Biogenesis and Function of Mitochondrial Outer Membrane Proteins

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1 Introduction

1.1 Mitochondrial structure and function

Most eukaryotic cells contain many mitochondria, which occupy up to 25 % of the cytoplasm. Each mitochondrion contains two highly specialized membranes, an outer and an inner membrane, that play a crucial part in its activities. These membranes define two separate mitochondrial compartments: the innermost matrix space and the intermembrane space. The outer membrane contains proteins that render the membrane permeable to molecules having molecular masses as high as 10,000 Daltons. The inner membrane, which is less permeable, is composed of approximately 20 % lipids and 80 % proteins- the highest ratio of proteins to lipids in cellular membranes (Lodish et al. 1999). The inner membrane is composed of two topologically continuous but distinct domains. The inner boundary membrane is closely juxtaposed to the outer membrane around the circumference and it appears to be the preferred region where nuclear encoded preproteins are imported into and across the inner membrane. The cristae, tubular or lamellar structures which protrude into the matrix, are connected to the inner boundary membrane by narrow tubular cristae junctions (Reichert and Neupert, 2002).

Mitochondria play essential rules in cell life and cell death. Besides being the main site of ATP production under aerobic conditions, these complex organelles carry out many other functions such as the synthesis of lipids, heme and amino acids. They have essential roles in the iron-sulfur cluster biogenesis (Muhlenhoff and Lill, 2000) and perform functions related to cell stress response, programmed cell death and aging (Jiang and Wang, 2004; Trifunovic et al., 2004). Mitochondria are also important for the maintenance of cellular Ca^{2+} homeostasis (Gunter et al., 2004). Mitochondrial dysfunction has been implicated in many different aspects of diseases. For example, a certain defect in the biogenesis of iron-sulfur cluster leads to the neurodegenerative disease Friedreich ataxia (Puccio and Koenig, 2000) and mutations in the OPA1 gene, which encode a dynamin-related mitochondrial protein, cause autosomal dominant optic atrophy (ADOA) (Alexander et al., 2000).

Mitochondria are highly dynamic organelles. They actively move along cytoskeletal tracks and frequently change their shape and size due to fission and fusion events. Mitochondrial motility, fission and fusion play important roles in the adaptation of the cell's energy requirements and in the inheritance of mitochondria by daughter cells during cell division (Reichert and Neupert, 2002; Yoon and McNiven, 2001). In line with

the previous notion, it is important to note that no *de novo* synthesis of the organelle occurs.

It is widely accepted that present day mitochondria represent the remnant of an α-proteobacterium that had become a partner in a symbiotic relationship with another cell early in the evolution of life on earth (Gray et al., 1999). As the symbiotic relationship evolved over time, it was accompanied by the loss of redundant genes and the transfer of prokaryotic genes to the eukaryotic nucleus. As a result, the present mitochondria are no longer autonomous and are totally dependent on their host. About 98-99% of mitochondrial proteins are nuclear encoded. For example it is estimated that the yeast *Saccharomyces cerevisiae*, contains 600-800 different mitochondrial polypeptides (Lithgow, 2000). Amongst these mitochondrial proteins, only eight are encoded and synthesized within the mitochondria itself, while the rest are encoded by nuclear genes and synthesized on ribosomes in the cytosol (Lithgow, 2000). Evidence from studies *in vitro* with isolated mitochondria shows that completely synthesized preproteins can be released from ribosomes and imported in a post translational manner (Neupert et al., 1990). The majority of preprotein import *in vivo* probably also occurs by a post-translational mechanism (Schatz and Dobberstein, 1996; Wienhues et al., 1991), although it cannot be ruled out that part of the import occurs contranslationally.

1.2 Mitochondrial biogenesis

1.2.1 Overview on protein translocation into mitochondria

Transport of nuclear-encoded proteins into the mitochondria is mediated by distinct multi-subunit translocation machineries located in the outer and inner membranes of mitochondria (Fig 1). The mitochondrial entry gate for these preproteins is formed by high molecular weight machinery, termed the translocase of the outer mitochondrial membrane (TOM). From the TOM complex, β-barrel precursors are relayed to another complex in the outer membrane which was termed TOB complex (topogenesis of mitochondrial outer membrane β-barrel proteins) or SAM complex (sorting and assembly machinery). The latter complex mediates the insertion of the β-barrel precursors into the outer membrane (Kozjak et al., 2003; Paschen et al., 2003; Wiedemann et al., 2003). For import of preproteins across or into the inner membrane, the TOM complex cooperates with TIM23 and TIM22 complexes in the inner membrane, which differ in their substrate specificity for preproteins (Fig. 1). Whereas the TIM22 complex mediates the membrane

potential-dependent insertion of the multitopic proteins (AAC proteins) into the inner membrane, the TIM23 complex mediates translocation of preproteins with a matrix-targeting signal into or across the inner membrane (Paschen and Neupert, 2001). Another pathway involves the export of proteins from the matrix into the inner membrane and is used by both proteins synthesized within the mitochondria, as well as by a subset of nuclear encoded proteins. The protein translocase involved in this pathway is the OXA1 complex (Hartl and Neupert, 1990).

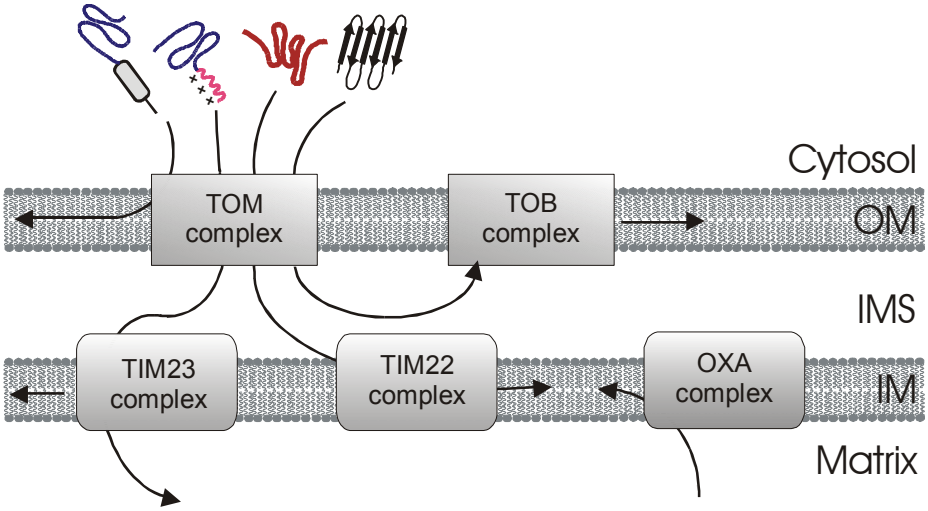

Figure 1. The general import pathways of mitochondrial preproteins
The TOM complex mediates the translocation of virtually all mitochondrial preproteins. From the TOM complex β-barrel proteins are relayed to the TOB complex which mediates their insertion into the outer membrane. Preproteins with a matrix targeting signal are translocated further via the TIM23 machinery. The inner membrane multispanning proteins use TIM22 complex for membrane insertion. OXA complex mediates the insertion into the inner membrane of proteins coming from the matrix side.

1.2.2 Mitochondrial targeting signals

Mitochondrial biogenesis is dependent upon the import of nucleus-encoded, cytoplasmically synthesized proteins. Thus, mitochondrial proteins must be targeted specifically to the organelle and imported into the correct sub-mitochondrial compartment. About half of mitochondrial precursor proteins possess a presequence at their N-terminus that contains sufficient information to be recognized by the mitochondrial import apparatus, leading to import into the mitochondria. Presequences of mitochondrial preproteins are commonly 10-80 amino acid residues in length, enriched in positively charged, hydrophobic and hydroxylated amino-acid residues (von Heijne,

1989). They are able to form amphipathic α-helices that present one positively charged surface and one hydrophobic surface (Abe et al., 2000; Epand et al., 1986). Specific primary sequence motifs have not been found. Previously, it was believed that the positive charges were required for recognition by the receptors and the amphipathic nature of the presequence favored insertion into the outer membrane. However, new studies show that different surfaces of the presequence are recognized by different receptors of the TOM complex: the hydrophobic side by Tom20 and the positively charged side by Tom22 (Brix et al., 1999). Furthermore, the NMR structure of presequence-receptor complex shows that hydrophobic residues of the presequence are required for the interaction with Tom20 (Abe et al., 2000). Presequences are cleaved, in most cases, upon import into the matrix by the mitochondrial processing peptidase, MPP (Braun et al., 1992). However, several matrix proteins such as rhodanese, 3-oxo-CoA-thiolase and chaperonin 10 (Hsp10) are synthesized with a non-cleavable N-terminal targeting signal which has characteristics very similar to those of the cleaved signals (Hammen et al., 1996; Jarvis et al., 1995; Waltner and Weiner, 1995). One matrix protein, the DNA helicase Hmil, has a presequence-like targeting signal at its carboxyl terminus. In contrast to the usual amino-to-carboxy terminal translocation, this preprotein seems to be translocated in the reverse orientation, showing that the mitochondrial import system is flexible (Lee et al., 1999).

Several other preproteins contain signals resembling presequences that are present mainly in proteins of the mitochondrial membranes and the intermebrane space. In these cases the positively charged sequences are followed by hydrophobic sorting signals that lead to the specific arrest of the preprotein in the outer or inner membranes (Gärtner et al., 1995; Glick et al., 1992a; McBride et al., 1992). Some inner membrane proteins (e.g. cytochrome c_1) and intermembrane space proteins (e.g. cytochrom b_2) are sorted via a bipartite presequence. This presequence consists of the N-terminal matrix targeting sequences followed by the hydrophobic sorting sequences which are preceded by a few positively charged residues. The sorting sequences are cleaved off at the outer surface of the inner membrane by the heterodimeric inner membrane peptidase (Imp1-Imp2) (Glick et al., 1992b). Two alternative mechanisms for sorting proteins into the inner membrane were proposed: the stop transfer route, where proteins are arrested in the inner membrane during import, and the conservative sorting pathway, where proteins are first translocated into the matrix and then directed to the inner membrane (Fölsch et al., 1996; Hartl et al., 1987). Two critical characteristics of the sorting signal were recently reported to play a

role in determining the insertion pathway of a protein. Accordingly, a strong hydrophobic character and the absence of proline residues in the transmembrane segment would favor sorting by the stop transfer mechanism (Meier et al., 2005).

Proteins of the metabolite carrier family of the inner membrane do not contain a cleavable presequence, but have multiple signals distributed over the entire length of the preprotein (Endres et al., 1999; Pfanner and Neupert, 1987; Smagula and Douglas, 1988). Other membrane proteins, such as components of the inner membrane translocase (Tim23, Tim17 and Tim22), also contain several internal targeting and sorting signals, including hydrophobic segments and positively charged loops (Davis et al., 2000; Káldi et al., 1998; Paschen and Neupert, 2001). The targeting signal of outer membrane proteins will be discussed in detail in the chapter dealing with the biogenesis of mitochondrial outer membrane proteins (see **1.3.2**).

1.2.3 Interaction of cytosolic chaperones with precursor proteins

The eukaryotic cytoplasm contains several different molecular chaperones that bind newly synthesized precursors and prevent their aggregation or misfolding. These chaperones include members of the 70 kDa heat shock protein family (hsp70), which maintain the newly synthesized preproteins in import competent conformations using an ATP-dependent mechanism (Mihara and Omura, 1996). The function of Hsp70 is not specific to mitochondrial preproteins; it is also involved in targeting preproteins to other subcellular compartments. The Ydj1 protein, a yeast homolog of Hsp40, also plays a role in mitochondrial protein import (Caplan et al., 1992a; Caplan et al., 1992b). It may assist cytosolic Hsp70 to cycle on and off unfolded (or partially folded) preproteins, and help to target preprotein-Hsp70 complexes to the surface of the outer membrane. Another cytosolic chaperone was reported to be dedicated to mitochondrial protein import; the heterodimeric protein termed mitochondrial import stimulating factor (MSF), which has been purified from rat liver cytosol. The ATPase activity of MSF is greatly stimulated by binding of non-native proteins (Hachiya et al., 1993). Recently, it was shown that the chaperone Hsp90, in cooperation with Hsp70, mediates the targeting of a subset of mitochondrial preproteins in mammals (Young et al., 2003).

One component that assists the co-translational import is the nascent polypeptide – associated complex (NAC), which was originally identified in mammalian cell extracts as a ribosome associated factor that interacts with nascent polypeptides (Wiedemann et al.,

2004a). In yeast, disruption of the genes encoding the subunits of the NAC heterodimer leads to defects in protein targeting to the mitochondria (George et al., 1998).

1.2.4 Cotranslational versus posttranslational import

The question of whether mitochondrial protein import *in vivo* occurs co- or post-tranlationally is still open. Cytosolic ribosomes were found to be associated with yeast mitochondria *in vivo* and *in vitro* under certain conditions and some biochemical data were taken as evidence of a cotranslational insertion of nascent polypeptide chains into mitochondria (Fujiki and Verner, 1991; Fujiki and Verner, 1993; Verner, 1993). When translation is slowed-down by the addition of cycloheximide, yeast mitochondria are covered with ribosomes, suggesting that ribosome–bound precursors are accumulated on the surface of mitochondria (Kellems et al., 1975). Thus it seems that the relative kinetics of translation and translocation probably determines the enrichment of polysomes encoding mitochondrial precursors on the surface of the organelle (Beddoe and Lithgow, 2002). Several recent observations support the idea that a co-translational process is involved in the mitochondrial import of at least some proteins. It was proposed that mRNA localization to the vicinity of mitochondria plays a critical role in organelle biogenesis (Knox et al., 1998; Marc et al., 2002; Stein et al., 1994). On the other hand, a large body of evidence provides convincing support for post-translational import. First, many mitochondrial precursor proteins synthesized in a cell-free system can be imported post-translationally into isolated mitochondria (Harmey et al., 1977; Neupert, 1997). Second, mitochondrial precursor proteins that accumulate in the cytosol can be chased subsequently into mitochondria (Hallermayer et al., 1977; Reid and Schatz, 1982), Third, translatable mRNAs for imported mitochondrial proteins are present in free as well as mitochondria-bound cytoplasmic polysomes (Suissa and Schatz, 1982). Fourth, some mitochondrial proteins contain targeting information at their C-terminus suggesting that import must occur after a complete synthesis of the protein (Borgese et al., 2003; Fölsch et al., 1998; Suissa and Schatz, 1982). Taken together, the translation of mitochondrial precursor proteins in the cytosol is generally not coupled to their import into the organelle, and the vast majority of precursor proteins can be imported post-translationally.

1.2.5 Translocation across the outer membrane

The TOM complex mediates the translocation across and insertion into the outer membrane of virtually all nuclear-encoded mitochondrial precursors. The TOM complex is a multi-subunit complex of ca. 450 kDa composed of seven subunits: Tom70, Tom40 Tom22, Tom20, Tom7, Tom6 and Tom5 (Neupert, 1997; Pfanner and Geissler, 2001). When purified without the receptor subunits Tom20 and Tom70, it is referred to the TOM core complex or the GIP (general import pore) (Ahting et al., 1999; Pfanner and Geissler, 2001). The two receptor proteins, Tom20 and Tom70, show different but partially overlapping specificities for preproteins (Lithgow et al., 1995). Single deletion of either receptor can be tolerated but double deletion is lethal (Ramage et al., 1993). Genetic and biochemical studies led to the idea that Tom20, together with Tom22 is a general receptor for most preproteins with a cleavable presequence (Harkness et al., 1994; Lithgow et al., 1995). In recent studies some proteins were found to interact with Tom20 although they lacked a mitochondrial presequence, like the outer membrane proteins porin (Schleiff et al., 1999), Tom40 (Rapaport and Neupert, 1999) and the intermembrane space protein cytochrome *c* heme lyase (Diekert et al., 1999). Tom70 forms a dimeric receptor for hydrophobic preproteins that contain internal targeting information, especially the carrier protein family (Brix et al., 1999; Schlossmann et al., 1994). An intriguing property of Tom20 and Tom70 is a repetitive, degenerate motif of 34 amino acid residues called the tetratricopeptide repeat (TPR). While Tom70 contains seven predicted TPR motifs, Tom20 contains one such domain (Haucke et al., 1996). This motif is present in the cytosol-exposed domains of these proteins and might have a role in protein–protein interaction (Haucke et al., 1996; Young et al., 2003).

Tom20 and Tom70 interact with cytosolic chaperones (Yano et al., 2003; Young et al., 2003). This interaction is essential to deliver a set of chaperone-associated preproteins to the receptor for subsequent membrane translocation. In yeast, Hsp70 interacts with Tom70. In mammals, preprotein in the cytosol is associated with both Hsp90 and Hsp70 in a multichaperone complex, and docking of both chaperones onto Tom70 is essential for preprotein targeting (Young et al., 2003). In the case of human Tom20, the extreme C-terminus of the receptor interacts with tetratricopeptide repeats of arylhydrocarbon receptor-interacting protein (AIP). It was further demonstrated that AIP specifically binds to mitochondrial preproteins, suggesting that AIP functions as a cytosolic factor that mediates preprotein import into mitochondria (Yano et al., 2003). In *S. cerevisiae* another receptor component was identified, Tom71. Despite the fact that Tom71 is closely related

to Tom70 (53% sequence identity and 70% similarity) the two receptors do not perform identical functions. Tom71 is expressed in minor amounts and it loosely associates with the TOM machinery. The import of Tom70 dependent preproteins is minimally affected by the deletion of Tom71, regardless of the presence or the absence of the Tom70 receptor (Schlossmann et al., 1996). Up to now the function of Tom71 has not been elucidated.

After recognition of the preproteins on the mitochondrial surface by the receptor subunits, the preproteins are transferred into the protein-conducting channel of the TOM core complex. In this context Tom22 plays an important role. The cytosolic domain of Tom22 not only functions as the receptor for preproteins but also acts as the docking point of the GIP complex to which the peripheral receptors Tom20 and Tom70 can associate (Brix et al., 1997; Pfanner and Geissler, 2001).

The preprotein conducting channel of the GIP is probably formed by several molecules of Tom40, where dimers were suggested to be the basic structure unit (Rapaport et al., 1998a). This protein spans the membrane presumably with several β-strands that form a β-barrel (Hill et al., 1998; Künkele et al., 1998). Reconstitution of purified Tom40 molecules into liposomes showed that Tom40 alone is able to form a cation selective high-conductance channel, which is voltage gated and binds mitochondrial presequences in a specific manner (Ahting et al., 2001; Hill et al., 1998; Stan et al., 2000). Tom40, the only Tom subunit that is essential for cell viability under all growth conditions, is present in about six copies per GIP complex. Electron microscopy analysis revealed that a complete GIP complex contains two to three channels (Ahting et al., 1999; Künkele et al., 1998; Model et al., 2002). The channel has a pore diameter of ~ 22Å, a size that is sufficient for the passage of up to two α-helical segments, but not folded domains. (Hill et al., 1998; Schwartz and Matouschek, 1999). Site-specific photocrosslinking studies indicate that Tom40 seems to interact preferentially with unfolded segments of the preprotein in the translocation channel. Furthermore, purified Tom40 binds to non-native proteins and suppresses their aggregation (Esaki et al., 2003). Taken together, the Tom40 channel offers an optimized environment for translocating non-native precursor proteins by preventing their aggregation.

The functions of small Tom proteins are only partially understood. Tom5 is closely associated with Tom40 and was proposed to represent the connecting link between import receptors and the general import pore. It is also needed subsequently for

polypeptide chain insertion into the translocation pore. Up to now, Tom5 is seen as a receptor, which functions by taking over preproteins from the Tom22 receptor (Dietmeier et al., 1997). However, new experimental evidence (including this thesis) contradicting the receptor function of Tom5 have accumulated in more recent years (Habib et al., 2003a; Horie et al., 2003; Schmitt et al., 2005a). Possible role(s) that Tom5 may play will be discussed in more detail in the discussion chapter.

Tom6 and Tom7 seem to be involved in the dynamic modulation of assembly and disassembly of receptor proteins with the GIP (Meisinger et al., 2001; Sherman et al., 2005). Both proteins were found in *N. crassa* to be in the vicinity of Tom40, while Tom6 probably forms the link between Tom40 and Tom22 (Dembowski et al., 2001). Tom7 plays a role in sorting and accumulation of the preproteins at the outer membrane and supports dissociation of the translocase components which may maintain the continuous exchange of the Tom proteins (Dekker et al., 1998; Rapaport, 2002).

The driving force for translocation across the outer membrane is not completely understood. It seems possible, if not likely, that the translocation across the outer membrane is driven by sequential, non-covalent interactions of presequences with different modules of the TOM complex. Presequences interact initially with the primary receptor Tom20. The cytosolic domain of Tom22 takes part in the formation of this surface binding site, termed *cis* (Honlinger et al., 1996; Mayer et al., 1995a; Rapaport et al., 1997). Cross-linking experiments suggest that the presequence is already in the vicinity of Tom40 at this early stage of import (Kanamori et al., 1999; Rapaport et al., 1997). Movement of the presequence to the inner side of the outer membrane results in the formation of a second intermediate bound at the *trans* site of the outer membrane. The *trans* site consists of Tom40, the C-terminal domain of Tom22 and Tom7 (Bolliger et al., 1995; Endo and Kohdab, 2002; Esaki et al., 2004; Meisinger et al., 2001; Moczko et al., 1997; Rapaport et al., 1997; Schatz, 1997). *Trans* site binding occurs with much higher affinity than *cis* site binding (Mayer et al., 1995b; Rapaport et al., 1998b; Stan et al., 2000). Binding of the preprotein to the *cis* or *trans* sites induces distinct structural alterations in Tom40 and influences the interactions of Tom6 with both Tom40 and Tom22 (Dembowski et al., 2001; Rapaport et al., 1998a). The current model suggests that a chain of presequence binding sites with increased affinity towards the presequence provide the driving force for translocation across the outer membrane and facilitates vectorial movement of the protein precursor.

After passage through the TOM complex, proteins are sorted either to the outer membrane, the intermembrane space, or to one of the translocases of the inner membrane (TIMs).

1.2.6 The TIM23 translocase

The TIM23 translocase and TOM complex form independent structural and functional units. If the outer membrane is ruptured, preproteins can be directly translocated across the inner membrane without a need for the TOM machinery (Hwang et al., 1989).

The main building blocks of the TIM23 machinery are the inner membrane channel for preproteins and an import motor located in the matrix (also known as PAM-presequence translocase associated motor). The translocase consists of the two phylogenetically related membrane-embedded subunits Tim17 and Tim23 to which six largely hydrophilic subunits, Tim50, Tim44, Tim16 (Pam16), Tim14 (Pam18), Tim21 and Pam17 are attached (Rehling et al., 2004).

Tim50 is the first Tim component that interacts with the preprotein merging from the TOM complex. This component has a large domain in the intermembrane space, which binds to Tim23. Tim50 seems to associate with presequence-containing proteins when they enter the intermembrane space and directs them to the TIM23 translocase (Geissler et al., 2002; Mokranjac et al., 2003a; Yamamoto et al., 2002). Tim21 is specific for a TIM23 form that cooperates with TOM and promotes inner membrane insertion (see below) (Chacinska et al., 2005). The 90 kDa core of the TIM23 translocase is formed by equimolar amounts of Tim23 and Tim17. Both proteins contain a membrane-integrated domain with four predicted transmembrane segments. Although the membrane spanning regions of Tim23 and Tim17 are homologous, they cannot substitute for each other; each protein is essential for cell viability (Emtage and Jensen, 1993; Kübrich et al., 1994; Maarse et al., 1994). Tim23 additionally exposes an amino terminal domain of approximately 100 amino acid residues, which can be subdivided into two parts. Residues ~50 to 100 dimerize and thereby form a presequence receptor domain that is exposed to the intermembrane space (Donzeau et al., 2000). The N-terminal domain (amino acids ~1-49) was shown to be integrated into the outer membrane. Tethering the inner membrane translocase to the outer membrane may facilitate the transfer of precursor proteins from the TOM complex and increases the efficiency of protein import.

Biochemical studies indicate that Tim17 and Tim23 are in close contact with a preprotein in transit (Kübrich et al., 1994; Ryan and Jensen, 1993). Accordingly, it is

thought that the intermembrane space domain of Tim23 forms the presequence receptor, and the integral membrane domains of Tim17 and Tim23 form the translocation channel for preproteins. Purified recombinant Tim23 forms a voltage-sensitive high-conductance channel with specificity for mitochondrial presequences. The reported channel, which is formed by the C-terminal domain alone, is cation selective with a width of ~13-24 Å (Truscott et al., 2001). Functional reconstitution of Tim17 alone has not been demonstrated yet. Thus, the role of Tim17 remains elusive. However, it is possible that Tim17 also forms a channel due to the homology between its membrane domain and that of Tim23. Recently it was demonstrated that two conserved negative charges in the amino terminus of Tim17 are critical for its function (Meier et al., 2004). It was suggested that these charged amino acids are required for the preprotein mediated opening of TIM23 translocase.

Preprotein translocation across the inner membrane requires two driving forces. One is a membrane potential (negative on the matrix side) for presequence translocation and activation of Tim23. The other is an ATP-dependent motor system to drive the translocation of the mature portion of preproteins into the matrix. The ATP-dependent import motor consists of a subcomplex that is represented by the membrane proteins Tim14 and Pam17 (van der Laan et al., 2005), the two peripheral associated components Tim16 and Tim44, the mitochondrial heat shock protein (mtHsp70) and the cochaperone mGrpE (Mge1). Tim44 functions as a membrane anchor for the ATPase domain of mtHsp70 directly at the exit site of the import channel, whereas, mtHsp70 is required for translocation of the remainder of the polypeptide precursor across the inner membrane in an ATP-dependent manner. The J protein Tim14 stimulates the ATPase activity of mtHsp70 and thereby enables efficient binding of mtHsp70 to preproteins (Mokranjac et al., 2003b; Truscott et al., 2003).

