger, and N. Pfanner. 2004. Biogenesis of the protein import channel Tom40 of the mitochondrial outer membrane: intermembrane space components are involved in an early stage of the assembly pathway. *J. Biol. Chem.* 279:18188–18194.

Wimley, W.C. 2003. The versatile beta-barrel membrane protein. *Curr. Opin. Struct. Biol.* 13:404–411.

Wu, T., J. Malinverni, N. Ruiz, S. Kim, T.J. Silhavy, and D. Kahne. 2005. Identification of a multicomponent complex required for outer membrane biogenesis in *Escherichia coli*. *Cell*. 121:235–245.