Yeast cells contain a close homologue of Tim14, known as Mdj2, which was also found to be a component of the mitochondrial import motor. This protein stimulates, *in vitro,* the ATPase activity of mtHsp70 to the same extent as Tim14 does (Mokranjac et al., 2005b). mGrpE is a nucleotide exchange factor that promotes the reaction cycle of mtHsp70. Two models were proposed to explain the role of mtHsp70 in the protein import, the Brownian Ratchet Model and the Translocation Motor Model. According to the Brownian Ratchet Model, a precursor polypeptide oscillates randomly within the translocation channel due to Brownian motion. After an inward oscillation, mtHsp70 passively traps a segment of the precursor chain. A series of such events would lead to

complete import of the precursor (Neupert and Brunner, 2002). Meanwhile, the Translocation Motor Model proposes that mtHsp70, bound to Tim44, interacts with the precursor polypeptide. mtHsp70 is an ATPase that undergoes conformational changes upon binding ATP. The conformational changes of membrane-bound Hsp70 generate a mechanical force that pulls the preprotein into the matrix (Matouschek et al., 2000). Recently a new Tim component was identified, Tim21 (Chacinska et al., 2005; Mokranjac et al., 2005a). It was shown that Tim21 interacts with the TOM complex and plays an important role in coordinating the TOM complex, the TIM23 translocase and the import motor. Two forms of TIM23 seem to occur. In one form Tim21 associates with TIM23 and the import motor dissociates. This form promotes the TIM23 dependent insertion into the inner membrane. The other form is required for translocation of matrix proteins. In the latter, Tim21 dissociates from the TIM23 tranlocase and the import motor is recruited. Tim17 plays a crucial role in this switch, as it is required for both sorting into the inner membrane and direct interaction with the import motor (Chacinska et al., 2005).

The journey of preproteins from the cytosol to the matrix ends in most cases by proteolytic removal of the preprotein presequence by the mitochondrial processing peptidase (MPP) (Gakh et al., 2002). Then, the critical step of protein refolding begins. A number of folding helpers (mtHsp70, the chaperonin Hsp60, Peptidyl-Prolyl cis/trans isomerase) in the matrix can assist in refolding the imported proteins. Depending on the type and the conformation of imported proteins, the dependence on the various folding helpers can be quite different (Cheng et al., 1989; Ostermann et al., 1989).

1.2.7 The TIM22 translocase

While the TIM23 complex inserts inner membrane proteins, which have presequences and contain only one transmembrane segment, the TIM22 complex is required for the insertion of multiple membrane-spanning domain proteins like Tim23, Tim17, Tim22 and the metabolite carried proteins family. This preprotein translocase inserts the proteins into the inner membrane in a membrane potential-dependent manner (Kerscher et al., 1997; Kerscher et al., 2000; Koehler et al., 2000; Sirrenberg et al., 1996). To pass through Tom40, the precursor adopts a hairpin-like conformation and the small soluble Tim proteins in the intermembrane space bind to it. Whereas the essential 70 kDa Tim9-Tim10 complex is required for the transport of carrier proteins, the non-essential 70 kDa Tim8-Tim13 complex was found to interact with Tim23 precursors (Bauer et al., 2000). Recently, it was demonstrated that in *N. crassa* the Tim23 precursor is also one of the

substrates of the Tim9-Tim10 complex (Vasiljev et al., 2004). The small Tim proteins function in a chaperone-like manner to prevent aggregation of the imported precursors and are required for further translocation from the outer membrane to the TIM22 complex. This complex is organized into peripheral and membrane integrated units. The peripheral unit, which consists of the 70 kDa Tim9-Tim10-Tim12, associates with the integral portion of the complex on the intermembrane-space side. The membrane-integrated unit consists of three proteins: Tim18, Tim54 and the essential Tim22. Little is known about the molecular roles of Tim18 and Tim54 in protein insertion, but Tim22 was shown to form a pore in the inner membrane. A single-particle, electron microscopy analysis of the 300 kDa TIM22 highlighted two stain filled pits that are reminiscent of two pores, each with a diameter of about 16 Å (Rehling et al., 2003). Electrophysiological analysis on the purified and reconstituted Tim22 showed characteristics of a single pore with a diameter of 12-17 Å, depending on the open state (Kovermann et al., 2002; Rehling et al., 2004). Furthermore, Tim22 was found to recognize internal targeting signals and responds to the membrane potential with rapid gating in a signal dependent manner.

1.2.8 The OXA1 translocase

The OXA1 translocase of the mitochondrial inner membrane facilitates the insertion of both mitochondrially and nuclear-encoded proteins from the matrix into the inner membrane. Examples for such precursors are the mitochondrially encoded subunit 2 of the cytochrome oxidase complex (Cox2p) which spans the inner membrane twice, and Oxa1p itself, a nuclear encoded polytopic protein (He and Fox, 1997; Hell et al., 1998; Hell et al., 2001; Nargang et al., 2002). Oxa1p is a member of the highly conserved Oxa1p/YidC/Alb3 protein family found throughout prokaryotes and eukaryotes (Kuhn et al., 2003). Oxa1 spans the inner membrane five times, exposing a long α-helical C-terminal domain into the matrix. The ability of this domain to bind mitochondrial ribosomes was suggested to tether the precursors to the site of its integration into the lipid bilayer (Jia et al., 2003; Szyrach et al., 2003).

1.3 Biogenesis of mitochondrial outer membrane proteins

1.3.1 Topologies of mitochondrial outer membrane proteins

The mitochondrial outer membrane harbors a variety of proteins that include porins, components of the protein translocation machinery, enzymes for the lipid biosynthesis and other processes as well as components that control the morphology of the organelle (Schmitt et al., 2006; Zahedi et al., 2006). The topology of these proteins can be divided in different classes (Fig 2):

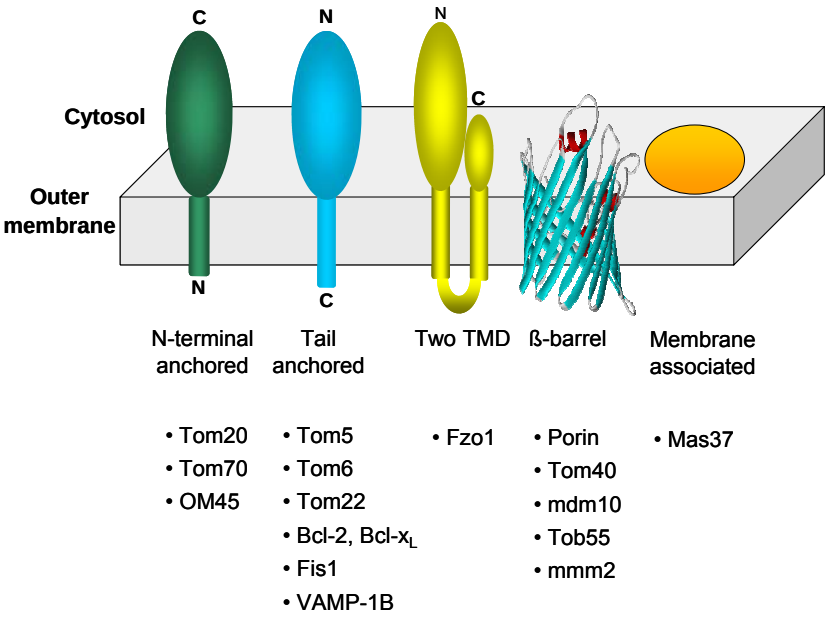

Figure 2. Topologies of outer membrane proteins of mitochondria.

(1) Signal anchor proteins like Tom70, Tom20 and OM45. These proteins expose a large domain to the cytosol and only a small N-terminal segment protrudes the outer membrane. (2) Tail-anchored proteins such as Tom5, Bcl-2 and Fis1, have a single transmembrane domain (TMD) at the carboxy terminus and their large N-terminal region is exposed to the cytosol. (3) Fzo1 (a component of the fusion machinery) which spans the outer membrane twice, exposing a small loop to the IMS. (4) β-barrel proteins that are predicted to traverse the outer membrane as a series of anti parallel β-strands that form a β-barrel structure. To date, only structures of membrane β-barrel proteins from bacteria are resolved (Tamm et al., 2004). Accordingly, the number of β strands varies from eight to 22, but is always an even number. On average, a β strand consists of 8-11 amino acid

residues, a number sufficient to span a biological membrane (Paschen, 2004; Rapaport, 2003). (5) Peripheral associated membrane proteins, like Mas37, which are attached to the cytosolic side of the outer membrane (Gratzer et al., 1995; Wiedemann et al., 2003).

1.3.2 Targeting sequences of mitochondrial outer membrane proteins

1.3.2.1 *The targeting sequence of signal anchored proteins*

Amino-terminally anchored proteins are also known as 'signal anchored' proteins because their transmembrane domain (TMD) and its flanking regions function both as an intracellular sorting signal and as an anchor to the membrane. The signal sequence of *S.* cerevisiae Tom70 was analyzed in detail. The targeting information resides in a linear sequence that include the predicted transmembrane domain (residues 10-29) as well as residues 1-9, which comprise a hydrophilic, positively charged segment (Shore et al., 1995). The TMD is required for both mitochondrial targeting and membrane anchoring of the protein, whereas the positively charged residues are required to enhance the import rate (McBride et al., 1992). Analysis of the signal anchor sequence of rat Tom20 revealed that both moderate TMD hydrophobicity and a net positive charge within five residues of the COOH-terminal flanking region were critical for mitochondria targeting. (Kanaji et al., 2000). Recently, it was also shown in yeast that moderate hydrophobicity of the TMD is the most important requirement for mitochondrial targeting and anchoring. Furthermore, signal anchor domains of outer membrane proteins were shown to be functionally interchangeable. Hence, they seem to play only a minor role in the specific function of these proteins but have a decisive role in topogenic signaling, although they do not posses sequence homology (Waizenegger et al., 2003).

1.3.2.2 *The sorting sequence of tail anchored proteins*

Like the N-terminally anchored proteins, tail anchored proteins do not share any sequence conservation in their tail region (Fig 3), and the mitochondrial targeting information is encoded instead in the structural features of this part. The importance of the positive charges in the TMD-flanking regions was emphasized in several proteins. The Bcl-2 family of proteins is central regulators of apoptosis. In cells, it was shown that only Bcl-x_L is specifically targeted to the mitochondrial outer membrane, whereas Bcl-2 is distributed on several intracellular membranes. The TMD of both proteins have the same length and hydrophobicity. However, Bcl-x_L contains a particular mitochondrial signal sequence. This signal requires two basic amino acids at both ends of the TMDs Bcl-2 lacks this signal, as it contains only one basic residue on either side, and thereby is

inserted into different membranes in non-specific manner (Kaufmann et al., 2003). Cyt*b*5 and VAMP1 are other examples of dual-membrane localization. Both proteins exist in two isoforms: one in the ER membrane and the other in the mitochondrial outer membrane. Proper targeting and insertion in the mitochondrial outer membrane require a short TMD within the tail domain and the lack of negatively charged amino acids in its C-terminal flanking region (Borgese et al., 2003; Isenmann et al., 1998).

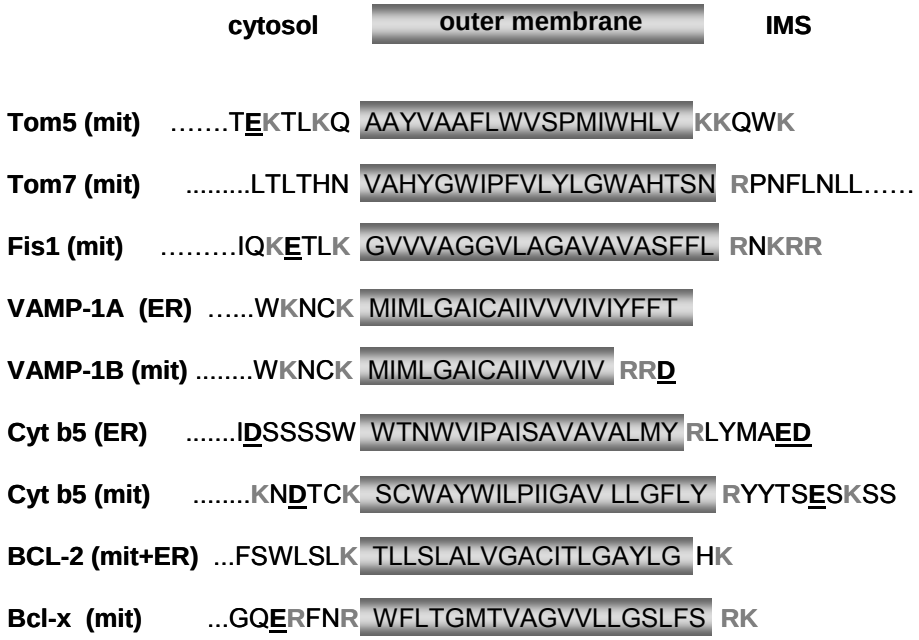

Figure 3. Sequences of tail–anchor domains of mitochondrial and endoplasmic reticulum proteins. Positively charged amino-acid residues are shown in grey and negatively charged residues are shown in bold, underlined letters.Cyt*b*5, cytochrome *b*5; ER, endoplasmic reticulum; IMS, intermembrane space; mit, mitochondrial; Net+, net positive charge; OM, outer membrane; Tom, translocase of the outer membrane of mitochondria; VAMP, vesicle-associated membrane protein/synaptobrevin (Rapaport, 2003)

Single residues within the TMD were also shown to play a role in the targeting of tail anchored proteins such as the components of the TOM core complex: Tom5, Tom6, Tom7 and Tom22. The TMD of these proteins harbour a conserved proline residue. In the case of Tom7, this residue was shown to be important for efficient targeting of the protein (Allen et al., 2002). It is possible that proline, which is known as an α-helical destabilizer, introduces flexibility within the TMD. This flexibility might further help the tail domain to be anchored in the membrane. Further information was obtained from studying the targeting of the tail anchored yeast Tom5 in mammalian system. This study revealed that the moderate length of the TMD, the positive charges in the C-segment, and the distance between or context of the TMD and C-segment are critical for the targeting signal (Horie

et al., 2002). Importantly, yeast Tom5 was targeted to the mammalian mitochondria but did not assemble into the TOM complex. In one part of this thesis, the possible roles of the TMD in the biogenesis of tail anchored proteins were investigated and the importance of positively charged residues in the flanking region of the TMD was accentuated.

1.3.2.3 *The targeting information of β-barrel proteins*

The targeting information of membrane proteins, such as porin and Tom40 is spread throughout different regions of the protein. In the case of *N.crassa* Tom40, deletion of the N- or the C-terminus did not affect protein targeting to mitochondria, thus indicating that the targeting information is not in the termini of the protein (Rapaport and Neupert, 1999). Studies on yeast porin show that deletions of residues 17-98 (Hamajima et al., 1988) or 9-156 (Smith et al., 1995) abrogate the import process. The import efficiency is also decreased when one of the two residues, Lys234 or Lys236, is mutated to a neutral or negatively charged amino acid (Smith et al., 1995). Additionally, deletion of the last 62 residues prevents its import (Hamajima et al., 1988). On the other hand, it appears that *N. crassa* porin contains import targeting and/or assembly information at its C-terminus, rather than at the N-terminus (Court et al., 1996). Taken together, these studies suggest that the targeting information in β-barrel proteins may be encoded in a structural element that involves different regions rather than a linear sequence (Rapaport, 2003).

1.3.3 Biogenesis of β-barrel membrane proteins

1.3.3.1 *The import pathway of mitochondrial β-barrel membrane proteins*

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. Despite their central role in bacterial and organelle biogenesis very little is known about how newly synthesized β-barrel proteins are sorted within the cell, integrated into lipid bilayers and assembled into oligomeric structures.

In the case of mitochondria, β-barrel precursors are initially recognized by the receptors Tom20 and Tom70. They are then translocated via the general pore of TOM complex (Krimmer et al., 2001; Model et al., 2001; Rapaport, 2002; Rapaport and Neupert, 1999; Schleiff et al., 1999). From the TOM complex β-barrel precursors are transferred to the

TOB complex which mediates the topogenesis of these proteins in the outer membrane. 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 residing in this compartment (Hoppins and Nargang, 2004; Wiedemann et al., 2004b).

1.3.3.2 *The TOB complex*

The major component of the TOB complex is Tob55 (also known as Sam50) (Gentle et al., 2004) (Kozjak et al., 2003; Paschen et al., 2003). Its sequence is similar to that of the highly conserved bacterial protein Omp85/YaeT, which was proposed to mediate the insertion of β-barrel proteins into the bacterial outer membrane (see below) (Voulhoux et al., 2003; Wu et al., 2005b). Furthermore, Tob55 apparently has homologous proteins throughout the entire eukaryotic kingdom. Tob55 was found to be essential for viability in yeast cells. Depletion of Tob55 or the growth of a conditional mutant at restrictive temperature leads to reduced levels of β-barrel membrane proteins in mitochondria. Furthermore, isolated mitochondria lacking Tob55 are unable to import β-barrel membrane proteins. Taken together, Tob55 plays a specific role in the biogenesis of mitochondrial β-barrel membrane proteins. The 55 kDa protein is composed of two parts: the membrane-integrated β-barrel domain in its C-terminal region and the predicted hydrophilic N-terminal domain which is exposed to the IMS. The N-terminal domain is rich in POTRA (polypeptide-transport-associated domain) repeats which are assumed to have a chaperone-like function (Gentle et al., 2005; Sanchez-Pulido et al., 2003a) Thus, this domain was proposed to present the interaction site for β-barrel precursors before there TOB-mediated insertion into the membrane (Pfanner et al., 2004). This proposal was investigated in this thesis in detail.

Electron microscopy analyses of the recombinant and the purified Tob55 show ring-shaped assemblies with an outer diameter of approximately 15 nm and an inner diameter of about 7–8 nm. The central density measured approximately 4–5 nm (Paschen et al., 2003). Tob55 is the main component of a complex of ~ 250 kDa, termed TOB/SAM complex. Mas37 is another component of this complex, and its role in the biogenesis of mitochondrial β-barrel membrane proteins has not yet been identified. Mas37 is not an essential protein in yeast. In its absence, a functional TOB core complex is present, yet imported β-barrel precursors accumulate as TOB-bound species (Paschen et al., 2003; Wiedemann et al., 2003). An important point which was addressed in this thesis together

with Waizenegger T. *et al* is the identification and characterization of a new component of the TOB complex. Furthermore, the assembly of the TOB complex into the outer membrane was analyzed in detail.

1.3.4 Biogenesis of bacterial β-barrel membrane proteins

Proteins present in the outer membrane (OM) of gram-negative bacteria belong to one of two classes: lipoproteins, which are anchored to the OM with an N-terminal lipid tail, and integral proteins that contain membrane-spanning regions that form β-barrel structures (OMPs, outer membrane proteins). All proteins destined to the OM are synthesized in the cytosol with an N-terminal signal sequence (Bos and Tommassen, 2004). The Sec machinery of the inner membrane recognizes the signal sequence and mediates the translocation of the proteins across the inner membrane. Several chaperons in the periplasm, such as Skp (Harms et al., 2001) and SurA (Rouviere and Gross, 1996) interact with the β-barrel precursor. The insertion of OMPs into the OM is mediated by Tob55 homolog, Omp85. This component was discovered in *N. meningitides* and was shown to be essential for viability (Voulhoux and Tommassen, 2004). Depletion of Omp85 from cells leads to accumulation of outer-membrane β-barrel proteins in the periplasm. Moreover, overlay experiments showed that Omp85 bound non-native porin, indicating a direct role of Omp85 in OMP assembly. It was also suggested that Omp85 has a role in lipid transport to the outer membrane (Genevrois et al., 2003). However, in a temperature-sensitive mutant of YaeT, the homolog of Omp85 in *E. coli*, the lipid synthesis and export was (essentially) not affected, whereas levels of β-barrel proteins were reduced (Doerrler and Raetz, 2005). Recently, YaeT was shown to be in a multimeric protein complex that contains additionally three conserved lipoproteins: YfgL, YfiO and NlpB (Wu et al., 2005a). Taken together, it seems that Omp85/YaeT exerts a similar function(s) to Tob55 in the biogenesis of β-barrel membrane proteins.

1.4 Aims of the present study

The main purpose of this study was to investigate the biogenesis of the TOB complex. In particular two points were addressed in detail: (1) The mechanisms by which Tob55 and Mas37 are targeted to mitochondria and become assembled into the TOB complex. (2) The role of the N-terminal domain of Tob55 in the topogenesis of mitochondrial β-barrel membrane proteins.

Another goal of this study was to investigate the targeting information of mitochondrial tail-anchored proteins and the role(s) that these domains play in the function of the individual protein.

2. Material and Methods

2.1 Methods in molecular biology

Standard molecular biology methods were performed according to Sambrook *et al.* (Sambrook et al., 1989).

2.1.1 Small and large scale isolation of plasmid DNA from *E. coli*

Small scale preparation of plasmid DNA was performed according to the alkaline lysis method (Birnboim and Doly, 1979). Two millilitres LB-medium (Luria- Bertani medium: 10 g/l bactotrypton, 5 g/l bacto-yeast-extract, 10 g/l NaCl supplemented with 100 mg/l Ampicillin) inoculated with a single bacterial colony and incubated (ON at 37°C) under vigorous agitation conditions. Cells from 1.5 ml culture were harvested by centrifugation (7,500xg, 30 sec, room temperature (RT)). The resulting cell pellet was resuspended in 300 µl buffer E1 (50 mM Tris-HCl, 10 mM EDTA, 100 mg/l RNase, pH 8.0) and cell lysis was performed by adding 300 µl buffer E2 (0.2 M NaOH, 1% (w/v) SDS). The samples were mixed by inverting the tubes 5 times and left for 5 min at RT. For neutralization, 300 µl buffer E3 (3.1 M KOAc, pH 5.5) was added, and the samples were mixed immediately by inverting the tubes 5 times. After centrifugation (35,000 x g, 10 min, RT), the supernatant (containing the plasmid DNA) was transferred to a new tube and the DNA was precipitated by adding 600 µl isopropanol (96%). The samples were pelleted via centrifugation, washed with 70% cold ethanol and, after drying at RT, resuspended in 30 µl H_2O and used for further analysis.

For large scale preparation of plasmid DNA (up to 0.5 mg) a "Jetstar" Kit (Genomed) was used. LB-medium (50 ml) supplemented with Ampicillin was inoculated with bacteria carrying the required plasmid and incubated (ON, 37°C) under vigorous agitation conditions. The bacteria were harvested by centrifugation (3,000 x g, 10 min, RT) and resuspended in 4 ml of buffer E1 and cells lysis was performed by adding 4 ml of buffer E2. The samples were mixed by inverting the tubes 5 times and left for 5 min at RT. After neutralization by adding 4 ml of buffer E3, samples were centrifuged (31,000xg, 10 min, RT). The supernatant was applied to an anion-exchange column, previously equilibrated with 10 ml buffer E4 (0.15% v/v Triton X-100, 0.6 M NaCl, 100 mM NaOAc, pH 5.0). The column was washed twice with 10 ml buffer E5 (0.8 M NaCl, 100 mM NaOAc, pH 5.0) and the plasmid was eluted by adding 5 ml buffer E6 (1.25 M NaCl, 100 mM Tris-HCl, pH 8.5). The DNA was precipitated with isopropanol,

sedimented (23,700xg, 30 min, 4°C), washed with 70% ethanol, dried at RT and resuspended in 100µl of water**.**

2.1.2 Preparation of yeast DNA

Isolation of yeast DNA was performed as described previously by Rose *et al*. (Rose et al., 1990). YPD-medium (see 2.2.2.1) (5 ml) was inoculated with *S. cerevisiae* cells and incubated overnight at 30ºC, while shaking (140 rpm). The cells were harvested by centrifugation (7,500xg, 30 sec, RT), washed with water, and resuspended in 200 µl of breaking buffer (2% Triton-X100, 1% SDS, 100 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0). Then, phenol/chloroform/isoamyl alcohol (25:24:1) mix (200 µl) and 0.3 g glass beads were added, and the samples were vortexed for 2 min. The probes were then centrifuged (36,670xg, 5 min, RT) and the supernatant (the aqueous phase) transferred to new tubes. DNA was precipitated by adding 2.5 vol. of 100% ethanol. Samples were incubated for 10 min at –20°C, centrifuged (36,670xg, 10 min, 2°C), and washed with 70% ethanol. Pellets were dried at RT, resuspended in 20 µl H_2O and stored at –20°C.

2.1.3 Polymerase chain reaction (PCR)

DNA sequences were amplified by Polymerase Chain Reaction (PCR), using thermostable DNA polymerase. PCR mix contains (total 100 µl): 1-2 U DNA polymerase (*Taq*-polymerase and/or *Pfu*-polymerase), 10 µl PCR-buffer (1% Triton X-100, 100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$, pH 8.8), 2 µl [10 mM] dNTPs, 100 pmol primers and 100 ng plasmid DNA template or 1 µg genomic DNA as template.

The following program was used:

1) 5 min, 94°C	nuclease inactivation and complete DNA denaturation;
2) 30 cycles:	1 min, 94°C DNA denaturation;
	1 min, 45-65°C annealing of oligonucleotide primers;
	1-3.5 min, 72°C new DNA synthesis (extension)
	(The duration of this step is determined by the length of the DNA fragment to be amplified)
	Taq-polymerase: 1 min/1 kb
	Pfu-polymerase: 2.5 min/1 kb;
3) 10 min, 72°C	completion of the last reaction.

The amplified DNA fragments were analyzed by agarose gel electrophoresis.

Annealing temperature for primers was calculated by arithmetically adding the number of A and T nucleotides (in primer's sequence), multiplied by two, to the number of G and C nucleotides multiplied by 4. The calculation includes only the part of primer which fully anneals with the template. Temperature that is ~5 degrees lower than the lowest calculated annealing temperature of the two primers was chosen.

2.1.4 Enzymatic manipulation of DNA

2.1.4.1 *Digestion of DNA with restriction endonucleases*

For analytical and preparative purposes plasmid DNA and PCR products were digested with specific restriction endonuclease (up to 5 U of enzyme for 1 µg DNA). The incubation time, temperature and the buffer used in different reactions were according to the manufacturer's recommendations. The fragments obtained were analyzed by agarose gel electrophoresis or directly isolated using anion-exchange chromatography (Qiagen).

2.1.4.2 *Ligation*

Linearized DNA vector (50-200 ng) and a 5 fold molar excess of DNA fragment to be inserted, were incubated in a 10 µl reaction with 1 µl of 10x ligation buffer (10 mM $MgCl_2$, 5% (w/v) PEG-8000, 1 mM DTT, 1 mM ATP, 50 mM Tris-HCl, pH 7.6), and 0.5 µl (1 U) T4-DNA ligase (Gibco-BRL). Reactions were performed at 14ºC for 16 h and 0.5-1 µl of this mixture was used for transformation into *E. coli* cells.

2.1.5 DNA purification and analysis

DNA fragments were separated by electrophoresis in a horizontal agarose gel (0.8-2%) according to their molecular weight. The samples were mixed with loading buffer (6% (v/v) glycerol, 0.05% bromphenolblue, 0.05% xylencyanol) and electrophoresis was performed in TAE-buffer (4.84 g/l Tris-Base, 1.14 ml/l acetic acid, 1 mM EDTA, pH 8.0). The agarose solution contained 0.5 µg/ml ethidium bromide, to allow visualization of DNA in gel under UV light. The agarose was stored at 65°C until use. The 1 kb and the 100 b DNA –ladders from NEB (New England Biolabs, Beverly, USA) were used as markers. DNA fragments required for further work were cut out from the gel with a clean scalpel and DNA was extracted from the gel and isolated using anion-exchange chromatography kit (Qiagen).

For DNA concentration measurements, the absorption of DNA solutions was measured at 260 nm. An OD of 1.0 corresponds to a concentration of 50 µg/ml of double

stranded DNA, 33 µg/ml single stranded DNA, 40 µg/ml RNA or 20 µg/ml oligonucleotides.

2.1.6 Preparation and transformation of *E. coli* competent cells

2.1.6.1 *Preparation of competent cells*

A small culture, usually 10 ml of LB-medium + Ampicillin (LBamp), inoculated with a single colony of the corresponding *E. coli* strain (MH-1 or XL-1 Blue), was grown overnight at 37ºC under moderate shaking conditions. The following day, 500 ml of liquid LBamp medium was inoculated with the overnight culture. The bacterial cells were grown further until they reached the logarithmic growth phase (OD_{600} ~ 0.5). Then, they were incubated on ice for 30 min, harvested by centrifugation (4,400 x g, 5 min, 4ºC) and washed sequentially with 500 ml, 250 ml, and 50 ml of 10% (v/v) glycerol. The competent cells were finally resuspended in 500 µl 10% (v/v) glycerol, aliquoted, and stored at –80ºC.

2.1.6.2 *Transformation of E. coli*

E. coli competent cells were incubated with 1-5 µl ligation mixture for 30 sec on ice. The suspension was transferred to a pre-chilled cuvette and the cuvette was introduced in an electroporation apparatus, Gene Pulser (BioRad). The instrument was set at 2.5 kV, 400 Ω, 25 µF, time constant 8-9 ms. After a brief application of a high electric voltage to the cells, the suspension was diluted with 800 µl LB-medium, and incubated for 30-60 min at 37°C under moderate shaking conditions. The transformed cells were harvested by centrifugation and plated on LBamp plates. The plates were incubated ON at 37°C.

2.1.7 Over-view of used Plasmids

Plasmid	**Reference**
MBP (pMal cRI)	New England BioLabs
MBP-Fis1 (1-98)	This thesis
MBP-Tob55 (1-120)	(Paschen et al., 2003)
pGEM4 (empty)	Promega
pGEM4-Mas37	(Habib et al., 2005)
pGEM4-Mdm10	(Paschen et al., 2003)
pGEM4-*N.c.* $F_1\beta$	(Rassow et al., 1990)

pGEM4-Porin	(Mayer et al., 1993)
pGEM4-Tim23	(Berthold et al., 1995)
pGEM4-Tob55	(Paschen et al., 2003)
pGEM4-Tob55Δ50	This thesis
pGEM4-Tob55Δ80	This thesis
pGEM4-Tob55Δ102	This thesis
pYX132-Fis1(2Gln)	This thesis;(Habib et al., 2003)
pYX132-Fis1(4Gln)	This thesis;(Habib et al., 2003)
pYX132-Fis1(cyt)-Tom5	This thesis; (Habib et al., 2003)
pYX132-Fis1(cyt)-Tom6	This thesis; (Habib et al., 2003)
pYX132- Tob55Δ50	This thesis
pYX132- Tob55Δ80	This thesis
pYX132- Tob55Δ102	This thesis

Cloning strategies

MBP-Fis1 (1-98)

The DNA sequence encoding the first 98 amino acids of Fis1 was amplified by PCR. The following primers were used:

Primer Name	Included cutting site	Primer sequence
Fis1-N-ter	EcoRI	5'-AAA GAA TTC ATG ACC AAA GTA GAT TTT TGG-3'
Fis1 (cyt)	HindIII	5'-AAA AAG CTT TTA CTT CTG GAT CTT ATC CTC TAC-3'

pGEM4-Tob55Δ50

The DNA sequence of Tob55-lacking the first 50 amino acid was amplified by PCR. The following primers were used:

Primer Name	Included cutting site	Primer sequence
Tob55 d50 FW	BamHI -EcoRI	5'- AAA GGA TCC GAA TTC_ATG TTA GAT GAT ACT ATT ATG AAA TCC -3'
Tob55 RW:	HindIII	5'- AAA AAG CTT TTA TAA AAA TGC CAG ACC AAG ACC -3'

pGEM4-Tob55Δ102

The DNA sequence of Tob55-lacking the first 50 amino acid was amplified by PCR. The following primers were used:

Primer Name	Included cutting site	Primer sequence
Tob55 d102 FW	BamHI -EcoRI	5'- AAA_GGA TCC GAA TTC_ATG CAT GAT GTG GTG CCT TTG ATG G -3'
Tob55 RW	HindIII	5'- AAA AAG CTT TTA TAA AAA TGC CAG ACC AAG ACC -3'

pYX132-Fis1(2Gln)

The DNA sequence of Fis1, where the Arg154 and Arg155 were replaced by glutamine residues, was amplified by PCR. The following primers were used:

Primer Name	Included cutting site	Primer sequence
Fis1-N-ter	EcoRI	5'-AAA GAA TTC ATG ACC AAA GTA GAT TTT TGG-3'
C-ter 2Gln	HindIII	5'- AAA AAA AAG CTT TCA TTG TTG CTT GTT TCT TAA GAA GAA ACT AGC-3'

pYX132-Fis1(4Gln)

The DNA sequence of Fis1, where Arg151, Lys153, Arg154,and Arg155 were replaced by glutamine residues, was amplified by PCR. The following primers were used:

Primer Name	Included cutting site	Primer sequence
Fis1-N-ter	EcoRI	5'-AAA GAA TTC ATG ACC AAA GTA GAT TTT TGG-3'
C-ter 4Gln	HindIII	5'- AAA AAA AAG CTT TTA TTG TTG TTG GTT TTG TAA GAA GAA ACT AGC C-3'

pYX132-Fis1(cyt)-Tom5

Description: Chimerical construct composed of the N-terminal region of Fis1 (residues 1-123) and the C-terminal region of Tom5 (residues 21-51). The N-terminal region of Fis1 was first amplified by PCR and cloned into the victor. The following primers were used:

Primer Name	Included cutting site	Primer sequence
Fis1-N-ter	EcoRI	5'-AAA GAA TTC ATG ACC AAA GTA GAT TTT TGG-3'
Fis1 123	HindIII	5'- AAA AAA GGA TCC CTT ATC CTC TAC CAT ACT CTT CAA AGC -3'

The C-terminal region of Tom5 was amplified by PCR and sub cloned in the vector containing Fis1 (1-123). The following primers were used:

Primer Name	Included cutting site	Primer sequence
Tom5_1	BamHI	5'- AAA AAA GGA TCC ACT GAG AAA ACC TTG AAA CAG G -3'
Tom5_2	HindIII	5'- AAA AAA AAG CTT TTA TTT CCA TTG CTT TTT CAC C -3'

pYX132-Fis1(cyt)-Tom6

Description: Chimerical construct composed of the N-terminal region of Fis1 (residues 1-123) and the C-terminal region of Tom6 (residues 31-60). The N-terminal region of Fis1 was first amplified by PCR and cloned into the victor as described above. The C-terminal region of Tom6 was amplified by PCR and sub cloned in the vector containing Fis1 (1-123) .The following primers were used:

Primer Name	Included cutting site	Primer sequence
Tom6_1	BamHI	5'- AAA AAA GGA TCC CCA CTA TAC ACA ATT GCA CTA -3'
Tom6_2	HindIII	5'- AAA AAA AAG CTT TTA TAA TTG TGG GGC CAA CAT GTC C -3'

In all cases the PCR product was digested with the appropriate restriction enzymes and cloned into the pre-digested vector. The recombinant plasmid DNA was isolated from

positive cultures and the construct was sequenced to confirm proper in-frame ligation and fidelity of the polymerase.

2.2 Methods in yeast genetics

2.2.1 Over view of used *S. cerevisiae* strains

Strain	Genotype	Reference
ADM551	*MATa ura3-52 leu2Δ1 trp1Δ63 his3Δ200*	(Mozdy et al., 2000)
ADM552	*MATa ura3-52 leu2Δ1 trp1Δ63 his3Δ200*	(Mozdy et al., 2000)
D273-10B	Wild type	German Collection for Microorganisms
YPH499	*MATa ade2-101 his3-* Δ200 *leu2-* Δ*1 trp1-* Δ*63 ura3-52 lys2-801*	(Sikorski and Hieter, 1989)
YPH501	*ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801*	(Dietmeier et al., 1997)
YSH1	YPH499, *tob55::HIS3 (pVTU-102-TOB55)*	This thesis
YTJB4	*MATa ura3 leu2 his4 lys2*	(Haucke et al., 1995)
YTJB64	YTJB4, *tom20::LEU2*	(Lithgow et al., 1994)
BY4743	*MATa/* α *his3/his3 leu2/leu2 ura3/ura3 met15/MET15 lys2/LYS2*	(Brachmann et al., 1998)
tim10-1		(Koehler et al., 1998)
Δtim8/13	MB2, *tim8::HIS3 tim13::URA3*	(Paschen et al., 2000)
Δtom5	YPH501, *tom5::HIS3*	(Dietmeier et al., 1997)
Δtom6	BY4743*, tom6::kanMX4*	Huntsville (AL, USA)
Δtom70	BY4743*, tom70::kanMX4/tom70::kanMX4*	Euroscarf (Frankfurt)
ΔMas37	YPH499, *mas37::HIS3*	(Habib et al., 2005)
KKY3	*MATa his3-* Δ200 *leu2-3,112 ade2-101 suc2-* Δ*9 trp1-* Δ*901 ura3-52 tom40::HIS3 (pRS316-TOM40)*	(Kassenbrock et al., 1993)
KKY3.3	KKY3 *(pRS314-tom40-3)*	(Kassenbrock et al., 1993)
KKY3.4	KKY3 *(pRS314-tom40-4)*	(Kassenbrock et al., 1993)

GAL-His_8-Tob55	YPH499, *tob55::HIS3-pGAL-His8-TOB55*	(Paschen et al., 2003)
GAL-Tob38	YPH499, *tob38::HIS3-pGAL-TOB38*	(Waizenegger et al., 2004)
Tob38-6HA	YPH499, *tob38::TOB38-6HA-HIS3*	(Habib et al., 2005)

Construction of TOB55 genomic disruption strain

The TOB55 gene was cloned into the yeast expression vector pVTU-102 which contains the selectable marker URA3. The resulting plasmid was transformed into the wild type strain YPH499. Next, the genomic TOB55 open reading frame was replaced with the HIS3 marker gene by homologous recombination. The HIS3 marker gene-containing the flanking regions of TOB55 gene was amplified from the plasmid pFA6s-His3MX6 (Wach et al., 1997) by using the following primers:

F1-OM55	5' TAC GTG GCA AAA GTT TTG ATG CCA AAT AGA CAA AAG TAG CTC AAT CGT ACG CTG CAG GTC GAC-3'
R1-OM55	5' AAT GGG AAG CTA GGC GAT AGC TTC ACC TTG ACA TTA AAA GGA ATG TAT TCT ATC GAT GAA TTC GAG CTC G-3'

The resulting His+Ura+ strain containing a complete coding sequence deletion of the genomic TOB55 gene and a wild type TOB55 gene on a 2μ plasmid was termed YSH1.

2.2.2 Cultivation of *S. cerevisiae* strains

2.2.2.1 *Media for S. cerivisiae*

Lactate medium: 3 g yeast extract, 1g KH2PO4, 1 g NH4Cl, 0.5 g CaCl2 x 2 H2O, 0.5g NaCl, 1.1 g MgSO4 x 6 H2O, 0.3 ml 1% FeCl3, 22 ml 90% lactic acid, H2O to 1 l, pH 5.5 (adjusted with 10 M KOH). Usually supplemented with 0.1% glucose or 0,1 % galactose.

YP –medium: 10 g yeast extract, 20 g bacto-pepton, 5.5 ml 90% lactic acid, H_2O to 930 ml pH 5.5 (adjusted with 10 M KOH) after autoclaving add 67 ml 2% glucose (YPD) or 100 ml 20% galactose (YPGal) or 100 ml 30% glycerol (YPG).

S-medium: 1.7 g yeast nitrogen base, 5 g ammonium sulfate, 20 g glucose, 1.5 g "Drop-out mix" powder" (mix containing equal weight of all amino acids; for selecting one auxothrophic marker, the corresponding amino acid was left out), H_2O to 900 ml. After

autoclaving add 100 ml 20% galactose (SGal) or 100 ml 20% glucose (SD) or 30% glycerol (SG). To eliminate wild type (WT)-*URA3*-allele, yeast were grown on SD medium containing 0.1% (w/v) 5-fluoro-orotic acid (5-FOA)

To prepare plates with solid media, 2% (w/v) agar was added before autoclaving. For selective media, amino acids solutions (His, Leu, Lys, all 10 mg/ml) and uracil and adenine solutions (both 2 mg/ml) were separately autoclaved for 20 min at 120°C, with the exception of tryptophan (10 mg/ml) which was filter sterilized. The amino acids were added to the mixture before pouring the plates.

2.2.2.2 *S. cerivisiae growth conditions*

S. cerevisiae growth was performed as described in Sambrook *et* al. (Sambrook et al., 1989) in YPD complete medium or, when a selection on the auxotrophic marker was necessary, on SD medium. The cells were incubated at 30ºC, under moderate shaking conditions. Temperature-sensitive mutants were grown at 24°C. For isolation of mitochondria, cells were propagated for ca. 3 days while the OD_{600} never exceeded 1. For depletion of an essential protein, a yeast strain harbouring the corresponding gene under *GAL* promoter was grown for ca. 3 days on galactose-containing media. Cells were then collected, washed with water and resuspended in glucose-containing media. Cells were grown in glucose medium till the gene of interest was not detected but the mitochondria still have basal import activity.

2.2.2.3 *Transformation of S .cerivisiae by the lithium acetate method*

The corresponding yeast strain was grown overnight in YPD-medium and diluted the next morning to 50 ml medium with an OD_{600} of 0.2. Cells were grown further, till they reached an OD_{600} of 0.8. Then, cells were transferred to a sterile centrifuge tube, and harvested by centrifugation (1,000 x g, 3 min, RT). After washing with 25 ml of sterile water, cells were recollected, resuspended in 1 ml 100 mM lithium acetate and transferred to an Eppendorf tube. Cells were centrifuged again (7,500 x g, 15 sec, RT) and were resuspended in 400 µl 100 mM lithium acetate. For each transformation 50 µl of the cell suspension was centrifuged (7,500 x g, 5 min, RT) and the supernatant removed. Next, a mixture was added to the cells in the following order: 240 µl PEG 3350 (50% v/v), 36 µl 1 M lithium acetate, 5 µl single stranded salmon sperm DNA (10 mg/ml; previously incubated for 5 min at 95ºC), 70 µl H_2O containing 0.1-10 µg of DNA

to be transformed. The mixture was vortexed for 1 min and incubated for 30 min at 30ºC, with moderate shaking, followed by another 20-25 min at 42ºC. The cells were harvested by centrifugation (7,000xg, 15 sec, RT), washed with sterile water, resuspended in a small volume of sterile water (150 µl), and spread on plates with the appropriate selective media. The plates were incubated for 2-4 days at 30ºC to recover transformants.

2.3 Methods in cell biology

2.3.1 Isolation of mitochondria from *S. cerevisiae*

Mitochondria were isolated from *S. cerevisiae* following the previously described method (Daum et al., 1982). Yeast cells were cultivated to OD_{600} of 1-1.5 and collected by centrifugation (4,400 x g, 5 min, RT). The pellets were washed with H_2O and resuspended to a final concentration of 0.5 g/ml in DTT buffer (100 mM Tris-SO_4, 10 mM dithiotreitol (DTT), pH 9.4). The cell suspension was incubated for 15 min at 30°C with gentle shaking, followed by a new centrifugation step and resuspended in 100 ml of 1.2 M sorbitol buffer (1.2 M sorbitol, 20mM potassium phosphate-KOH, pH 7.4). The cell wall was digested by 2.5 mg Zymolyase per gram yeast dissolved in Sorbitol buffer. Cells were incubated for 30-45 min at 30°C, under moderate shaking conditions. To test the cell wall digestion (obtaining of spheroplasts), 50 µl cell suspension was diluted with 2 ml H_2O or into a solution of 1.2 M sorbitol. Formation of spheroplasts was complete when the OD of the H_2O dilution was 10-20% of the OD of the sorbitol dilution. The solution of spheroplasts in pure H_2O becomes clear because spheroplasts burst under these conditions. All the subsequent steps were performed at 4°C. The spheroplasts were isolated by centrifugation (3,000 x g, 5 min, 4°C), resuspended (0.15 g/ml) in homogenizing buffer (0.6 M sorbitol, 10 mM Tris-HCl, 1 mM EDTA, 0.2% (w/v) BSA, 1 mM PMSF, pH 7.4) and homogenized 10 times in a Dounce-Homogeniser. The cell remnants and unopened cells were sedimented by double centrifugation (2,000 x g, 5 min, 4°C). The supernatant was spun (17,400xg, 12 min, 4°C) and the sedimented mitochondria were resuspended in SEM buffer (10 mM MOPS/KOH, 250 mM sucrose, 1 mM EDTA, pH 7.4) and separated again from cell's remnants (2,000xg, 5 min, 4ºC). The mitochondria were sedimented again as above (17,400xg, 12 min, 4°C). Finally, mitochondria were resuspended in a small volume of SEM buffer to a concentration of 10 mg/ml protein, aliquoted, frozen in liquid nitrogen, and stored at – 80ºC till use.

2.3.2 Preparation of mitoplasts

Mitochondria whose outer membrane has been disrupted are known as mitoplasts. To obtain mitoplasts, the outer mitochondrial membrane is ruptured by swelling of mitochondria in a hypotonic solution which keeps the inner membrane intact. Mitochondria in SEM buffer were diluted 1:10 in 20 mM HEPES-KOH, pH 7.3 containing 2 mM EDTA and incubated on ice for 30 min. If necessary, mitoplasts (or mitochondria) were reisolated by centrifugation (10 min, 17,000xg, 4ºC) and resuspended in a desired buffer.

2.3.3 Isolation of crude mitochondria from *S. cerevisiae*

Cells corresponding to 10-20 OD units were harvested by centrifugation (3,000 x g, 5 min, RT) and washed with water. The cells were resuspended in 400 µl SEM buffer and 0.3 g glass beads (diameter 0.3 mm) were added. The samples were vortexed four times 30 sec each, with 30 sec breaks in between (during this break the samples were incubated on ice). After centrifugation (1,000 x g, 3 min, 4ºC), the supernatant was transferred to a new tube and the protein concentration was determined. The desired amount of protein was centrifuged (10,000xg, 10 min, 4ºC), resulting in crude mitochondrial pellets.

2.3.4 *In vitro* synthesis of radioactive labelled proteins

Transcription/Translation in a cell-free system

For *in vitro* synthesis of ^{35}S labelled proteins, the constructs contained in pGEM4 plasmid were first transcribed in mRNA using SP6-RNA-polymerase. The transcription mixture (100µl) contained: 10-20 µg DNA, 20 µl 5x transcription buffer (200 mM Tris-HCl, 50 mM $MgCl_2$, 10 mM spermidine, pH 7.5), 10 µl 0.1 M DTT, 4 µl RNasin nuclease inhibitor (40 U/µl), 20 µl 2.5 mM rNTP, and 5.2 µl 2.5 mM M7G(5`)ppp(5`)G. After adding 3 µl of SP6-Polymerase (25 U/ml) the mixture was incubated for 1 h at 37°C. The RNA was precipitated by adding 10 µl of LiCl (10 M) and 300 µl ethanol (96%), centrifuged, and washed with 70% ethanol. After ethanol evaporation at RT, the RNA was resuspended in water supplemented with 1 µl RNasin (40 U/µl), aliquoted and kept at – 80°C till use.

For translation using rabbit reticulocyte lysate, the following mix was incubated for 60 min at 30°C: 25 µl RNA, 3.5 µl amino acid mix (without methionine), 7 µl 15 mM MgOAc, 12 µl ^{35}S (10 mCi/ml) and 100 µl rabbit reticulocyte lysate (Promega). At the

end of the translation reaction 5 mM of cold methionine and 250 mM sucrose were added.

TNT Coupled reticulocyte lysate system

This system combines transcription and translation in the same reaction mixture. TNT mix (50 µl) contained: 25µl TNT rabbit reticulocyte lysate (Promega), 2 µl TNT reaction buffer, 1 µl TNT SP6 RNA polymerase, 1 µl amino acid mix without methionine, 2 µl ^{35}S 45 methionine (10 mCi/ml), 1 µl RNasin ribonuclease inhibitor (40 U/µl) and 2 µl DNA template (0.5 µg/µl). The reaction was incubated for 90 min at 30ºC, and further treatment was as described for the translation reaction alone.

2.3.5 Import of preprotein into isolated mitochondria

Precursor proteins were synthesized in the presence of ^{35}S-methionine as mentioned above. Mitochondria were resuspended at 0.5-1 mg/ml in F5 import buffer (0.03-3% (w/v) fatty acid- free BSA, 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 2 mM ATP, 2 mM NADH, 100 µg/ml ceratin kinase, 5 mM ceratine phosphate, 10 mM MOPS-KOH, pH 7.2). Import reactions were performed by addition of lysate (1-3% (v/v)) for the indicated time periods at 25°C (or in some cases at 15°C). Samples were divided and diluted in SEM buffer. In some experiments protease treatment was performed by incubation of the import reaction with 15-100 µg/ml proteinase K or 100 µg/ml trypsin for 15 min on ice. Protease treatment was stopped by addition of 1 mM PMSF. Mitochondria were reisolated and the import reaction was analyzed by SDS-PAGE, transferred to nitrocellulose membrane, autoradiography and quantification.

In some experiments import was competed by addition of recombinant proteins like: pSu9 (1-69)-DHFR (fusion protein of mitochondrial targeting sequence of subunit 9 of F_o-ATPase from *N. crasa* and dihydrofolate reductase (DHFR) from mouse) were added to 50 µg mitochondria or MBP-Tob55(1-120) (maltose binding protein fused to the first 120 amino acids of Tobb55).

2.3.6 Carbonate extraction

Carbonate extraction was used to check whether a protein is inserted into a membrane or it is in a soluble form. For this purpose, mitochondria were pelleted via centrifugation, resuspended in buffer containing 10 mM HEPES-KOH, 100 mM Na_2CO_3, pH 11.5 and incubated for 30 min at 0ºC. The probes were then centrifuged for 30 min at 125,000 x g, 2ºC. The soluble proteins were found in the supernatant and were precipitated with

Trichloroacetic acid (TCA) and membrane-embedded proteins were found in the pellet. The samples were analyzed by SDS-PAGE.

2.3.7 Antibody shift

The radiolabelled preproteins were imported into mitochondria and the organelles were sedimented. The resulting pellet was resuspended in 30-45 µl buffer N (20 mM Tris-HCl, 50 mM NaCl, 10% (v/v) glycerol, 0.1 mM EDTA, 1 mM PMSF, pH 7.4) supplemented with 1% (w/v) digitonin and incubated for 15 min on ice. Next, 5 µl of the desired immunoglobulin G (IgGs) or the purified anti-bodies were added and the mixture was further incubated for 1 h at 4ºC with gentle shaking (over-head shaker). Insoluble materials and aggregates were removed by centrifugation step (36700 x g, 20 min, 4ºC). The samples were further analysed by BNGE.

2.3.8 Fluorescence microscopy

Yeast cells were transformed with plasmid pVT100U-mtGFP expressing mitochondria-targeted green fluorescent protein (Westermann and Neupert, 2000). Cells were grown to exponential phase in liquid selective glucose medium at 30°C. Fixation of the samples was achieved by mixing 7 µl transformed cells together with 7 µl of 0.5 % (w/v) low melting point agarose. The samples were analyzed by Axioplan microscope with Plan-Neoflur 100 x/1,30 oil-objective using 100 W Quecksilber-lamp (Carl zeiss Gmbh, Jena). Classification and quantification of the morphology phenotypes were performed without knowledge of strain identity at the time of analysis. For quantification of the phenotype, at least 100 cells were analyzed in three independent experiments.

2.3.9 Pull down of radiolabeled preprotein via His-tagged Tob38

Mitochondria harbouring a hepta-histidin tag at the C-terminus of Tob38 were used to study the interaction of Tob38 with radiolabeled precursors of the TOB complex components. Mitochondria (150 µg) were incubated with radiolabeled precursors for 15 min at 0°C. Mitochondria were then reisolated and lysed in 300 µl buffer N containing 0.25% digitonin and 10 mM Imidazole for 1 h at 0°C. Aggregates and insoluble membranes were removed by clarifying spin (36,700xg, 20 min, 4°C). A portion of 20 % was taken from the resulted supernatant as a control. The rest of the supernatant was incubated with 50 µl pre-equilibrated Ni-NTA beads (equilibration with buffer N) for 1 h at 4°C. Finally the beads were washed 3 times with buffer N (0.05% (w/v) digitonin) and

one time with $SEMK^{20}$ (SEM + 20 mM KCl). Bound proteins were eluted with 2x Laemmli buffer and analysed by SDS-PAGE followed by autoradiography.

2.3.10 Interaction of radiolebeled preprtoeins with the N-terminal domain of Tob55

The DNA sequences 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), and expressed in E. coli BL21 cells as soluble fusion proteins with maltose binding protein (MBP). Purification of the protein was according to the manufacturer instructions (see 2.5.1). For *in vitro* binding assays, 4 ml induced *E. coli* cells were lysed and proteins were applied to 40 µl amylose resin (New England Biolabs). Next, unbound proteins were washed twice with 1 ml MBP-column buffer (20 mM HEPES, 100 mM NaCl, 2 mM EDTA, 1 mM PMSF, pH 7.4). To minimize unspecific binding the resin was washed with 500 µl import buffer containing 3% BSA, followed by 20 min incubation of the resin with 50 µl reticulocyte lysate diluted in 500 µl F5 import buffer containing 0.03 % BSA. The resin was washed twice with 1 ml F5 import buffer and then incubated with 50 µl reticulocyte lysate containing the radiolabeled proteins 500 µl in import buffer for 20 min at 4°C. At the end of the incubation period the resin was washed twice with import buffer and eluted with 1 M NaCl. The eluted material was precipitated by TCA and analyzed on SDS-PAGE.

2.3.11 Binding assay with water soluble porin

Initially, all the eppindorf tubes were washed with 340 µl 0.25% BSA to prevent unbound and aggregated proteins to bind to the surface of the tube. The procedure of binding MBP or MBP Tob55 to amylose resin is according to (2.3.10).Briefly, lysed cells were applied to 15-25 µl (1:12) amylase resin which were prewashed with MBP column buffer containing 0.25% BSA. Unbound proteins were washed twice with 1 ml MBP-column buffer. For binding experiments, various amounts of radiolabeled ws-porin (see 2.4.4) were added in binding buffer (100 mM KCl, 0.025% BSA, 10% glycerol, 100 mM sodium phosphate, pH 6.8) to MBP or MBP-Tob55 pre-bound to amylose beads. After incubation at 4°C for 35 min the beads were washed once with binding buffer, then 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 ws-porin were analyzed directly by SDS-PAGE and autoradiography.

2.4 Methods in protein biochemistry

2.4.1 Purification of recombinant MBP-fusion proteins expressed in *E. coli*

The first 120 amino acids residues of Tob55 or the first 98 amino acids residues of Fis1 fused to maltose binding protein (MBP) were expressed in *E.coli* from pMAL-cRI vector (New England Biolabs). Purification of the recombinant proteins out of *E. coli* was done as described before (Luciano et al., 1997) and according to the manufacturer instructions. An overnight *E. coli* culture (10 ml) were diluted into 250 ml LB-Amp and grown until OD_{600} of ca. 0.5- 0.8 at 37°C. Expression of the recombinant protein was induced by 1 mM isopropyl-β, D-tiogalactopyranoside (IPTG) and the culture grown for additional 3 h at 37°C. Cells were pelleted by centrifugation (4,400xg, 10 min, RT) and resuspended in 20 ml MBP-column buffer. In order to degrade the cell wall and to obtain spheroplasts, 1 mg/ml Lysozyme was added and the mixture was incubated for 45 min at 4°C. Cells were completely broken by sonication (10x12 s, Branson sonifier, setting 4, 80% duty cycle). After centrifugation (27,200xg, 15 min, 4°C), clear supernatant was loaded on a 5 ml Amylose-column (New England Biolabs) preequilibrated in MBP-column buffer. Column was washed with 30 ml of MBP-column buffer and bound proteins eluted with 10 mM maltose in MBP-column buffer. Expression and purification were monitored by SDS-PAGE.

2.4.2 Purification of porin from *N. crassa*

Native porin from *N. crassa* was isolated by modification of a published procedure (Pfaller et al., 1985). Shortly, 5 mg outer membrane vesicles (OMV) which were isolated as described elsewhere (Schmitt et al., 2006) were solubilized in 1 ml buffer containing 50 mM HEPES-KOH, 1 mM PMSF, 10% glycerol and 2% Triton X-100 for 1h at 4ºC. After a clarifying spin (36670xg, 10 min, 2 °C), the supernatant was applied to anion-exchange column (ResQ, Amersham). The flow-through (4ml) which contains porin was collected.

2.4.3 Preparation of water –soluble porin.

Water soluble porin was obtained according to a published protocol (Pfaller et al., 1985). After addition of 1/10 volume of 3 M TCA and 1/3 volume of methanol, purified

porin was precipitated for 1 h at 0 ºC. The precipitated porin was pelted (48,800xg, 1h, 4ºC) and washed twice with ethanol (48,800xg, 30 min, 4ºC). The resulting protein pellet was solubilzed by vortixing with 1 volume of 0.1 M NaOH for 60 s and immediately neutralized by an equal volume of 0.2 M NaH_2PO_4 . This procedure yielded water-soluble porin in 100 mM NaPi, pH 6.8. the final concentration was determined to be 0.007 µg/ml, 21 µg/3 ml)

2.4.4 Reductive Methylation of water-soluble porin.

Reductive methylation was carried out according to published protocols (Jentoft and Dearborn, 1979; Pfaller et al., 1985) employing [^{14}C] formaldehyde as a radioactive label and sodium cyanoborohydride as reducing agent. [^{14}C] Formaldehyde (specific activity 52.5 µCi/µmol, PerkinElmer LAS GmbH) at a concentration of 270 nmol/nmol of porin in 100 mM NaPi, pH 6.8, and sodium cyanoborohydride (Sigma, 334 nmol/nmol of formaldehyde, 48 mM stock solution in 100 mM NaPi, pH 6.8) were consecutively added to a sample of water-soluble porin 0.007 µg/ml. After 2 hours incubation at 25 ºC, the reaction was stopped by adding 100 mM Tris.HCl, pH 7.0 followed by 10 min incubation at 25 ºC. Then water soluble porin was precipitated with 3 M TCA and methanol for 1 h at 0 ºC and centrifuged, and the pellet was washed once with ethanol and once with acetone. The protein pellet was resolubilized as described to a final protein concentration of 0.007 µg/ml

2.4.5 Protein precipitation with trichloroacetic acid (TCA)

Proteins from aqueous solutions were precipitated by adding TCA to a final concentration of 12% (w/v). The samples were incubated for 10 min on ice or at –20ºC, and then centrifuged (36,700xg, 20 min, 2ºC). The precipitated proteins were washed with acetone (kept at –20ºC), and re-centrifuged (36,700xg, 10 min, 2ºC). Protein pellet was shortly dried at RT and dissolved in 2 x Laemmli buffer.

2.4.6 Protein precipitation with ammonium sulphate

Aqueous solutions containing proteins were mixed with 2 volume of saturated solution of ammonium sulphate (4°C). The samples were incubated for 30 min at 4°C and then centrifuged (36,700xg, 10 min, 4°C). The pellets containing the precipitated proteins were dissolved in the appropriate buffer. To prepare the saturated ammonium sulphate solution, 76.7 g of ammonium sulphate was dissolved in 100 ml 100 mM Tris-HCl pH

7.0, was heated until it dissolved and then chilled to 4°C, so that the ammonium sulphate crystals sediment.

2.4.7 Determination of protein concentration

Protein concentration was determined according to (Bradford, 1976) using the "Bio-Rad- Protein assay" reagent. Protein solution was mixed with 1 ml reagent (1:5 dilution) and incubated for 10 min at RT. The absorbance was measured at 595 nm using a 1-cm-path length microcuvette. Protein concentration was calculated according to a standard curve (Bovine from IgGs (Biorad) were used as standard proteins).

2.4.8 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated via one-dimensional vertical slab SDS Polyacrylamide gel electrophoresis (SDS-PAGE), under denaturing conditions, as described (Laemmli, 1970). Two different electrophoresis systems were used. Normally, big gels were used (running gel: 9 x 15 x 0.1 cm; stacking gel: 1 x 15 x 0.1 cm) and in some cases Mini gels (Mini-PROTEAN II, Bio-Rad) (running gel: 7 x 7.2 x 0.075 cm; stacking gel: 1 x 7.2 x 0.075 cm). The concentration of acrylamide and bis-acrylamide in the separating gel was chosen according to the molecular size of the proteins to be separated. Glass plates of 160 x140 mm and spacers of 1 mm thickness were used. The samples were dissolved in 35 µl 1 x Laemmli buffer (60 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% glycerol, 5% (v/v) β-mercaptoethanol, 0.05% (w/v) bromphenol-blue) and incubated at 95°C for 5 min, before loading. The electrophoresis was performed at 25 mA for 3 h (1 h for mini gels). Protein molecular weight standards were used in each gel.

Bottom gel	2% (w/v) agar in running buffer
Separating gel	8-16% (w/v) acrylamide, 0.16-0.33% (w/v) bis-acrylamide, 375 mM Tris-HCl (pH 8.8), 0.1% (w/v) SDS, 0.05% (w/v) APS, 0.05% (v/v) TEMED
Stacking gel	5% (w/v) acrylamide , 0.1% (w/v) bis-acrylamide, 60 mM Tris-HCl (pH 6.8), 0.1% (w/v) SDS, 0.05% (w/v) APS, 0.05% (v/v) TEMED
Running buffer	50 mM Tris-HCl (pH 8.3), 384 mM Glycin, 0.1% (w/v) SDS

2.4.9 Semi native SDS-PAGE

Recombinant proteins were dissolved in 1x Laemmli buffer (containing 0.05% SDS) at 4°C and separated via Semi native SDS-PAGE (Stacking gel and running buffer do not contain SDS). The electrophoresis was performed at 15 mA for 40 min at 4°C.

2.4.10 Blue-Native gel electrophoresis (BNGE)

For separation of proteins under non-denaturing (native) conditions, Blue-Native gel electrophoresis (BNGE) was use (Schägger et al., 1994). For this purpose, mitochondria (50-150 µg) were pelleted, resuspended in 30-45 µl of buffer N containing 0.4-1% digitonin. The solubilisation was performed at 4°C for 15 min. After a clarifying spin (36,700 x g, 10 min, 4°C), 5 µl of sample buffer (5% (w/v) Coomassie-Brilliant-blue G-250, 100 mM bis-Tris pH 7.0, 500 mM 6-amino-n-Capronic acid) were added to the soluble material, mixed and finally loaded onto a 6%-13% gradient gel. The electrophoresis was performed at 4ºC. For the first step of electrophoresis, cathode buffer containing Coomassie-Brilliant-blue and anode buffer were used and the voltage was set for 1 h at 100V and then shifted to 500V. When the blue front had migrated to about two thirds of the separation distance, the cathode buffer with Coomassie-Brilliant-blue was replaced by a cathode buffer without Coomassie-Brilliant-blue. Marker proteins like Apoferritin (440 kDa), Alcohol dehydrogenase (ADH) (monomer: 50 kDa, dimer: 100 kDa, trimer: 150 kDa), and bovine serum albumin (BSA) (monomer: 66 kDa, dimer: 136 kDa) were used.

Bottom gel	20% (w/v) acrylamide, 0,13% (w/v) bis-acrylamide, 0.5 M 6-amino-n-Capronic acid, 50 mM bis Tris-HCl, pH 7,0, 0.05% (w/v) APS, 0,025% (v/v) TEMED
Separating gel	0.15-0.3% (w/v) bis-acrylamide, 5% (v/v) glycerol, 0.5 M 6-amino-n-Capronic acid, 50 mM bis Tris-HCl, pH 7,0, 0.04% (w/v) APS, 0.04% (v/v) TEMED
Stacking gel	3.8% (w/v) acrylamide, 0,12% (w/v) bis-acrylamide, 0.5 M 6-amino-n-Capronic acid, 50 mM bis Tris-HCl, pH 7.0, 0.08% (w/v) APS, 0.08% (v/v) TEMED
Cathode buffer	15 mM bis-Tris, pH 7.0, 50 mM Tricine, with or without 0.02% Coomassie-Brilliant-blue G250
Anode buffer	50 mM bis Tris, pH 7.0

2.4.11 Staining SDS-PA gels with Coomassie brilliant blue

After SDS-PAGE, and removal of the bottom and stacking gels, separating gel was stained at RT for 30-40 min, with an aqueous solution containing 30% (v/v) methanol, 10% (v/v) acetic acid, and 0.1 (w/v) Coomassie-Brilliant-blue R250. The gel was destained with destaining solution (30% (v/v) methanol and10% (v/v) acetic acid in H_2O) until the protein bands appeared against a clear background. The contrast between proteins background was enhanced through further 15 minutes incubation with 7% (v/v) acetic acid solution. This step was followed by further 10-15 minutes incubation in 50% (v/v) methanol solution in water. The gel was dried overnight between two gel-drying-films (Promega).

2.4.12 Transfer of proteins to nitrocellulose or PVDF membrane (Western-blot)

Proteins separated via SDS-PAGE were transferred onto nitrocellulose membrane using the semi-dry blotting method (Kyshe-Anderson, 1984; Towbin et al., 1979). The gel, the membrane, and six sheets of Whatman filter paper (3MM) were incubated in transfer buffer (20 mM Tris, 150 mM glycine, 20% (v/v) methanol, 0.08% (w/v) SDS). Three sheets of filter paper were placed on the anode electrode followed by the membrane and on top of it the gel. This was covered with other three filter papers and with the cathode electrode. The transfer was performed at 200 mA for 1.5 h or at 110 mA for 40 min (for mini gels). To verify transfer efficiency, the nitrocellulose membranes were reversibly stained with Ponceau S solution (0.2% (w/v) Ponceau S in 3% (w/v) TCA).

Proteins separated via BNGE were transferred onto a PVDF membrane using the semi-dry blotting method described above. Before blotting, the PVDF membrane was activated for 5 min in methanol and 10 min in transfer buffer. After electrophoresis the PVDF membrane was reversibly stained with Coomassie-Brilliant-blue R solution (30% (v/v) methanol, 10% (v/v) acetic acid, 0.1 (w/v) Coomassie-Brilliant-blue R250) for 2 min in order to visualize the marker proteins. For destaining, a solution of 30% (v/v) methanol and 10% (v/v) acetic acid was used. After completely removing the methanol by washing with TBS buffer (9 g/l NaCl, 10 mM Tris/HCl, pH 7.4) the membrane was immunodecorated or the radioactive material was visualized by autoradiography.

2.4.13 Autoradiography and quantification

Radiolabeled proteins were detected by autoradiography. A dried PVDF or a nitrocellulose membrane was exposed to Röntgen film (Kodak Bio Max MM) for different time points (ranging between few hours to 15 days). Period of exposure depended on signal intensities. Next, the exposed Film was developed in a developing machine (Gevamatic 60, AGFAGevaert). The films were scanned and the intensity of the bands was quantified using theAida Image Analyzer 3.4.3 software.

2.5 Methods in immunology

2.5.1 Generation of Fis1-polyclonal antisera in a rabbit

The first 98 amino acids of Fis1 fused to maltose binding protein (MBP) were expressed in and purified from *E. coli* as mentioned before (see 2.5.1) Polyclonal antisera were made in rabbits. Purified recombinant proteins were analyzed by SDS-PAGE, blotted on nitrocellulose membranes and stained with Ponceau S. Pro injection, 3-7 bands (ca. 200 µg protein) were cut and the nitrocellulose dissolved in 300 µl DMSO (Knudsen, 1985). Dissolved proteins were mixed with 300 µl Freunds incomplete adjuvant (or Injection TiterMaxTM for the first injection) until a stable emulsion was obtained. This emulsion was subcutaneously injected into a rabbit (nr. 309). The antigen was injected twice within ten days before the first bleeding was taken. Ten days after each injection, 25 -35 ml blood was taken from the ear vane and left at RT for ca. 3 h. After coagulation was complete, serum was obtained by two centrifugation steps (3,000 x g, 5 min, RT and 27,200 x g, 15 min, RT). Inactivation of the complement was achieved by 20-30 min incubation at 56°C and the serum was stored at -20°C.

2.5.2 Immunoblotting

To visualize the immobilized proteins on PVDF or nitrocellulose membrane, immonodecoration with specific antibodies was carried out. Membranes were first incubated for 1 h in 5% (w/v) milk powder in TBS (135 mM NaCl, 10 mM Tris-HCl, pH 7.5) to block all non-specific binding sites. The immunodecoration was done for 1 h at RT or over night at 4°C, with specific antiserum (1:200 to 1:1000 dilutions in milk/TBS). The membrane was then washed 3 times (each wash lasts 10 min), with TBS, TBS/0.05% (w/v) Triton X-100 and again with TBS, and incubated for 1 h with horseradish peroxidase coupled to secondary goat anti-rabbit-IgG or anti-mouse-IgG (Bio-Rad)

(diluted 1:10.000 dilutions in milk/TBS). The membrane was again washed (as above) and treated with ECL reagents: luminol (2.5 mM 3-aminophtalhydrazide and 0.4 mM p-cumaric acid in 0.1 M Tris-HCl, pH 8.5), mixed with equal volume of H_2O_2 (0.018% (v/v) in 0.1 M Tris-HCl, pH 8.5). The luminescence reactions were detected with Röntgen films (FujiNewRX).

For detection of HA-tagged Tob38, anti-HA antibody (Santa Cruz, Roche) was used. Blots were blocked in the supplied blocking solution and secondary antibody (goat anti-mouse) was diluted 1:5000 in the blocking reagent in TBS. Further treatment was as described above.

2.5.3 Binding of water soluble porin to MBP–Tob55(1-120) on a blot

An overlay method described in (Voulhoux and Tommassen, 2004) was used to evaluate the binding of water soluble porin to the first 120 amino acids residues of Tob55 on blot. Different amounts of purified fusion protein MBP-Tob55 (1-120), and the control proteins MBP-Fis1(1-98) and MBP were separated by SDS-PAGE under seminative conditions and the proteins were blotted onto nitrocellulose membranes. The membrane was blocked in TBS pH 7.6 containing 2.5% non-fat dried milk for 30 min and incubated 2h at 4°C with water soluble porin (11 µg/ml). The membrane was washed twice with 100 mM NaCl and 90 mM Tris base followed by 3 time standard washes as mentioned above. The immunodecoration was done for 1 h at RT with antibodies against *N. crassa* porin. Further treatments were as described in (2.5.2)

2.5.4 Purification of immunoglobulin G (IgG)

Antiserum (4 ml) was centrifuged (20,000xg, 20 min, 4°C) and the white surface film (corresponding to aggregated lipids) was removed. The antiserum was diluted with 10 ml of buffer C (100 mM KPi, pH 8.5), filtrated, and loaded onto a 5 ml Protein A-Superose column (Pharmacia), previously equilibrated with buffer C. The column was washed with 25 ml buffer C and the bound IgGs were eluted with buffer E (100 mM Citrate/NaOH, pH 3.0). The elute was immediately neutralized with 2 ml 2 M Tris-HCl, pH 8.0, dialyzed ON against 5 l of water and concentrated via lyophilization. The IgGs were resuspended in 10 mM MOPS-KOH, pH 7.2 to a final protein concentration of 10-50 mg/ml, aliquoted, and stored at – 20ºC till use.

3. Results

3.1 Structural and functional characterization of tail anchor domains of mitochondrial outer membrane proteins

Tail anchored proteins form a distinct class of integral membrane proteins that reside in the outer membrane of mitochondria. These proteins are anchored to the outer membrane via a single transmembrane domain (TMD) at the C-terminus and expose a hydrophilic N-terminal domain of variable size to the cytosol. The TMDs of mitochondrial tail-anchored proteins are relatively short, moderately hydrophobic and do not exhibit sequence similarity (Fig. 3). Both flanking regions of the TMDs have positively charged residues (Borgese et al., 2003).

3.1.1 A net positive charge at the C terminus of Fis1 is crucial for mitochondrial targeting

The importance of the positive charges in targeting tail-anchored proteins to mitochondria was investigated. To that end, the tail-anchored protein Fis1 was used as a model protein. Fis1 is anchored to the outer membrane through a 20 amino acid residues C-terminal TMD. A short segment of five residues is facing the IMS whereas four of those are basic ones (Fig. 4A). To study the role of these basic residues, two charge variants were constructed. In the first case, two arginine residues Arg154 and Arg155, were replaced by glutamine residues reducing the net positive charge to +2 (Fis1(2Gln), Fig. 4A). In the second case, the net charge in the C-terminal segment was made neutral by mutating all four basic amino acids to glutamine residues (Fis1(4Gln), Fig. 4A).

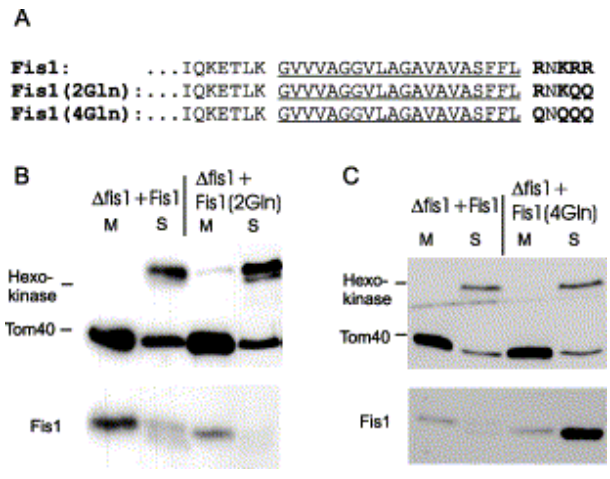

Figure 4. A net positive charge at the C terminus of Fis1 is crucial for mitochondrial targeting. A. The sequences of the tail domains of native Fis1 and two charge-mutation variants are presented. **B.** *Fis1* null cells transformed with a vector encoding either authentic Fis1 or the indicated variant were ruptured by vortexing in the presence of glass beads. A mitochondrial and a post-mitochondrial fraction were obtained by differential centrifugation (M and S, respectively) and were subjected to SDS–PAGE and immunoblotting. The antibodies used were directed against the cytosolic domain of Fis1, a control marker protein for the cytosol (hexokinase), and the mitochondrial outer membrane protein Tom40. **C.** As B, but the variant was Fis1(4Gln).

The Fis1(2Gln) variant was targeted to mitochondria *in vivo* as was demonstrated by subcellular fractionation (Fig. 4B). In contrast, the vast majority of the Fis1(4Gln) protein was in non-mitochondrial compartments (Fig. 4C). Hence, a net positive charge in the C-terminal segment appears to be crucial for mitochondrial targeting of Fis1.

Fis1 is involved in mitochondrial fission and thus, deletion of this protein causes changes in mitochondrial morphology. In the absence of Fis1, mitochondria form a net-like structure instead of the normal reticulum structure (Mozdy et al., 2000). When Fis1(2Gln) variant was transformed into *fis1* null strain, the wild type morphology of mitochondria was completely restored (Table 1). The small amount of Fis1(4Gln) protein that was targeted to mitochondria was sufficient to complement the morphology phenotype of *fis1* null strain (Table 1). These results suggest that the positive charge at the C-terminal segment is required for targeting to mitochondria but not for the function of Fis1, once it is present in the membrane.

Recently, the involvement of human Fis1 (hFis1) in the fission machinery of mammalian mitochondria was investigated. It was demonstrated that high expression levels of hFis1 strongly promote fission and the resulting mitochondria are fragmented (Yoon et al., 2003). Notably, the expression levels of Fis1(4Gln) in cells were higher than those of Fis1(2Gln) and Fis1wt (Fig. 4B,C). The remarkable differences in the expression levels among the various variants of Fis1 in yeast may hint to a tight regulation of the functional levels of Fis1 in the outer membrane. Over-expression of the functional variants Fis1wt and Fis1(2Gln) might harm the cells; therefore their expression is regulated. Since only a small fraction of Fis1(4Gln) is functional in the outer membrane of mitochondria, this variant can be expressed in high amounts in the cell.

Table 1. Complementation analysis of Fis1 variants

Yeast strain: wild type or Δfis1 transformed with a plasmid encoding the indicated protein	% of cells with normal reticular mitochondrial morphology[a]
Wild type	96
Δfis1 + Fis1	96
Δfis1 + Fis1(4Gln)	95
Δfis1 + Fis1(2Gln)	89
Δfis1 + Fis1(cyt)-Tom5C	79
Δfis1 + Fis1(cyt)-Tom6C	69
Δfis1 + empty plasmid	0

[a] The percentage of cells with wild type-like mitochondrial morphology was determined using fluorescence microscopy

3.1.2 The tail anchor-domain of Fis1 does not have a sequence-specific role

To explore some features of tail anchor domains we asked whether these domains could functionally replace each other. To address this point, the first 123 amino acid residues of Fis1 (located in the cytosol) were fused to the tail anchor domain of either Tom5 (residues 21-51) or Tom6 (residues 31-60). Subcellular fractionation shows that both variants, Fis1(cyt)–Tom5C and Fis1(cyt)-Tom6C were targeted to mitochondria (Fig. 5A). Thus, tail anchor domains of different proteins can be exchanged without losing their targeting and anchoring functions. As the cytosolic domain of Fis1 does not contain a mitochondrial targeting signal (Mozdy et al., 2000), these results imply that the signal-anchor domains of both Tom5 and Tom6 are sufficient for mitochondrial targeting.

Since Tom5 and Tom6 are components of the TOM core complex (Ahting et al., 1999; Dekker et al., 1998), the ability of their tail anchor domains to assemble into the TOM complex was tested. The TOM core complex from strains expressing either Fis1(cyt)–Tom5C or Fis1(cyt)-Tom6C, was analyzed by BNGE. In both cases, the vast majority of the fusion protein assembled into the 410 kDa TOM core complex and only a minor fraction (~6%) migrated as unassembled low-molecular-weight species (Fig. 5B). Taken together, the tail anchor domains of Tom5 and Tom6 are sufficient for the assembly of the fusion proteins into the TOM complex. Based on quantitative Western blotting, mitochondria expressing Fis1(cyt)-Tom5C or Fis1(cyt)-Tom6C were estimated to harbour 7-8 fold more Fis1 molecules as compared to Fis1wt. Although only minor amounts of these molecules were not assembled into the TOM complex (see above), both proteins were able to partially complement the morphology phenotype of *Δfis1* strain (Fig. 5C and Table 1). It seems that those molecules of Fis1 fusion proteins, which were not assembled into the TOM complex, can be as active as native Fis1 in mediating fission of mitochondria. The tail anchor domain of Fis1 is probably not involved in sequence-specific interactions that are essential for the function of the protein. These conclusions are in line with data suggesting that the region mediating mitochondrial fission resides within the first 31 amino acids of the N-terminal cytosolic domain of human Fis1 (Yoon et al., 2003).

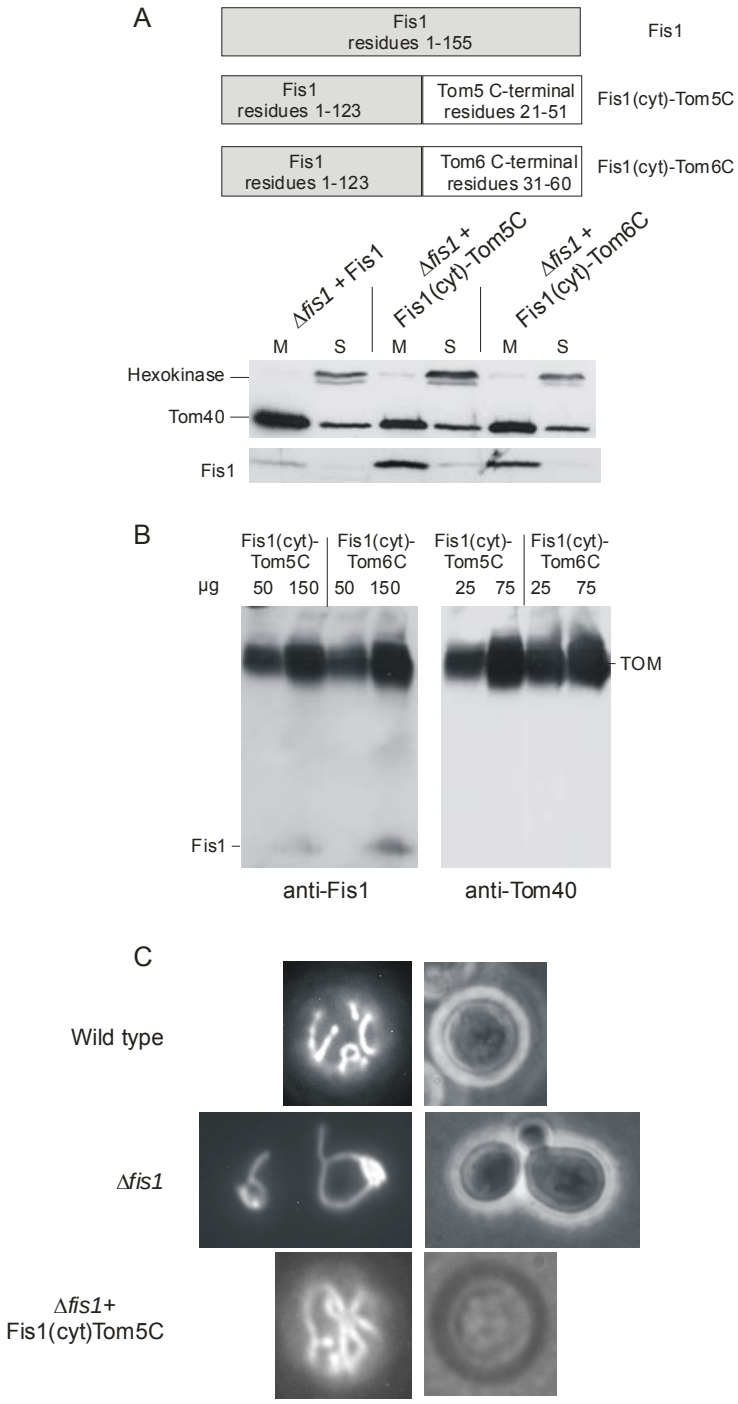

Figure 5. The tail anchor domains of Tom5 and Tom6 are sufficient for targeting and assembly of the proteins. A. *Fis1* null cells transformed with native or with the indicated variants of Fis1 were sub-fractionated as described in the legend to Fig. 4. Fis1(cyt), the cytosolic domain of Fis1. **B.** Mitochondria isolated from a *fis1* null strain transformed with either Fis1(cyt)-Tom5C or Fis1(cyt)-Tom6C were lysed with digitonin and analyzed by BNGE. The amounts of mitochondrial proteins applied (µg) are indicated. Blots were immunodecorated with antibodies against either Tom40 or Fis1. The TOM complex (TOM) and the unassembled Fis1 variants (Fis1) are indicated. **C.** Fis1(cyt)-Tom5C can partially complement the mitochondrial morphology phenotype of the ΔFis1 strain. Cells of the indicated strains (containing mitochondria-targeted GFP) were analyzed by fluorescence (left) and phase contrast (right) microscopy.

3.1.3 The tail anchor domain of Tom6 plays a role in the stability of the TOM complex

Tom6 was suggested to function as an assembly factor of the TOM core complex. Deletion of Tom6 has a strong effect on the stability of the GIP complex. The interaction between Tom40 and Tom22 is destabilized (Alconada et al., 1995; Dekker et al., 1998; Dembowski et al., 2001), and the proteins are preferentially found in the lower-molecular-mass range in blue native gel electrophoresis; Tom40 in an 100kDa subcomplex and Tom22 below 60kDa. Tom5 and Tom7 remain associated with Tom40 in the 100kDa subcomplex (Dekker et al., 1998). Since Fis1(cyt)-Tom6C could assemble into the TOM complex, we asked whether this hybrid protein could functionally replace the native Tom6. To that end, Fis1(cyt)-Tom6C was transformed into a Δ*tom6* strain and the TOM complex was analyzed by BNGE. Western blotting and immunodecoration with antibodies directed against Tom40 shows that most of the TOM core complexes regained their stability (Fig. 6). Thus, the tail anchor domain of Tom6 is sufficient to fulfil the stabilization role of the protein.

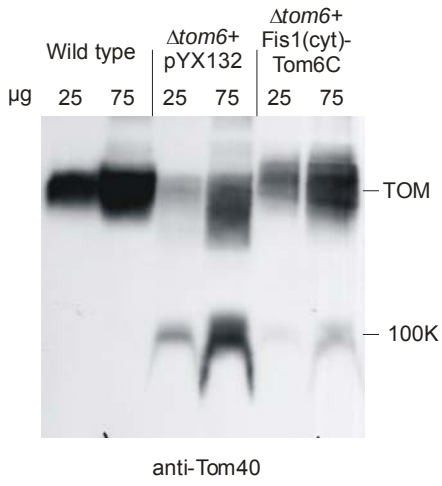

Figure 6. The tail anchor domain of Tom6 is sufficient for the TOM-stabilizing function of the protein. Mitochondria isolated from a wild type strain and from a *tom6* null strain transformed with either empty vector (Δtom6+pYX132) or vector encoding Fis1(cyt)-Tom6C (Δtom6+Fis1(cyt)-Tom6C) were lysed with digitonin and analyzed by BNGE. The amounts of mitochondrial proteins applied (µg) are indicated. The blot was immunodecorated with antibodies against Tom40. The native 410 kDa TOM core complex (TOM) and the 100 kDa form of Tom40 (100K) are indicated.

3.1.4 The tail-anchor domain of Tom5 plays an essential role in the function of the protein

Deletion of the gene encoding Tom5 was reported to be lethal at 37°C (Dietmeier et al., 1997). Based on this observation, the importance of the TMD for the function of Tom5 was examined. The fusion protein Fis1(cyt)–Tom5C was transformed into a *ΔTom5* strain, and its ability to complement the *ts* phenotype of this strain was tested.

Interestingly, a complete restoration of growth at 30°C and partial restoration of growth at 37°C was observed (Fig 7A). Tom5 was suggested to be a functional link between the surface receptors and the GIP, as mitochondria lacking Tom5 were observed to abrogate the import of preproteins (Dietmeier et al., 1997). Mitochondria lacking Tom5 but harbouring Fis1(cyt)–Tom5C displayed partial alleviation of this import defect (Fig. 7B). Hence, the cytosolic domain of Tom5 appears not to be essential for the function of the protein.

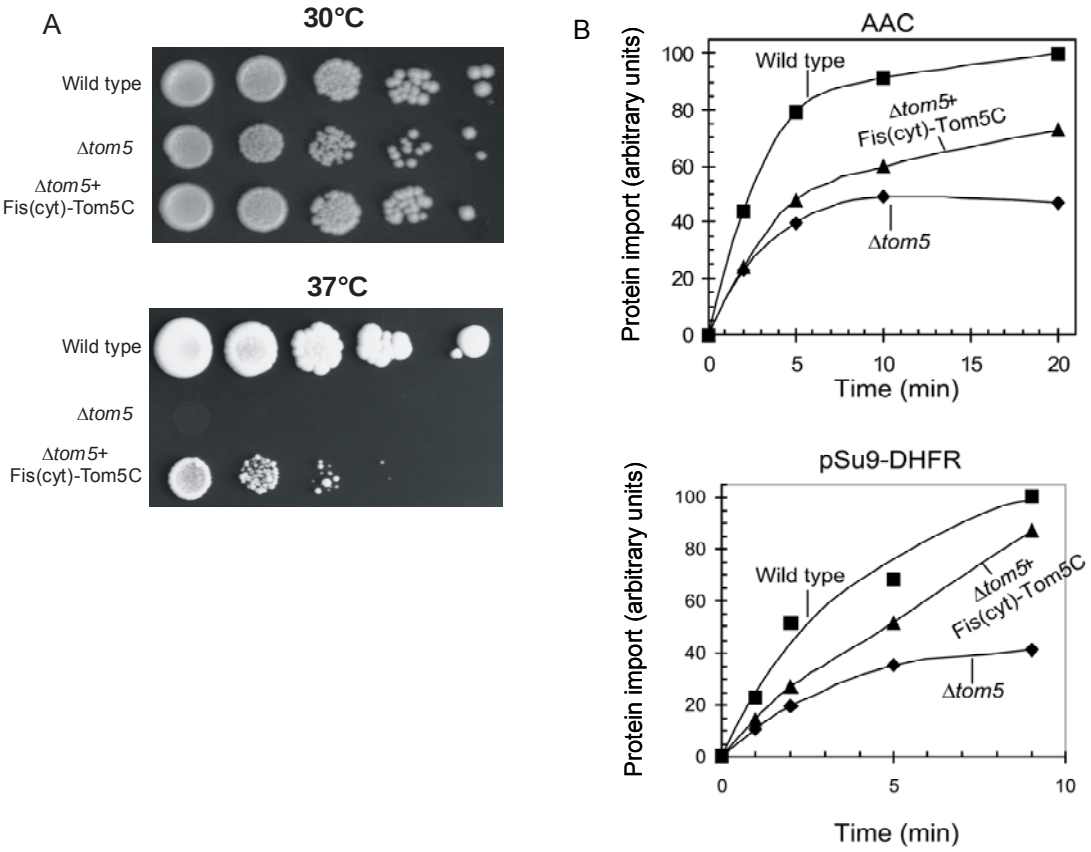

Figure 7. The tail-anchor domain of Tom5 can partially complement the function of authentic Tom5. A. The fusion protein Fis1(cyt)-Tom5C can partially rescue the temperature-sensitive phenotype of a *tom5* null strain. Δ*tom5* cells, their isogenic wild type, and Δ*tom5* cells transformed with a plasmid encoding Fis1(cyt)-Tom5C were tested by dilution in 10-fold increments for their ability to grow on YPD medium at either 30°C or 37°C. **B.** Fis1(cyt)-Tom5C can partially rescue the import phenotype of *tom5* null strain. Radiolabeled precursor proteins of the ADP/ATP carrier (AAC) and of pSu9-DHFR (pSu9-DHFR) were incubated at 25°C with mitochondria isolated either from wild type, Δtom5 or Δtom5+ Fis1(cyt)-Tom5C cells for the indicated time periods. After import the samples were treated with proteinase K and mitochondria were re-isolated. Imported proteins were analyzed by SDS–PAGE and autoradiography. The amount of precursor proteins imported into wild type mitochondria for the longest time period was set to 100.

3.2 Tob38, a novel essential component of the TOB complex

3.2.1 Identification of Tob38

The major aim of this thesis was to study the biogenesis of mitochondrial outer membrane. This included identification and characterization of novel components of the newly discovered TOB complex. This part of my work was carried out in collaboration with Thomas Waizenegger. To find interacting partners of Tob55, mitochondria from both a wild-type yeast and a strain carrying a His8-tag at the N terminus of Tob55 ($Tob55_{His}$;(Paschen et al., 2003) were lysed with digitonin and Ni-NTA beads were added. Proteins retained on the beads selectively with the $Tob55_{His}$ mitochondria were identified by mass spectrometry (Protein analysis unit of Adolf-Butenandt Institute, LMU, Munich). In addition to Mas37 and $Tob55_{His}$, a protein of 38 kDa was identified and it was termed Tob38 (Waizenegger et al., 2004). Tob38 is encoded by an essential gene (ORF Yhr083w) (Niedenthal et al., 1999) and is predicted to consist of 330 amino acid residues.

3.2.2 Tob38 is part of the TOB complex and essential for the biogenesis of β-barrel proteins

Tob38 is a peripheral membrane protein of the TOB complex and is associated with the cytosolic surface of the outer membrane. Combined with Tob55, Tob38 forms a functional TOB core complex (Waizenegger et al., 2004). Due to the essential nature of Tob38, a yeast strain was constructed in which the *TOB38* gene was under the control of the *GAL10* promoter. In the presence of galactose, these cells grew at a similar rate to that of the wild type cells. In contrast, in the presence of glucose, growth of the *GAL10-TOB38* strain was slowed down after 12 h and was strongly reduced after 19 h. Mas37 and Tob55 were present at strongly reduced levels as early as 12 h after the shift to glucose. Depletion of Tob38 for 19 h resulted in even lower amounts of Mas37 and Tob55 and in reduced amounts of the β-barrel proteins Tom40, Mdm10 and porin. In contrast, other proteins of the various mitochondrial subcompartments were present at roughly the same levels as those in the wild type.

Next, the involvement of Tob38 in the membrane insertion of β-barrel preproteins was checked. Precursors of Tom40 and Mdm10 bind to the TOB complex, and thereby an import intermediate of about 250 kDa is formed (Model et al., 2001; Paschen et al., 2003; Wiedemann et al., 2003). In the case of mdm10, this import intermediate was strongly

reduced when Tob38-depleted mitochondria were used (Fig 8A). The import of another β-barrel protein, porin, (Gentle et al., 2004; Kozjak et al., 2003; Wiedemann et al., 2003), was analysed by the formation of assembled complexes . The formation of these oligomeric species was inhibited in Tob38-depleted cells (Fig 8B). Import of porin, however, shows less dependency of Tob38 than import of Tom40 and Mdm10. In contrast to the β-barrel precursors, other proteins of the various mitochondrial subcompartments, such as Tim23, (Fig 8C) were imported into mitochondria from Tob38-depleted cells with similar efficiencies as into mitochondria from wild-type cells. Taken together, Tob38 is a peripheral membrane protein of the TOB complex and has an essential function in the biogenesis of β-barrel proteins.

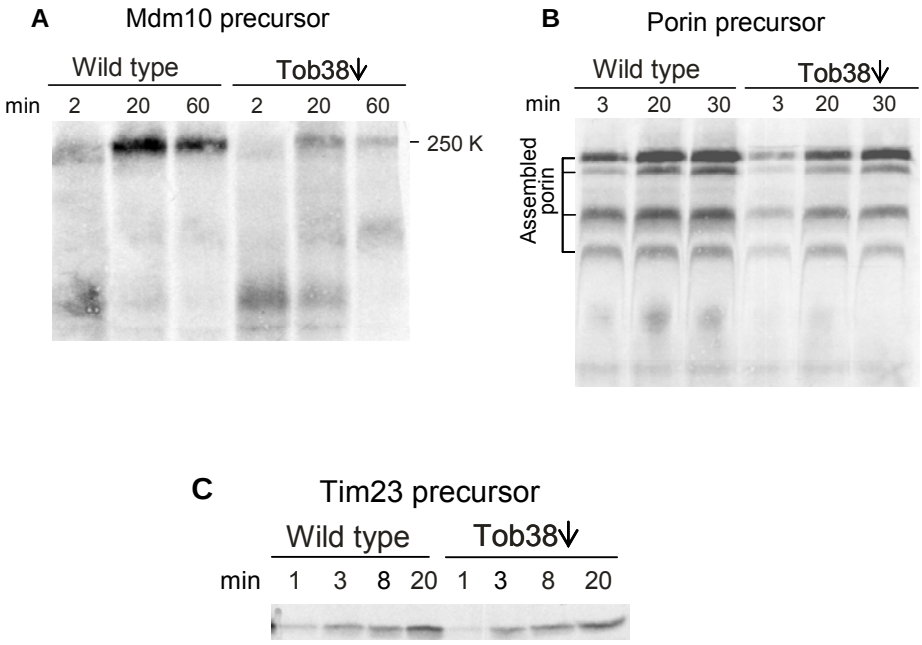

Figure 8. Tob38 is required for the insertion and assembly of β-barrel precursor proteins. **A, B.** Mitochondria isolated from either wild-type or Tob38-depleted cells (Tob38↓) were incubated with radiolabelled precursors of Mdm10 (**A**) and porin (**B**) for the indicated time periods. Mitochondria were re-isolated and analysed by BNGE followed by autoradiography. The 250 kDa assembly intermediate of Mdm10 (250K) and the assembled complexes of porin are indicated. **C.** A radiolabeled precursor of Tim23 was imported into the indicated mitochondria. Samples were re-isolated, treated with PK and analysed by SDS–PAGE and autoradiography.

3.3 Assembly of the TOB complex

The TOB complex of mitochondria is composed of three identified components: the major one, Tob55, is an essential β-barrel membrane-embedded protein. Tob55 mediates the insertion of β-barrel proteins into the outer membrane. The other known components of the TOB complex are the peripheral membrane proteins Mas37 and Tob38. Mas37 interacts with Tob55 and so far plays an unidentified role in the biogenesis of β-barrel proteins. Tob38, as mentioned above, is essential for viability in yeast, and together with Tob55 forms a functional TOB core complex. Despite the central role of the TOB complex in the biogenesis of β-barrel proteins, little is known about the biogenesis of the complex itself. In this part of the study, the mechanism by which Tob55 and Mas37 are targeted to mitochondria and assembled into the TOB complex was elucidated.

3.3.1 The establishment of an *in vitro* import assay

To investigate the import and assembly pathways of Tob55 and Mas37, the precursors were individually synthesized in rabbit reticulocyte lysate and labelled with [^{35}S] methionine. The precursors were then incubated with isolated mitochondria, and a protease accessibility assay was used as a criterion for correct insertion into the outer membrane. In the case of Tob55, treatment of mitochondria with proteinase K (PK) resulted in the formation of a specific proteolytic fragment of 30 kDa (Paschen et al., 2003). This fragment was also observed upon insertion of a radiolabeled Tob55 precursor into the outer membrane of isolated mitochondria (Fig. 9A). A second fragment with an apparent molecular mass of 20 kDa was also observed. These fragments were completely degraded when mitochondria were solubilized with the detergent Triton X-100, and were also absent when reticulocyte lysate containing the Tob55 precursor was treated with PK (Fig. 9A). The two fragments are embedded in the outer membrane, as they could not be extracted by alkaline solution (Fig. 9B). Thus, this protection assay can be employed to investigate the mechanism of insertion of Tob55 into the mitochondrial outer membrane. The intensity of the 30 kDa fragment served in further experiments to quantify membrane insertion of Tob55. In order to study the association of Mas37 with the mitochondrial outer membrane, the generation of two types of Mas37 fragment (14 and 20 kDa) upon treatment of mitochondria with PK (Wiedemann et al., 2003) was utilized. The 14 kDa fragment was recognized by antibodies against a carboxy terminal region of Mas37 (Wiedemann et al., 2003). The intensity of this band served as a measure for the association of Mas37 with the outer membrane.

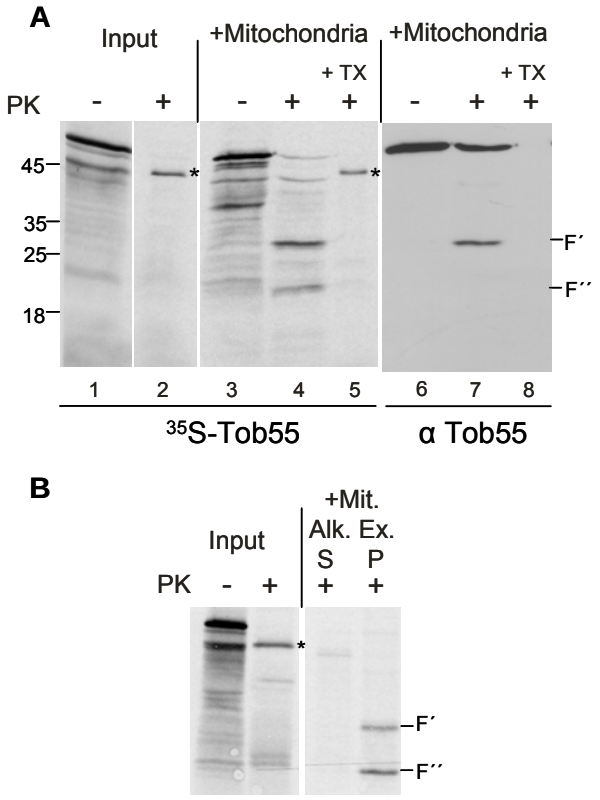

Figure 9. An assay to study the *in vitro* insertion of Tob55. A. Two specific proteolytic fragments are formed upon the correct insertion of Tob55 into the outer membrane. Radiolabeled Tob55 was incubated for 20 min at 25°C in an import buffer in the absence or presence of isolated mitochondria. After the incubation, the samples were divided into aliquots. Two aliquots were left intact (*lanes 1* and *3*), while the other aliquots were treated with 50 µg/ml PK (*lanes 2, 4*, and *5*) in the absence or presence of Triton X-100 (+*TX*). Proteins in the sample that contained Triton X-100 were precipitated with trichloroacetic acid. Mitochondria in the other samples were pelleted. Samples were subjected to SDS-PAGE and autoradiography. The membrane containing *lanes 3–5* was immunodecorated with antibodies against the N-terminal peptide of Tob55 (*lanes 6–8*). *F*' and *F*'', specific proteolytic fragments of Tob55. *, a nonprecursor protein expressed from mRNA in the reticulocyte lysate. **B**. Import reaction was performed as above. Mitochondria were suspended in 0.1 M Na_2CO_3 for alkaline extraction (*Alk. Ex.*). After 30 min on ice, the sample was centrifuged, and the pellet (*P*) and supernatant (*S*) were analyzed. As a control, 100% of the input lysate for the import reactions was treated with PK. *, a nonprecursor protein as in A

3.3.2 The TOM machinery is involved in the import of Tob55 but is dispensable for the import of Mas37

The TOM machinery is the central entry gate for mitochondrial precursor proteins. Generally, preproteins are initially recognized by the mitochondrial surface receptors Tom20 or Tom70 (Rapaport and Nargang, 2004). The requirement of these receptors for the import of Tob55 and Mas37 was examined. Mitochondria isolated from strains lacking either Tom20 or Tom70 were incubated with radiolabeled Tob55, and the amounts of inserted precursors were analyzed by SDS-PAGE. Tob55 was inserted into

these mutated mitochondria with a strongly reduced efficiency as compared to wild type organelles (Fig. 10).

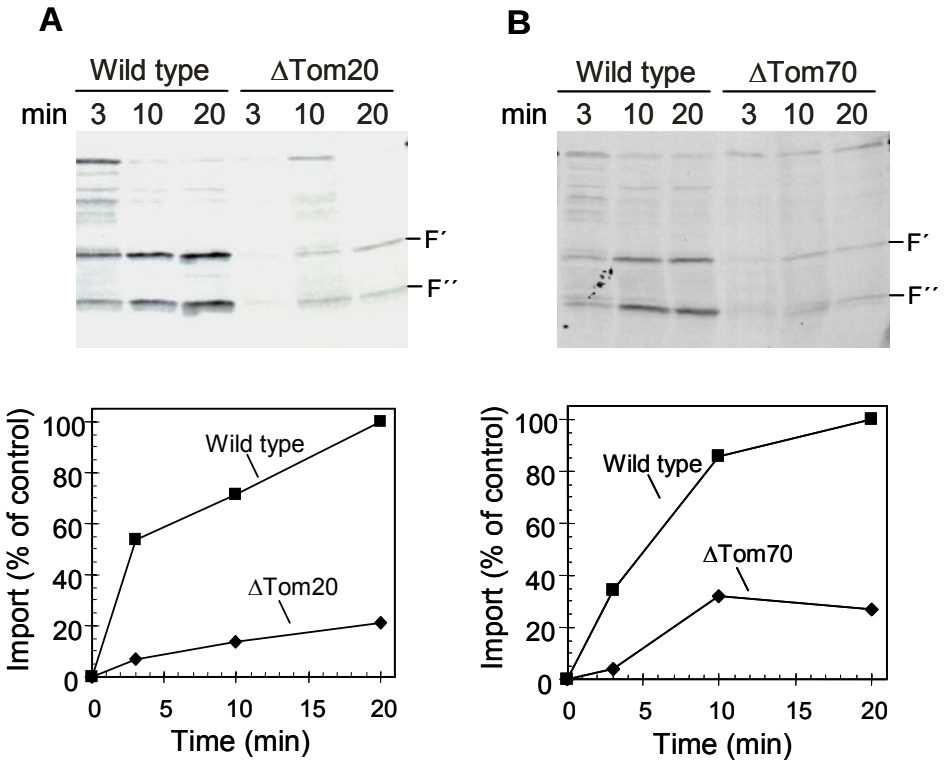

Figure 10. The insertion of Tob55 requires the import receptors. The radiolabeled precursor of Tob55 was incubated at 25°C for the indicated time periods with mitochondria isolated from either the wild type strain or from the strain lacking Tom20 (**A**) or Tom70 (**B**). After import, the samples were treated with proteinase K, and mitochondria were reisolated. Inserted proteins were analyzed by SDS-PAGE and autoradiography. The proteolytic fragments of Tob55 (*F*' and *F*'') are indicated. The insertion of Tob55 was quantified by analyzing the formation of the 30 kDa fragment (*F*'). The amount of precursor protein imported into wild type mitochondria for the longest time period was set to 100%.

In contrast, deletion of the import receptors did not affect the association of Mas37 with mitochondria (Fig. 11). Similar results were obtained when the import experiments were performed at 15°C. Hence, although the import receptors Tom20 and Tom70 appear to play only a minor role, if any, in the association of Mas37 with the outer membrane, they have a crucial function in the recognition of Tob55 precursor.

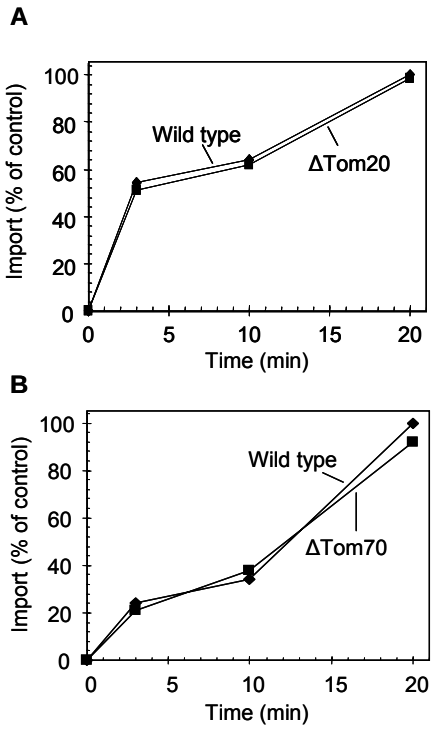

Figure 11. The association of Mas37 with mitochondria is independent of import receptors. The radiolabeled precursor of Mas37 was incubated at 25°C for the indicated time periods with mitochondria isolated either from the wild type strain or from the strain lacking Tom20 (**A**) or Tom70 (**B**). After import, the samples were treated with proteinase K (15 µg/ml), and mitochondria were re-isolated. Associated proteins were analyzed by SDS-PAGE and autoradiography. The association of Mas37 was quantified by analyzing the formation of the 14 kDa fragment. The amount of precursor protein associated with wild type mitochondria for the longest time period was set to 100%.

Do Tob55 and Mas37 use the general import pore of the TOM complex? To shed light on this question, the import pore was blocked by addition of an excess of recombinant pSu9(1-69)-DHFR, a precursor consisting of the presequence of ATP synthase subunit 9 fused in front of dihydrofolate reductase. A similar set of experiments had shown previously that the import of radiolabeled matrix-destined precursors as well as precursors of outer membrane β-barrel proteins was competed out by recombinant pSu9(1–69)-DHFR (Krimmer et al., 2001; Paschen et al., 2003; Rapaport and Neupert, 1999). Consistent with these observations, the insertion of Tob55 was strongly reduced upon the addition of excess preproteins (Fig. 12A). In contrast, excess recombinant pSu9(1–69)-DHFR did not reduce the association of radiolabeled Mas37 with mitochondrial outer membrane (Fig. 12A).

The involvement of Tom40 in the import of both precursor proteins was further investigated. To that end, mitochondria isolated from strains harbouring temperature-sensitive alleles of *tom40* (*tom40-3* and *tom40-4*) were used. It was shown previously that the mutation in the *tom40-3* strain did not affect the insertion of porin, whereas those in *tom40-4* resulted in lower efficiency of porin insertion into the membrane (Fig. 12B)(Krimmer et al., 2001). The insertion of Tob55 into the Tom40-4 mitochondria was significantly less efficient in comparison to the insertion into wild-type mitochondria, whereas no difference was observed with Tom40-3 mitochondria (Fig. 12B). The association of Mas37 with mitochondria from the mutated strains was similar to that with

the wild type mitochondria (Fig. 12B). In conclusion, Tom40 has a direct role in the insertion of Tob55, whereas the same mutational alterations of Tom40 do not affect the association of the Mas37 precursor with mitochondria.

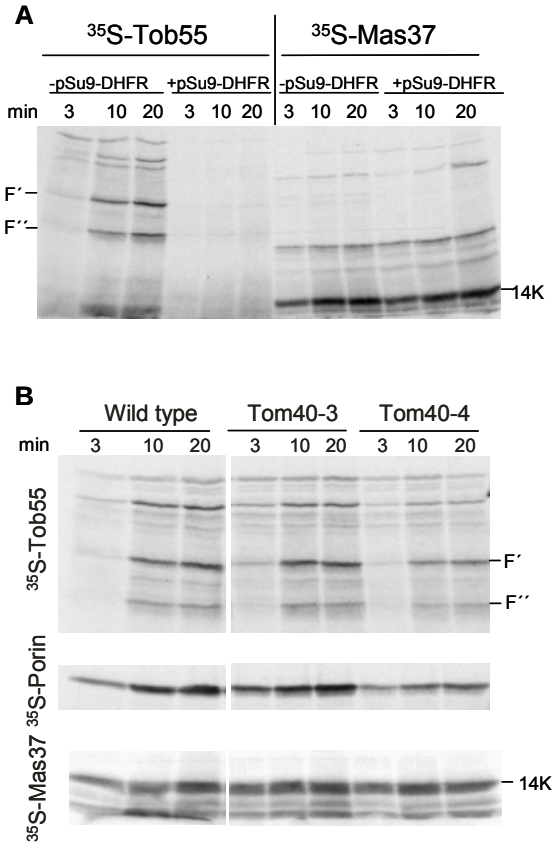

Figure 12. The TOM complex is not involved in the association of Mas37 with mitochondria. A. The association of Mas37 is not affected by an excess of matrix-targeted precursors. Radiolabeled precursors of Tob55 and Mas37 were added alone, or after mixing with recombinant pSu9-DHFR, to isolated mitochondria. The samples were incubated at 25°C for the indicated time periods. At the end of the import reactions, proteinase K was added, and proteins were analyzed by SDS-PAGE. The proteolytic fragments of Tob55 (*F*' and *F*'') and of Mas37 (*14K*) are indicated. **B.** Association of Mas37 with mitochondria is not affected in *tom40* mutant mitochondria. Radiolabeled precursors of Tob55, porin, and Mas37 were added to mitochondria isolated from the yeast mutant strains *tom40-3, tom40-4*, and the corresponding wild type strain and were incubated at 25°C for the indicated time periods. After import, the samples were treated with proteinase K, and mitochondria were reisolated. Imported proteins were analyzed by SDS-PAGE and autoradiography. The proteolytic fragments of Tob55 and Mas37 are indicated as in A.

3.3.3 The small Tim proteins in the IMS are involved in the import pathway of Tob55

To investigate the possible downstream events following the translocation of Tob55 via the general import pore, mutants of different components in the IMS were used. Since the C-terminal domain of Tom22 protrudes into the IMS, the import of radiolabeled

Tob55 was analyzed in mitochondria isolated from strain-expressing Tom22 molecules without this domain (Fig. 13A) (Moczko et al., 1997). The deletion had no effect on the insertion efficiency. Thus, the C-terminal domain of Tom22 does not play an important role in the insertion process of Tob55. Next, the involvement of the small Tim proteins (Tim9/10 and Tim8/13 complexes) residing in the IMS was examined. The import of radiolabeled Tob55 was carried out in the presence of mitochondria isolated from either the *ΔTim8/13* strain or a strain with a mutated *TIM10* allele (Fig. 13B and C). In both cases, an impaired insertion as compared with the wild-type organelles was observed. This reduction is similar to the previously observed one, when Tom40 was imported into mitochondria containing the mutated allele of *TIM10* (Wiedemann et al., 2004b). Notably, the effect of the mutations in *tim10* on the import of the ADP-ATP carrier protein, a *bona fide* substrate of the Tim9/10 complex, was much more profound as compared with the moderate effect on the insertion of Tob55 (Fig. 13C). In summary, both complexes Tim8/13 and Tim9/10 appear to be involved in the import of Tob55.

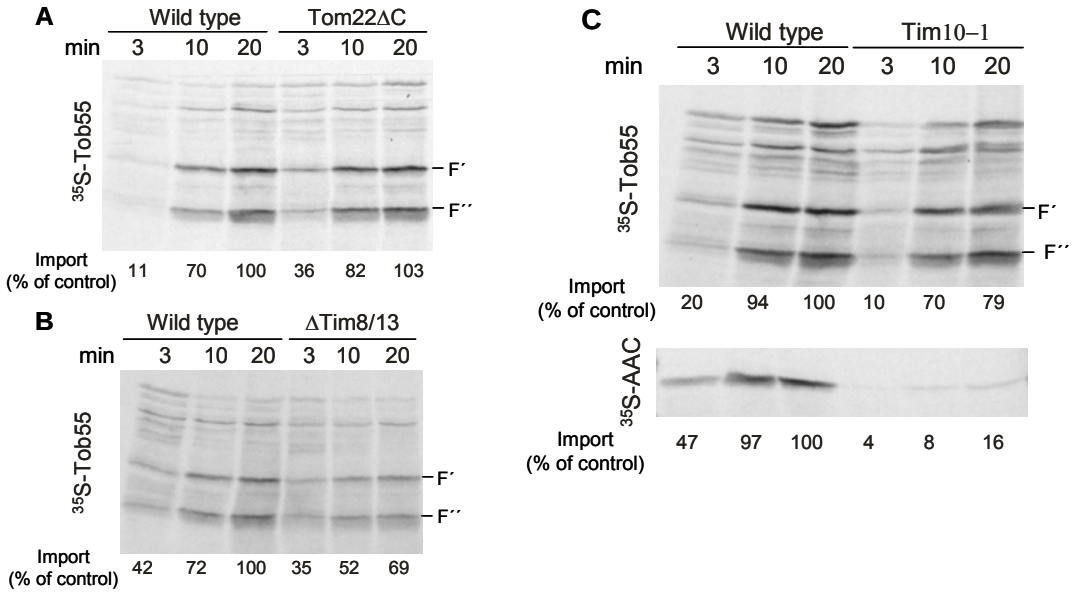

Figure 13. The insertion of Tob55 into the outer membrane involves components in the intermembrane space. The radiolabeled precursor of Tob55 was incubated at 25°C for the indicated time periods with mitochondria isolated from either the wild type strain or from the indicated strains. **A.** A strain containing a Tom22 variant where the C-terminal domain is deleted (Tom22ΔC). **B.** A strain lacking *tim8* and *tim13* (ΔTim8/13). **C.** A strain containing a mutated allele of *tim10* (Tim10–1). The radiolabeled precursor of ADP-ATP carrier protein (*^{35}S-AAC*) was also imported into the latter mitochondria for comparison. After import, the samples were treated with proteinase K, and mitochondria were re-isolated. The proteolytic fragments of Tob55 (*F*' and *F*") are indicated. The bands corresponding to F' in *panels B* and *C* and the protected AAC precursor were quantified, and the amount of precursor protein imported into wild type mitochondria for 20 min was set to 100%.

3.3.4 Analyzing the import intermediates of Tob55 by blue native gel electrophoresis

In order to elucidate further the biogenesis of Tob55, its assembly into the TOB complex was monitored by blue native gel electrophoresis (BNGE). This approach permits the separation of membrane protein complexes in their native state. Radiolabeled Tob55 was incubated with mitochondria isolated from either the wild type or a strain harbouring an HA-tagged version of Tob38 ($Tob38_{HA}$). At the end of the import reactions, the mitochondria were re-isolated, solubilized in a buffer containing 1% digitonin, and subjected to BNGE. At short incubation periods, a high molecular mass species of ~350 kDa was observed (Fig. 14A, *I*). At longer incubation periods, additional species with lower molecular masses (Fig. 14A, *II–IV*) were monitored. The two upper species (Fig. 14A, *I* and *II*) contained the Tob55-interacting partner, Tob38, since they migrated higher in $Tob38_{HA}$ mitochondria than in wild type organelles (Fig. 14A). Further support for the presence of Tob38 in the upper species (Fig. 14A, *I*) was obtained by an antibody shift experiment. Radiolabeled Tob55 was imported into $Tob38_{HA}$ mitochondria and antibodies against the HA-tag were added after solubilization of the mitochondria. This addition resulted in a shift of the upper band to higher molecular masses (Fig. 14B) by formation of a super complex between the antibody molecules and the TOB complex.

To obtain further information about the various species observed upon import of radiolabeled Tob55, the membrane was decorated with antibodies against Tob55. Because the radiolabeled Tob55 proteins are in amounts that are too low to be detected by the antibodies, this immunodecoration can be used to analyze the migration of the preexisting Tob55 protein. The endogenously assembled Tob55 migrates in BNGE in 3–4 distinct bands that represent various oligomeric conformations (Ishikawa et al., 2004b; Meisinger et al., 2004; Wiedemann et al., 2003). The exact composition and stoichiometry of these oligomeric states are not resolved yet. Nevertheless, the upper and lower bands of the radiolabeled Tob55 (Fig. 14C, *I* and *IV*) co-migrate with the upper and lower species of the assembled Tob55 (Fig. 14C). Hence, at least a fraction of the newly imported radiolabeled Tob55 molecules behave like the endogenously assembled Tob55.

To further assess the involvement of the IMS in the assembly of Tob55, radiolabeled precursors were imported into swollen mitochondria, in which the outer membrane was ruptured. The assembly of radiolabeled Tob55 precursor was strongly reduced under these conditions in comparison to intact organelles (Fig. 14D). As a control, the assembly

of Tom20 into the TOM complex was not affected by rupturing the outer membrane. Hence, opening of the outer membrane interferes with the assembly of Tob55.

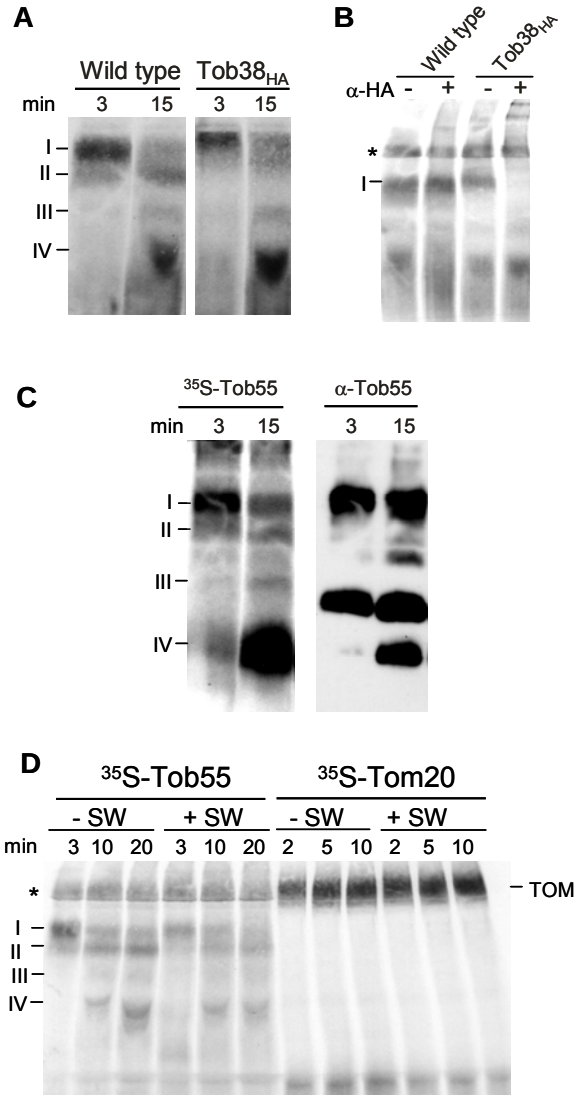

Figure 14. The assembly of Tob55 is inhibited upon opening of the outer membrane. A. The radiolabeled precursor of Tob55 was incubated with wild type and $Tob38_{HA}$ mitochondria at 25°C for the indicated time periods. Mitochondria were re-isolated and analyzed by BNGE followed by autoradiography. Species containing the radiolabeled Tob55 are indicated (*I, II, III*, and *IV*). **B.** Tob38 interacts with the precursor of Tob55. Wild type and $Tob38_{HA}$ mitochondria were incubated with the radiolabeled Tob55 precursor for 3 min at 25°C. Mitochondria were re-isolated and resuspended in a buffer containing 1% digitonin. One half of the samples was analyzed directly by BNGE, whereas the other half was incubated before analysis with antibodies against the HA-tag. The radiolabeled bands are indicated as in A. The unspecific band, which is often observed in BNGE, is indicated with an *asterisk* (Meisinger et al., 2004; Paschen, 2004). **C.** The radiolabeled precursor of Tob55 was incubated with wild type mitochondria at 25°C for 3 or 15 min. Mitochondria were re-isolated and analyzed by BNGE followed by autoradiography and immunodecoration with antibodies against Tob55. Species containing the radiolabeled Tob55 are indicated as in A. **D.** Opening of the outer membrane blocks the assembly of Tob55. Isolated mitochondria were preincubated in isotonic buffer (*–SW*) or in hypotonic buffer (*+SW*) for 30 min on ice. The mitochondria were re-isolated and incubated with radiolabeled precursors of Tob55 and Tom20 for the indicated time periods. At the end of the import reactions, mitochondria were analyzed by BNGE and autoradiography. The TOM complex and the assembly intermediates of Tob55 are indicated. *, unspecific band.

3.3.5 Assembly of Tob55 and Mas37 precursors into preexisting TOB complexes

The last stage in the import pathway of Tob55 and Mas37 is the assembly of these precursors into preexisting TOB complexes. To assess this notion, radiolabeled Tob55 or Mas37 were imported into mitochondria harbouring a His-tagged version of Tob38 (Waizenegger et al., 2004). After the import reaction was finished, mitochondria were reisolated, solubilized in a buffer containing digitonin and incubated with Ni-NTA beads. The beads were washed, bound material was eluted and analysed onto SDS-PAGE. Both precursors were co-isolated with $Tob38_{His}$, whereas no such interaction was observed with the control precursor of Tom20 (Fig. 15A). To further investigate the role of preexisting TOB machinery in the biogenesis of newly synthesized Tob55 and Mas37, mitochondria harbouring mutated TOB complexes were used. In the absence of Tob38, or upon depletion of preexisting Tob55, the insertion of Tob55 was strongly impaired (Fig. 15B and C). This reduction could be the outcome of the essential role of Tob55 in the assembly of all β-barrel proteins in the outer membrane and/or the result of the requirement of newly synthesized Tob55 molecules to assemble with preexisting Tob55 molecules. Only moderate reduction was observed when Tob55 was imported into mitochondria lacking Mas37 (Fig. 15C). These results are consistent with the observations that within the TOB complex, Tob55 interacts tightly with Tob38 and to a lesser extent with Mas37 (Ishikawa et al., 2004b; Waizenegger et al., 2004).

To gain further insights into the interaction between Tob38 and Tob55, the BNGE approach, in combination with carbonate extraction, was used. Tob55 is embedded in the outer membrane, whereas its partner protein, Tob38, is only associated with the membrane and can be extracted by an alkaline solution (Waizenegger et al., 2004). Radiolabeled precursor of Tob55 was imported into mitochondria. The organelles were reisolated, alkaline extraction was performed and the membrane pellet was analyzed by BNGE. Interestingly, in mitochondria subjected to alkaline extraction no assembly intermediates of Tob55 were observed (Fig. 15D). In addition, the endogenous TOB complex was hardly detected upon immunostaining with antibodies against Tob55, whereas the TOM complex and porin assemblies were not affected (Fig. 15E). Taken together, these results lend further support to the importance of Tob38 in the assembly and stability of the TOB complex. It might be that the extraction of Tob38 results in destabilization and aggregation of its partner protein Tob55; therefore Tob55 cannot be detected by BNGE.

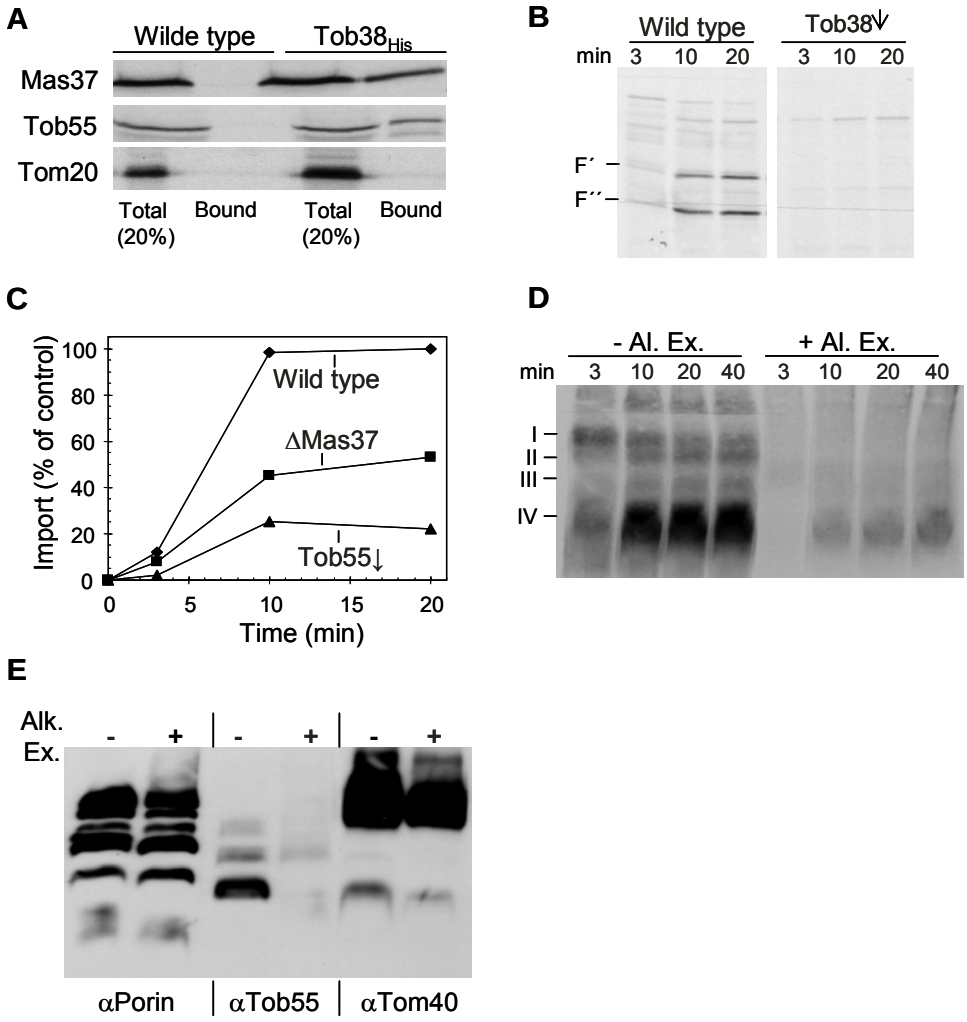

Figure 15. Tob55 assembles into preexisting TOB complexes. A. wild-type and $Tob38_{His}$ mitochondria were incubated with the indicated radiolabeled precursors. Mitochondria were re-isolated and lysed in a buffer containing 1% digitonin. After lysis, a portion (total 20%) was removed. The rest was incubated with Ni-NTA beads. These were washed and eluted with an SDS-containing buffer (*Bound*). Fractions were analyzed by SDS-PAGE and autoradiography. **B.** mitochondria isolated from wild type and Tob38-depleted cells (*Tob38*↓) were incubated at 25°C with radiolabeled precursors of Tob55 for various time periods. After import, the samples were treated with proteinase K, and mitochondria were re-isolated. Inserted proteins were analyzed by SDS-PAGE and autoradiography. The proteolytic fragments of Tob55 (*F'* and *F''*) are indicated. **C.** mitochondria isolated from wild-type, *Δmas37*, and Tob55↓ cells were incubated with radiolabeled Tob55 precursor. Further treatment and quantification were as described in the legend of Fig. 11. **D.** the assembly intermediates of Tob55 are not stable under alkaline conditions. Mitochondria were incubated with radiolabeled precursors of Tob55 for the indicated time periods. Samples were halved and re-isolated. One half was suspended in 100 µl of 20 mM Hepes-KOH, pH 7.4, while the other portion was suspended in 100 µl of 10 mM Hepes-KOH, 0.1 M Na_2CO_3 for alkaline extraction (*Alk. Ex.*). After 30 min on ice, the samples were centrifuged, and pellets were analyzed by BNGE. The radiolabeled bands are indicated as in Fig. 14A. **E.** The TOB complex is not stable under alkaline conditions. Mitochondria isolated from the wild type strain were analyzed directly by BNGE or were treated with alkaline solution as in *D* before such analysis. Immunodecoration with the indicated antibodies was performed.

Next, the association of Mas37 with the TOB complex and the requirement of each component of this complex were investigated. Surprisingly, when mitochondria lacking Mas37 were used, the association of Mas37 precursor molecules with mitochondria and their assembly into the TOB complex were increased by at least 2-fold (Fig. 16A, B). However, depletion of either Tob38 or Tob55 had an opposite effect. Mas37 precursor was not assembled into high molecular mass complexes when either Tob38 or Tob55 were depleted (Fig. 16B). Thus, newly synthesized Mas37 molecules do not need preexisting Mas37 molecules on mitochondria but rather require Tob55 and Tob38 for their proper targeting and assembly. These results are consistent with the previous observation that the endogenous levels of Mas37 in mitochondria depleted for either Tob38 or Tob55 are clearly reduced (Waizenegger et al., 2004). It appears that precursors of Mas37 assemble directly with the TOB core complex, which is composed of Tob38 and Tob55.

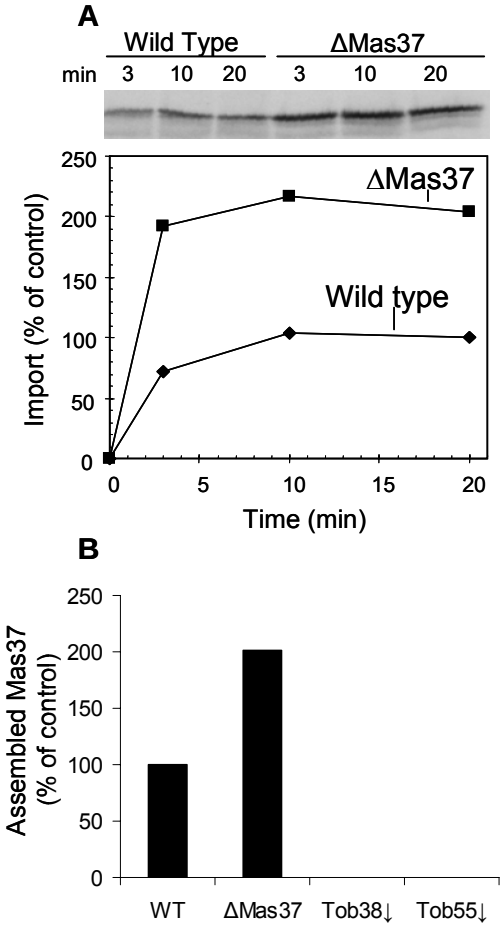

Figure 16. Mas37 associates directly with the TOB core complex. **A.** mitochondria isolated from wild-type and *Δmas37* cells were incubated at 25 °C with radiolabeled Mas37 precursor for the indicated time periods. Further treatment and quantification were as described in the legend of Fig. 11. **B.** mitochondria isolated from wild-type (*WT*), *Δmas37,* Tob38↓, and Tob55↓ cells were incubated at 25 °C for 20 min with radiolabeled Mas37 precursor. Mitochondria were re-isolated and analyzed by BNGE and autoradiography. The bands corresponding to the assembled Mas37 (Wiedemann et al., 2003) were quantified, and the amount of assembled material in the wild-type strain was set to 100%.

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

In the previous chapters, I described the various components that compose the TOB complex, as well as the import and the assembly pathways of Tob55 and Mas37 into the mitochondrial outer membrane. Despite some progress in understanding the assembly and the structure of the TOB complex, many questions as to the function of its subunits and their domains remain to be answered.

Tob55 is anchored in the outer membrane by the conserved C-terminal β-barrel domain which most probably mediates the insertion function of this protein. The N-terminal domain of Tob55, comprising about 100 amino acid residues, is predicted not to be part of the β-barrel structure (http://www.imtech.res.in/cgibin/betatpred2/). It appears to be exposed to the intermembrane space (Paschen et al., 2003). This suggests that this domain might interact with precursors of β-barrel membrane proteins that are on their way from the TOM to the TOB complex to become inserted into the outer membrane. In this part of the study, I investigated the contribution of the N-terminal domain of Tob55 to the function of the protein.

As the location of this domain is crucial for such a working model, its topology was analyzed. To this end, a Tob55 variant that carried a His_8-tag at the N-terminus was employed (Paschen et al., 2003) (Fig. 17A) and the proteolytic assay mentioned in 3.3.1 was used. Treatment of mitochondria carrying His_8-tagged Tob55 with PK resulted, as expected, in the formation of a 30 kDa fragment which could be immunodecorated with antibodies against the His-tag (Fig. 17B). Formation of this fragment was abolished when the outer membrane was ruptured by either osmotic shock or by solubilizing the membrane with the detergent Triton X-100 (Fig. 17B). It is unlikely that the protection of the His-tag against PK resulted from a stretch of eight Histidine residues buried within the hydrophobic core of the membrane. Hence, these results imply that the N-terminus of Tob55 is indeed exposed to the IMS.

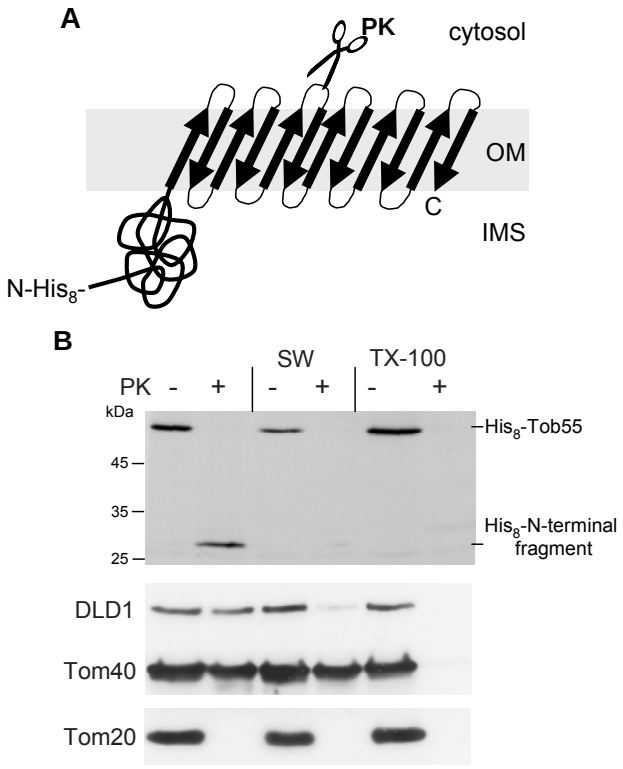

Figure 17. The N-terminal domain of Tob55 is facing the intermembrane space. **A.** Schematic representation of cleavage of Tob55 by proteinase K (PK) resulting in two proteolytic fragments. **B.** Mitochondria from cells expressing His-Tob55 were isolated and were either left intact, subjected to osmotic swelling (SW) or solubilized with Triton X-100 (TX-100). Mitochondria were incubated for 15 min on ice in the absence or presence of 50 µg/ml PK. All samples were subjected to SDS-PAGE and immunoblotting with antibodies against His-tag (upper panel) or against the marker proteins DLD1 (IMS), Tom40 (embedded in the outer membrane) and Tom20 (an outer membrane protein with a large cytosolic domain). Full-length Tob55 and the N-terminal fragments are indicated.

3.4.1 Tob55 precursor devoid of its N-terminal domain is targeted to and assembled into the outer membrane of mitochondria

To study the possible functions of the N-terminal domain of Tob55, constructs were created in which 50, 80 or 102 of the N-terminal amino acid residues were deleted resulting in Tob55Δ50, Tob55Δ80 and Tob55Δ102, respectively. First, the targeting and the insertion of these proteins into the outer membrane of mitochondria were checked *in vivo*. Accordingly, Tob55 variants were cloned into a yeast expression vector, and wild type cells were transformed with the resulting plasmids. Upon sub-cellular fractionation all Tob55 variants were found in the mitochondrial fraction, like the mitochondrial marker proteins Tom20 and porin (Fig. 18A), demonstrating the correct sub cellular localization of these variants.

Mitochondrial targeting and membrane integration of Tob55 variants were further studied using the *in vitro* import assay (see above and 3.3.1) with radiolabeled precursor

proteins and isolated mitochondria. Correct *in vitro* membrane insertion 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 were expected to remain unchanged. Indeed, upon incubation of radiolabeled Tob55 truncated variants with isolated mitochondria, smaller N-terminal proteolytic fragments were formed. Thus, all the variants appear to be targeted to mitochondria and become inserted into the outer membrane to reach a native topology (Fig. 18B).

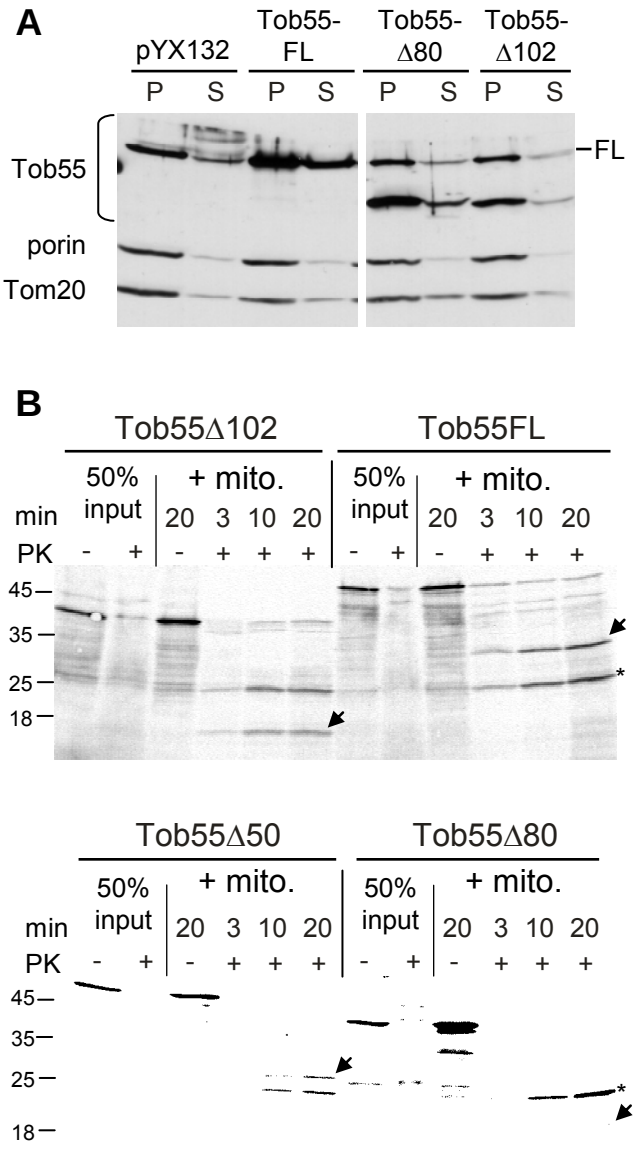

Figure 18. The N-terminal domain of Tob55 precursor molecules is not required for targeting and membrane insertion. A. Wild type yeast cells were transformed with either empty yeast expression vector (pYX132) or with vector encoding the indicated variant of Tob55. Cells were ruptured by vortexing in the presence of glass beads. Crude mitochondria were pelleted by differential centrifugation (P) and were analyzed together with the supernatant fraction (S) by SDS-PAGE. The membrane was immunodecorated with polyclonal antibodies against Tob55 or with antibodies against the outer membrane marker proteins Tom20 and porin. **B.** Radiolabeled Tob55 variants were incubated at 25°C 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), the other aliquots were treated with 50 µg/ml PK at 0°C (+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 (arrowhead), and C-terminal proteolytic fragments (asterisk) are indicated.

The import of the newly synthesized Tob55 precursors resembles the insertion route of other β-barrel precursors. Accordingly, efficient insertion requires the import receptor Tom20, the translocation pore of the TOM complex and the preexisting TOB complex. Using the *in vitro* assay, the ability of the Tob55 N-terminal variants to follow this common pathway was confirmed. First, the import efficiency of all three N-terminal truncated variants was reduced upon deletion of Tom20 (Fig. 19A and B). Second, blocking the TOM channel with a large molar excess of the recombinant matrix-destined preprotein pSu9(1-69)-DHFR strongly inhibited the import and membrane insertion of the variants of Tob55 precursors (Fig. 19C). Third, to study the involvement of preexisting TOB complexes, the insertion of Tob55 variants into wild type mitochondria and into mitochondria depleted of Tob55 was compared. All Tob55 radiolabeled variants were inserted with strongly reduced efficiency into the Tob55-depleted mitochondria (Fig. 19D). A similar inefficiency of insertion was observed for all Tob55 variants when mitochondria depleted of Tob38 were used (not shown). These results demonstrate that preexisting TOB complexes are essential for the membrane insertion of all Tob55 variants. Taken together, Tob55 precursors follow the insertion pathway of β-barrel proteins even when lacking their N-terminal domain.

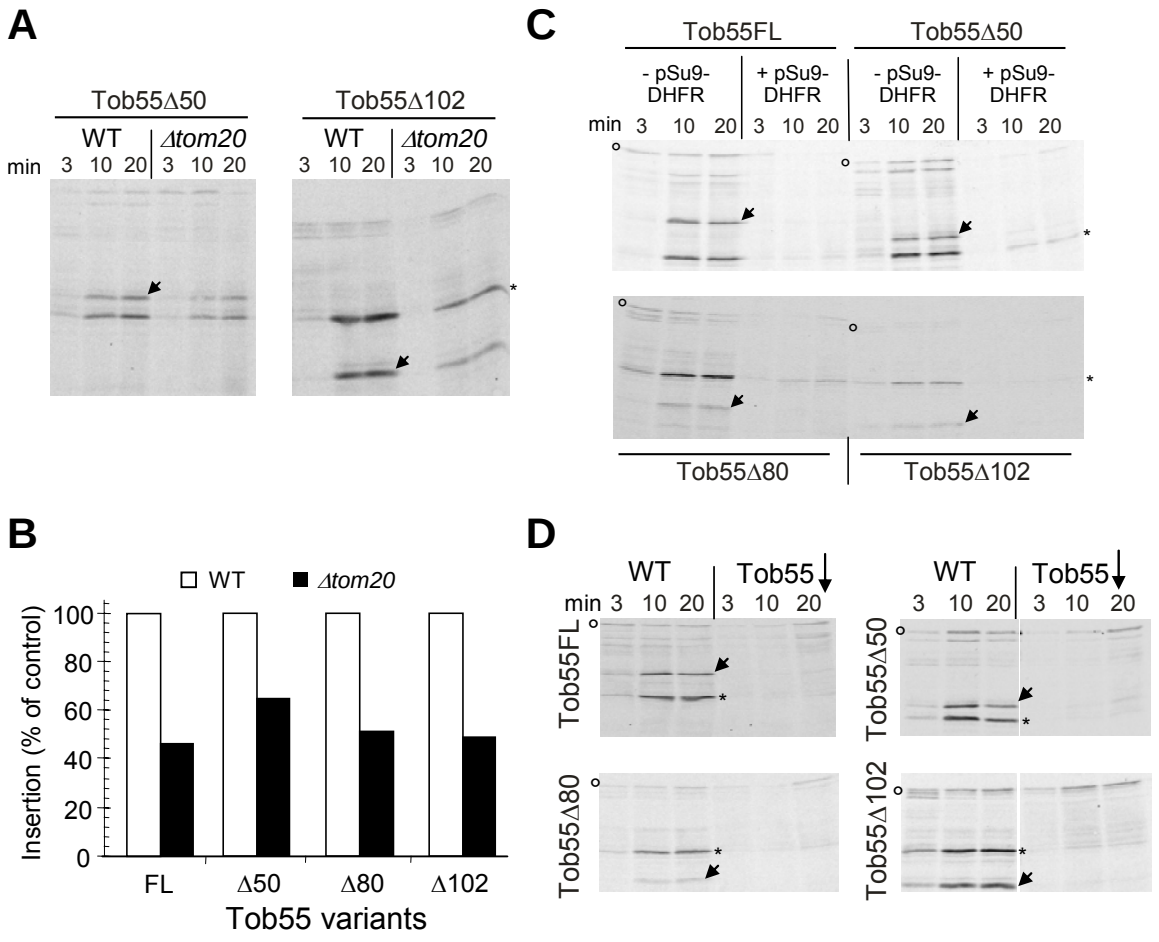

Figure 19. Truncated Tob55 variants use the common insertion pathway of β-barrel precursors.
A. Efficient insertion of Tob55 variants is dependent on Tom20. Radiolabeled precursors of Tob55 variants were incubated at 25°C for the indicated time periods with mitochondria from either wild type strain or from strain lacking Tom20. After import the samples were treated with PK (50 µg/ml) and mitochondria were re-isolated. Inserted Tob55 was analyzed by SDS-PAGE and autoradiography. Proteolytic fragments are indicated as described in the legend to Fig. 18B. **B.** Insertion of Tob55 variants after 20 min incubation with mitochondria isolated from WT and Δtom20 strain was quantified by monitoring the formation of the 30 kDa N-terminal fragment. The amount of precursor protein inserted into wild type mitochondria was set to 100%. **C.** The insertion of Tob55 variants is affected in the presence of excess matrix-targeted precursor. Radiolabeled precursors of Tob55 variants were added to isolated mitochondria in the absence or presence of recombinant pSu9-DHFR. The samples were incubated at 25°C for the indicated time periods. PK was added and proteins were analyzed by SDS-PAGE and autoradiography. Proteolytic fragments are indicated as described in the legend to Fig. 18B. Non-cleaved radiolabeled precursors are indicated by a circle. **D.** Insertion of Tob55 variants requires preexisting Tob55. Mitochondria isolated from either wild type or Tob55 depleted cells (Tob55↓) were incubated with radiolabeled precursors at 25°C for various time periods. Samples were treated with PK and mitochondria were re-isolated. Inserted proteins were analyzed by SDS-PAGE and autoradiography. Proteolytic fragments are indicated as described in the legend to Fig. 18B. Non-cleaved radiolabeled precursors are indicated by a circle.

3.4.2 The truncated variants of Tob55 become assembled into pre-existing TOB complexes

The assembly of Tob55 truncated variants into the pre-existing TOB complexes was monitored by BNGE. As described above (see 3.3.4), the TOB complex migrates upon BNGE as several species in which newly imported Tob55 becomes assembled (Habib et al., 2005b; Ishikawa et al., 2004a; Meisinger et al., 2004). Initially, the radiolabeled molecule of Tob55 migrate preferentially with the uppermost one (specie I) whereas upon an extended incubation period (which may allow an assembly into the pre-existing TOB complexes) the precursor molecules migrate mostly with another specie (specie II). This specie migrates at an apparent molecular mass smaller than the intermediate of Tom40 precursor associated with the TOB complex. The three truncated variants assembled into the same complex as that of the full-length protein (Fig. 20A). Moreover, the resistance of this assembly to treatment with either high salt concentrations or 2 M urea was not affected by the deletion of the N-terminal domain (not shown). Next, radiolabeled Tob55 variants were incubated with mitochondria isolated from either wild type or a strain containing HA-tagged version of Tob38 ($Tob38_{HA}$). The TOB complex from the latter mitochondria migrates slower in BNGE than in the native complex (see 3.3.4). The radioactive Tob55 species imported into the $Tob38_{HA}$ mitochondria showed the same reduced electrophoretic mobility (Fig. 20B) and were in a complex which was recognized by antibodies against the HA tag (Fig. 20C). This supports the notion that all studied Tob55 variants become assembled into preexisting TOB complexes.

To obtain additional independent support for this conclusion antibody shift experiments were performed (Paschen et al., 2003; Wiedemann et al., 2003). Antibodies raised against a peptide comprising amino acid residues 1-15 of Tob55, which do not recognize the two truncated variants, Tob55Δ80 and Tob55Δ102 were used. Mitochondria with inserted radiolabeled Tob55 variants were lysed, incubated with antibodies against the N-terminal peptide of Tob55 (or against Tim23 as control) and then analyzed by BNGE. The addition of the anti-Tob55-N antibody (but not of the control antibody) resulted in a shift of the upper precursor-containing band to a higher apparent molecular mass by formation of a supercomplex with the antibodies (Fig. 20D). Taken together, the absence of the N-terminal domain of the Tob55 precursor did not impair its ability of Tob55 precursor to become inserted into the outer membrane and assembled into preexisting TOB complexes.

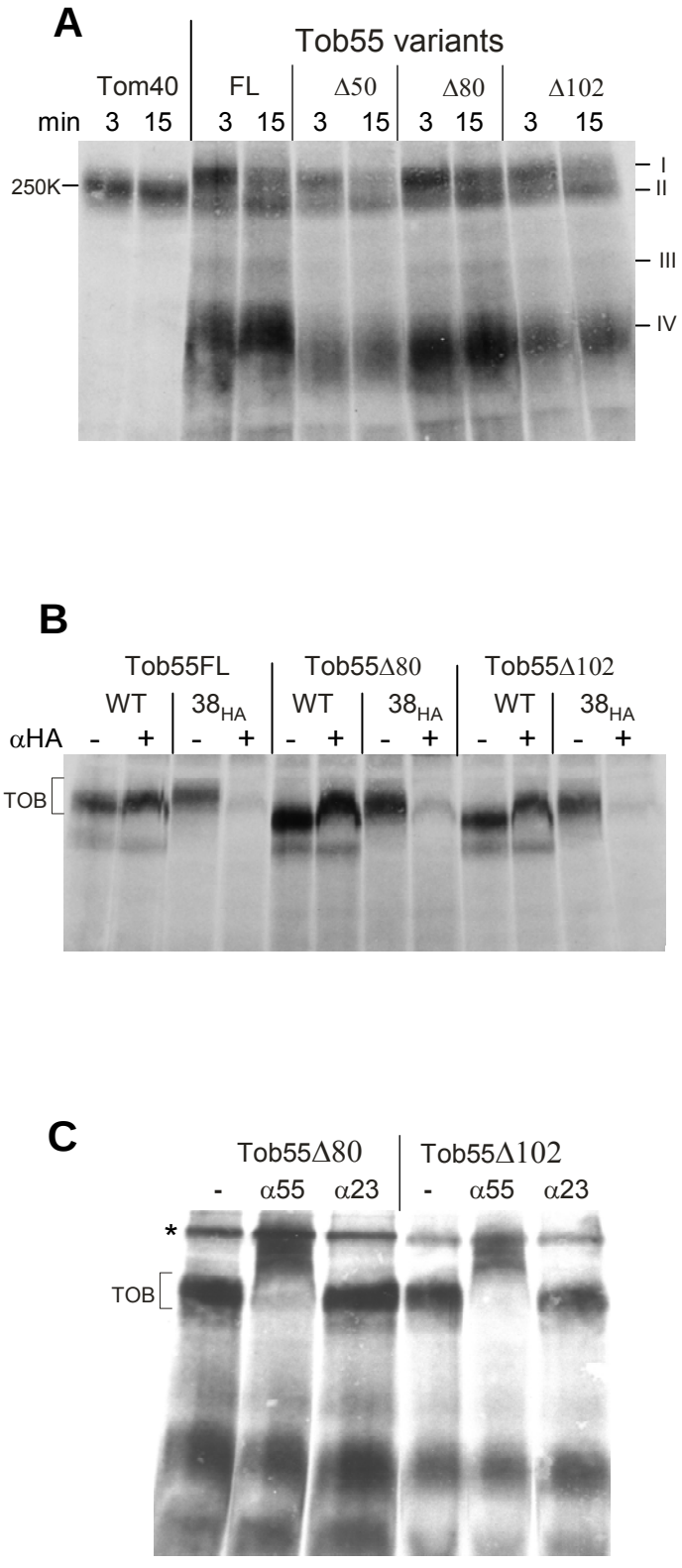

Figure 20. Tob55 variants are assembled into preexisting TOB complexes. A. Radiolabeled precursors of Tob55 variants were incubated at 25°C with wild type mitochondria for the indicated time periods. Mitochondria were re-isolated and analyzed by BNGE followed by autoradiography. Species containing the radiolabeled Tob55 are indicated as in Habib et al., 2005 (I, II, III and IV). **B.** Tob38 interacts with precursors of Tob55 variants. Wild type mitochondria (WT) and mitochondria from cells expressing an HA-tagged form of Tob38 (38HA) were incubated with radiolabeled Tob55 precursors for 3 min at 25°C. Mitochondria were re-isolated, resuspended in a buffer containing 1% digitonin and divided into two halves. One aliquot was analyzed directly by BNGE while the other was incubated with antibodies against the HA-tag before such an analysis. The gel was further analyzed by autoradiography. The TOB complex is indicated. **C.** Tob55 variants interact with preexisting Tob55. Radiolabeled Tob55 variants were incubated with isolated mitochondria for 3 min at 25°C. Mitochondria were re-isolated, resuspended in a buffer containing 1% digitonin and divided into three aliquots. One aliquot was analyzed directly by BNGE while the other two were incubated before analysis with antibodies against either an N-terminal peptide of Tob55 (amino acid residues 1-15 which are missing in Tob55Δ80 and Tob55Δ102) or Tim23. The TOB complex is indicated. An unspecific band which is often observed in BNGE is indicated with an asterisk (Habib et al., 2005; Paschen et al., 2003).

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

Does the absence of the N-terminal domain of Tob55 hinder the function of the protein? In order to test the ability of the truncated variants to complement the function of the endogenous molecules, the "plasmid shuffling" method was applied. Accordingly, an *URA3* plasmid expressing full-length Tob55 was transformed into a wild type haploid strain. Next, the genomic *TOB55* open reading frame in this strain was deleted resulting in a strain where Tob55 is plasmid-encoded (strain YSH1). This strain was then transformed with *TRP1* plasmid expressing the full-length or truncated versions of Tob55, and cells containing both plasmids were selected. Cells harboring both plasmids were grown on plates containing 5-FOA to eliminate the wild type copy of Tob55 which is encoded on the URA3-containing plasmid. Those strains that harbored a *TRP1* plasmid encoding either a full length or truncated form of Tob55 were tested for their ability to grow on glycerol- and glucose-containing medium at various temperatures (Fig. 21A). 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°C 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 non-fermentable carbon source where yeast cells are dependent on mitochondria for energy production (Fig. 21A). Thus, already the first 80 amino acid residues of Tob55 are required for optimal function of Tob55 and subsequently for normal growth of yeast cells.

3.4.4 Deletion of the N-terminal domain of Tob55 results in impaired biogenesis of β-barrel proteins

Due to the growth phenotype caused by the variants of Tob55, the biogenesis of β-barrel proteins was investigated. Mitochondria were isolated from cells harboring plasmid-encoded full-length Tob55 or its truncated variants and the amounts of expressed proteins were controlled by immunodecoration. The levels of the β-barrel proteins Tom40, Mdm10 and porin were strongly 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 to mitochondria containing full-length Tob55 as analyzed by either SDS-PAGE or BNGE (Fig. 21B and 21C). In contrast, other proteins

of the various mitochondrial sub-compartments like Fis1 and Tom70 (OM), Tim70 and AAC (IM) and Yah1 (matrix) were present at roughly control levels (Fig. 21B). Thus, the N-terminal domain of Tob55 appears to have an important role in the biogenesis of β-barrel proteins.

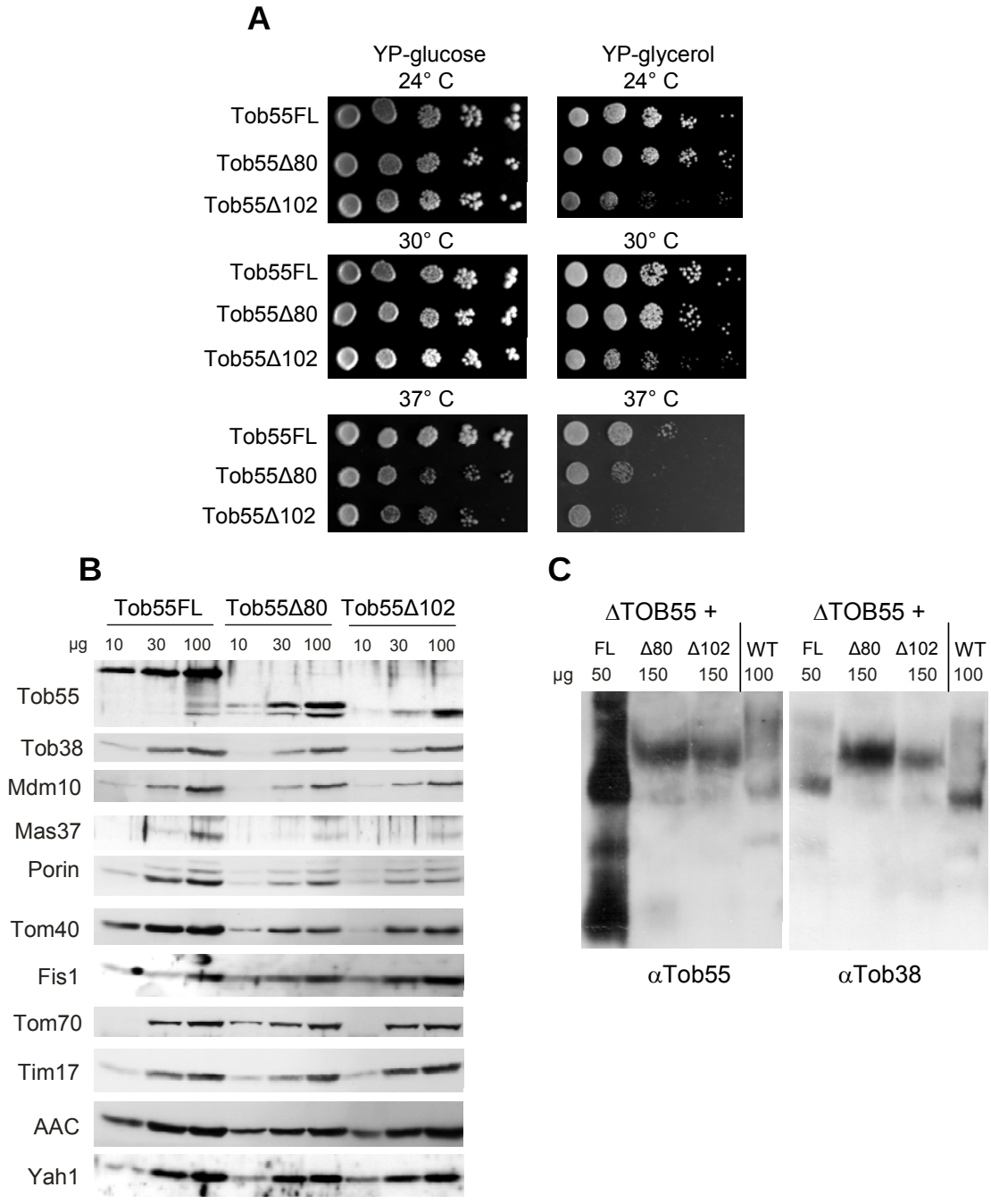

Figure 21. The N-terminal domain of Tob55 is required for optimal growth. **A.** Cells harboring plasmid-encoded Tob55 variants were constructed as described in Materials and Methods. The cells were tested by dilution in 10-fold increments for their ability to grow at the indicated temperature on mediums containing either glycerol or glucose. **B-C.** Mitochondria were isolated from cells harboring plasmid-encoded full-length Tob55 (Tob55FL) or its truncated variants. The indicated amounts of mitochondria were analyzed by SDS-PAGE or BNGE and immunodecoration with antibodies against the indicated mitochondrial proteins.

The assembly state of the TOB complex in the various mitochondria which harbor the truncated version of Tob55 was analyzed by BNGE, a method which usually results in several observed species of TOB complex. Due to the fact that mitochondria harboring the truncated versions of Tob55 contain reduced amounts of this protein, larger amount of these mitochondria were analyzed. The TOB complex from mitochondria harboring the truncated versions migrated mainly as the higher molecular specie of the TOB complex. Of note, all the Tob38 molecules in these mitochondria were assembled with Tob55 (Fig. 21C), excluding the possibility that due to the reduced levels of Tob55, Tob38 and Mas37 build partial complexes which exert a dominant negative effect. The full length Tob55 displayed a different migration pattern where a significant portion of it was found as low molecular weight unassembled species (Fig. 21C). This behavior probably resulted from the fact that, like the other Tob55 variants, it was expressed from an over-expression plasmid whereas the interacting partners Tob38 and Mas37, are not over-expressed.

To emphasize the involvement of the N-terminal domain in biogenesis of β-barrel proteins, *in vitro* protein import experiments were performed. Concomitantly to the *in vivo* data, the import efficiencies of newly synthesized β-barrel precursors like Tom40 and porin were significantly reduced in mitochondria containing the truncated versions (Fig. 22A). Other precursor proteins such as the inner membrane protein Tim23, and the matrix-destined pSu9-DHFR were only moderately affected (Fig. 22A). This reduction is probably due to the reduced level of the major subunit of the general import pore, Tom40, in the mitochondria harboring the truncated variants. As mentioned above (see 3.3.5), the assembly of newly synthesized Tob55 and the association of the Mas37 precursor with mitochondria require preexisting Tob55 molecules. To check the involvement of the N-terminal domain in the biogenesis of both proteins, *in vitro* proteins import were performed. As expected, due to the reduced levels of Tob55 a moderate reduction in the association of Mas37 was observed upon import into mitochondria with truncated variants of Tob55 (Fig. 22B). A stronger reduction was observed upon import of Tob55 precursor, a β-barrel protein itself (Fig. 22B). Thus, whereas the association of Mas37 with mitochondria is probably reduced due to 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 impaired biogenesis of β-barrel proteins could be due to the reduced level of the endogenous truncated Tob55 variants in comparison to the full length, *in vitro* control import experiments were performed. Radiolabeled

precursor proteins were incubated with either 50 µg of mitochondria harboring plasmid-encoded full length Tob55, 150 µg of mitochondria harboring Tob55Δ80 or with 100 µg wild type (YPH499) mitochondria. Under these conditions equivalent amounts of TOB and TOM complexes were present in import reactions with the two former types of mitochondria (Fig. 22C). The matrix-destined protein, pSu9-DHFR, was imported with similar efficiency in all reactions under these conditions. In contrast, the import of the β-barrel precursor porin (Fig 22D) into mitochondria carrying the truncated Tob55 variant was still impaired. Of note, although the samples with wild type mitochondria contain far less Tob55 molecules than those containing Tob55Δ80, the efficiency of porin import into the former mitochondria was significantly higher (Fig. 22D). Furthermore, the assembly of the β-barrel precursors Mdm10 (Fig. 22E) and Tom40 (Fig. 22F) was strongly impaired in mitochondria harboring the truncated version of Tob55. Thus, the reduced import of β-barrel precursors into mitochondria harboring truncated Tob55 variants is caused by the impaired function of the corresponding Tob55 molecules and is not due to a reduced level of Tob55.

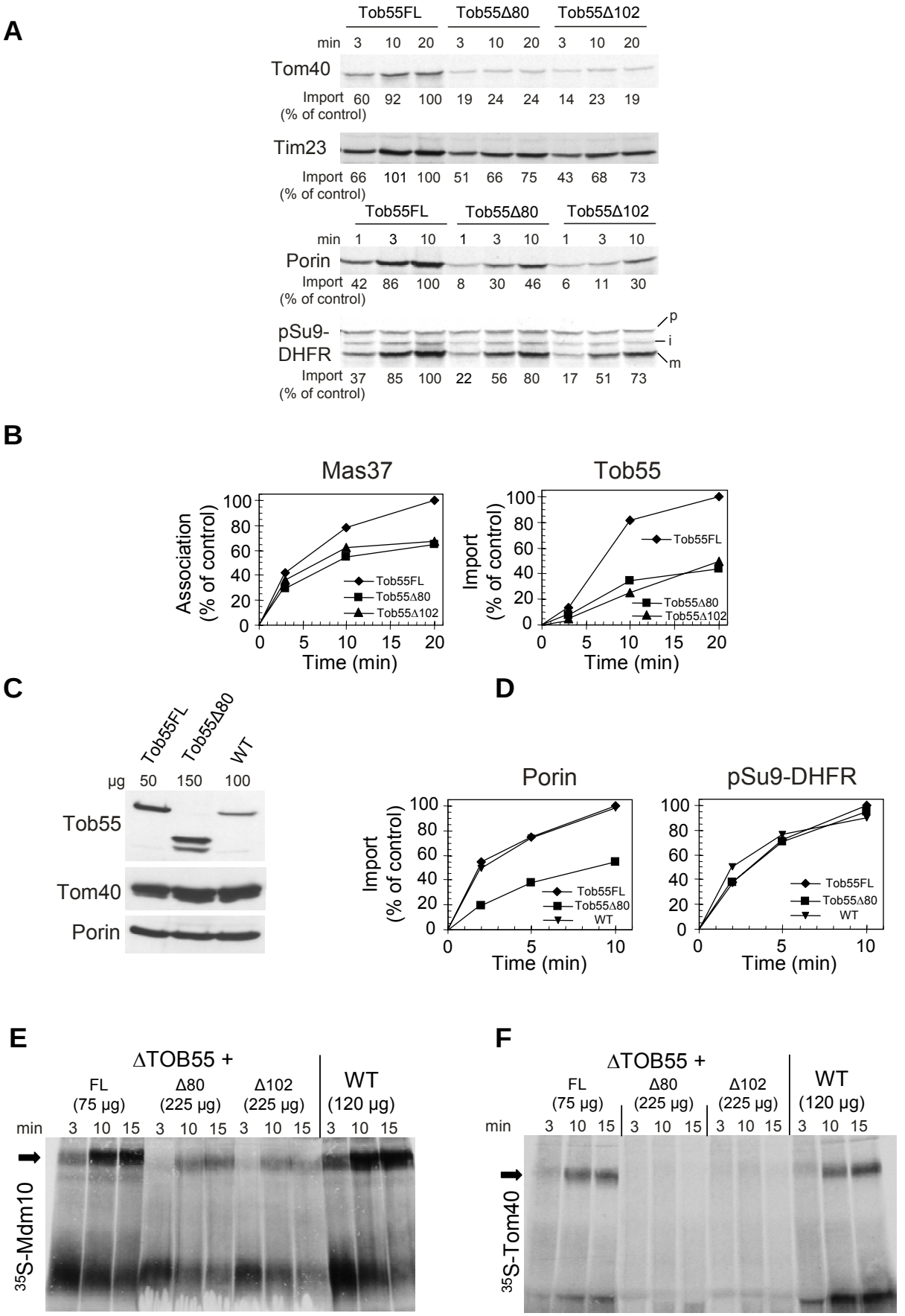

Figure 22. Mitochondria containing Tob55 truncated variants are impaired in import of β-barrel precursors. A. Mitochondria (50 μg) isolated from cells harboring plasmid-encoded full-length Tob55 (Tob55FL) or its truncated variants were isolated and incubated for 10 min at 37°C. Then the indicated radiolabeled precursor proteins were added and incubation continued at 25°C for various time periods. Samples

were treated with PK and mitochondria were re-isolated. Imported proteins were analyzed by SDS-PAGE and autoradiography. Membrane insertion of porin and Tom40 as well as translocation of the 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 mitochondria (50 µg) as in (A). The import (Tob55) and association (Mas37) of the precursor proteins were assayed by the formation of proteolytic fragments (Fig. 2 and (Habib et al., 2005b)). 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 cells harboring plasmid-encoded full-length Tob55 or Tob55Δ80 or from wild type cells. The indicated amounts of mitochondria were analyzed by SDS-PAGE and immunodecoration with antibodies against the indicated outer membrane mitochondrial proteins. **D.** Mitochondria (50 µg Tob55FL, 150 µg Tob55Δ80, and 100 µg WT) were incubated for 10 min at 37°C. Radiolabeled precursor proteins were added and incubation continued at 25°C for various time periods. The samples were then treated with PK and the mitochondria were re-isolated. Imported proteins were analyzed by SDS-PAGE and autoradiography. The insertion of porin was quantified by analyzing the protease protected precursor, whereas for pSu9-DHFR the bands corresponding to the mature protein were quantified. The amount of precursor proteins imported into mitochondria harboring plasmid-encoded Tob55FL for the longest time period was set to 100%. **E, F.** Radiolabeled precursor of Mdm10 or Tom40 (E and F, respectively) was incubated with the indicated mitochondria for various time periods. Imported proteins were analyzed by BNGE and autoradiography. The β-barrel precursor-TOB intermediate is indicated by an arrow.

3.4.5 Purified N-terminal domain of Tob55 binds β-barrel precursors

To gain insights into the function of the N-terminal domain of Tob55, its ability to bind β-barrel precursors was tested. A fusion protein consisting of the N-terminal 120 amino acid residues of Tob55 and maltose binding protein, MBP-Tob55, was expressed in and purified from *E. coli*. As controls, MBP alone and MBP-Fis1 (MBP fused to the cytosolic domain of the mitochondrial outer membrane protein, Fis1) were expressed and purified in parallel (Fig. 23A). The three fusion proteins were purified and bound to amylose resin. Next, radiolabeled precursors were added and the interactions with the recombinant proteins were analyzed. MBP-Tob55 but none of the control proteins, MBP alone and MBP-Fis1, could bind significant amounts of radiolabeled porin or Mdm10 precursors (Fig. 23B). Only background levels of a matrix destined precursor, pSu9-DHFR, were bound (Fig. 23B). To further verify that the binding to the N-terminal domain is specific and saturable, increasing amounts of radiolabeled Mdm10 precursor were added to equal small amounts of MBP-Tob55 or MBP alone as a control. In each added amount several fold more Mdm10 molecules were bound to the MBP-Tob55 as compared to the control protein. Notably, a saturation of binding was observed when large amounts of precursor were used. 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, the ability of the purified N-terminal domain to compete out the import of porin and Mdm10 was tested. To that end, radiolabeled precursors of porin or Mdm10 were incubated in a buffer in the absence or the

presence of excess amounts of the purified MBP-Tob55 or control proteins. Then, isolated mitochondria were added and import reactions were carried out. The presence of the N-terminal domain of Tob55 significantly abrogated the import of the β-barrel precursors while the control proteins (MBP and MBP-Fis1) did not have any significant effect (Fig. 23D,E). Notably, the level of inhibition depended on the amount of added recombinant MBP-Tob55 (Fig. 24A). This effect was just observed when the N-terminal domain was in a native state since pre-incubation of the latter protein with urea impaired its ability to compete out the import of porin (Fig. 24B). MBP-Tob55 did not compete out the import of other precursor proteins which are translocated through the TOM pore, such as pSu9-DHFR and Tim23 (Fig. 23D and Fig. 24B). Hence, the possibility that this inhibitory effect is entirely due to the ability of residues 1-120 of Tob55 to cross the TOM pore, and thereby jam the import channel is unlikely. Moreover, as shown above, residues 1-102 are not required for import of Tob55 through the TOM complex. Along the same line, when radiolabeled Tob55(1-120) was incubated with isolated mitochondria, it did not become protected from degradation by added proteases (data not shown). 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 out the import into mitochondria lacking either Tom20 or Tom70 (Fig. 25).

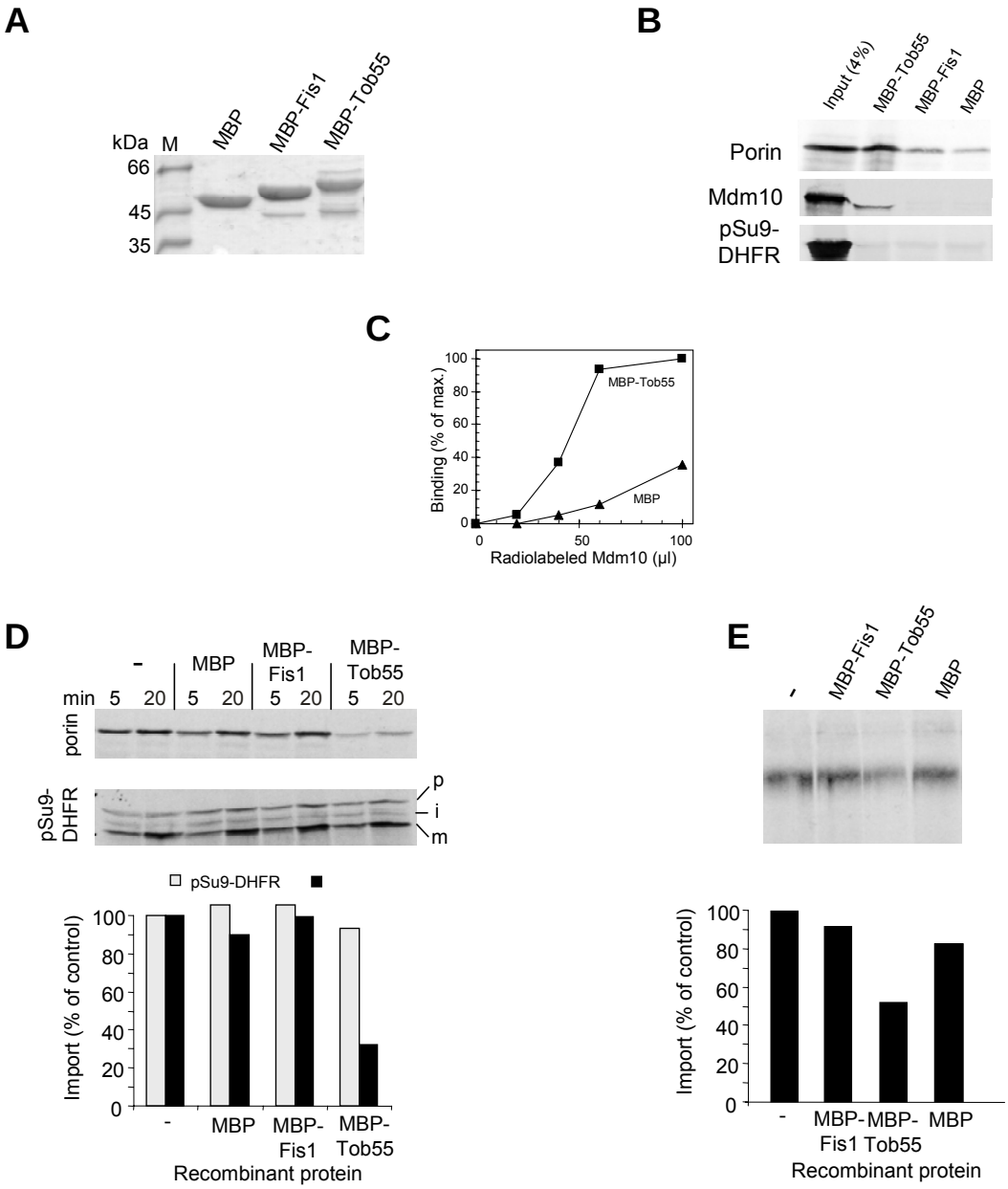

Figure 23. The N-terminal domain of Tob55 can bind β-barrel precursors *in vitro*. A. Maltose binding protein (MBP) alone or fused either to the cytosolic domain of Fis1 (MBP-Fis1) or to residues 1-120 of Tob55 (MBP-Tob55) were expressed in *E. coli* and purified. Proteins (5 µg each) were analyzed by 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. lysate were incubated with 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 another 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 re-isolated and inserted 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 for 10 min at 0°C 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 another 10 min at 25°C. The import reactions were analyzed by BNGE as in Fig. 22E and the bands representing Mdm10-TOB intermediate were quantified. The import without MBP-fusion protein was taken as 100%.

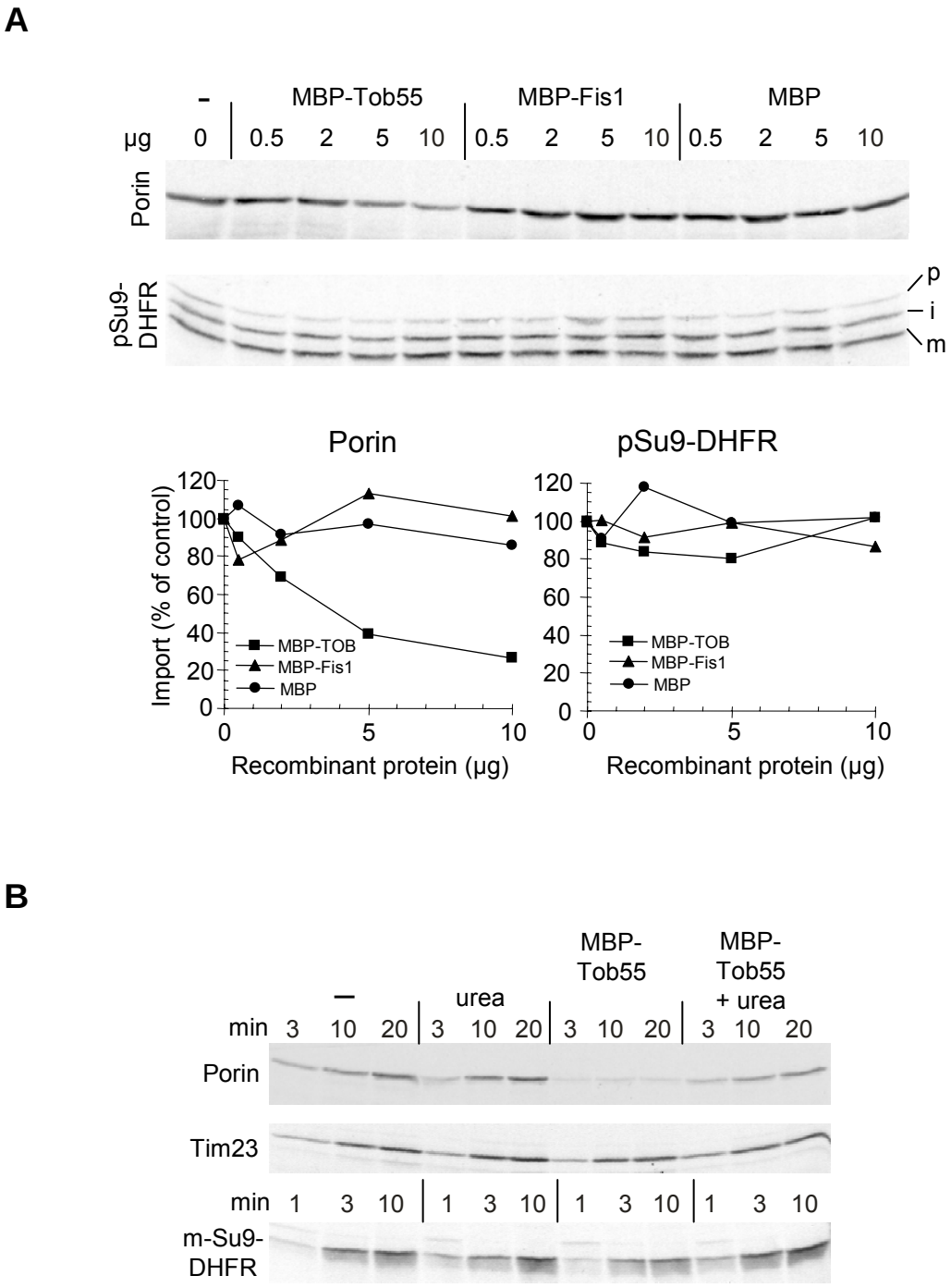

Figure 24. The inhibition by MBP-Tob55 is concentration dependent and requires folded structure. **A.** Inhibition of porin import depends on the concentration of added recombinant MBP-Tob55. Radiolabeled precursors of porin or pSu9-DHFR were incubated in import buffer in the presence or absence of the indicated amounts of purified MBP-fusion proteins for 10 min at 0°C. Mitochondria were then added and the mixture was incubated for another 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. 23D. **B.** Unfolded N-terminal domain of Tob55 cannot inhibit porin import. Mitochondria were incubated for 2 min on ice in import buffer with either native MBP-Tob55, 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 as described in the legend to Fig. 23D.

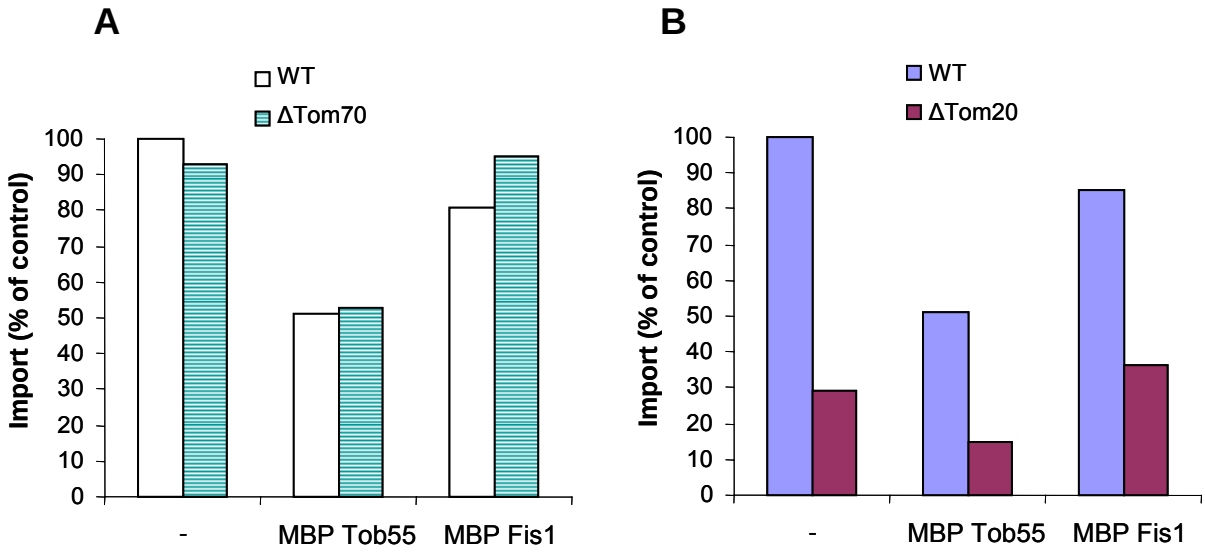

Figure 25. **The absence of Tom receptors does not affect the inhibition of porin import caused by purified N-terminal domain of Tob55**. Radiolabeled precursors of porin were incubated in import buffer in the presence or absence of the indicated purified MBP-fusion proteins for 10 min at 0°C. Mitochondria isolated from ΔTom70 (**A**) or ΔTom20 (**B**) and the corresponding wild type strains were then added and the mixture was incubated for 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 re-isolated and inserted proteins were analyzed by SDS-PAGE and autoradiography. The bands corresponding to protease protected porin were quantified. The amount of precursor protein imported into wild type mitochondria was set to 100%.

In order to further test the ability of the N-terminal domain to bind β-barrel precursors, its capacity to bind to a water-soluble form of porin was analyzed (Pfaller et al., 1985). This water-soluble porin, isolated from detergent-purified porin from *N. 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). As all the experiments described so far were conducted in the yeast *S. cerevisiae*, the ability of *N. crassa* porin to be imported into and assembled in the outer membrane of yeast mitochondria was investigated. Indeed, the *N. crassa* ortholog was imported into yeast mitochondria in the pathway that involved the general insertion pore. Blocking this pathway with excess recombinant matrix-destined preprotein, pSu9-DHFR, inhibited membrane integration (Fig. 26A) (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 abrogated in mitochondria lacking Tom20 (Fig. 26B) or harbouring reduced levels of Tob38 (Fig. 26C) (Ishikawa et al., 2004a; Krimmer et al., 2001; Milenkovic et al., 2004; Waizenegger et al., 2004). Porin is known to form several oligomeric structures which can be observed by BNGE (Gentle et al., 2004; Krimmer et al., 2001; 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. 26D). Taken together, *N. crassa* porin can use the yeast machinery for biogenesis of β-barrel proteins and follows the same pathway as its yeast ortholog.

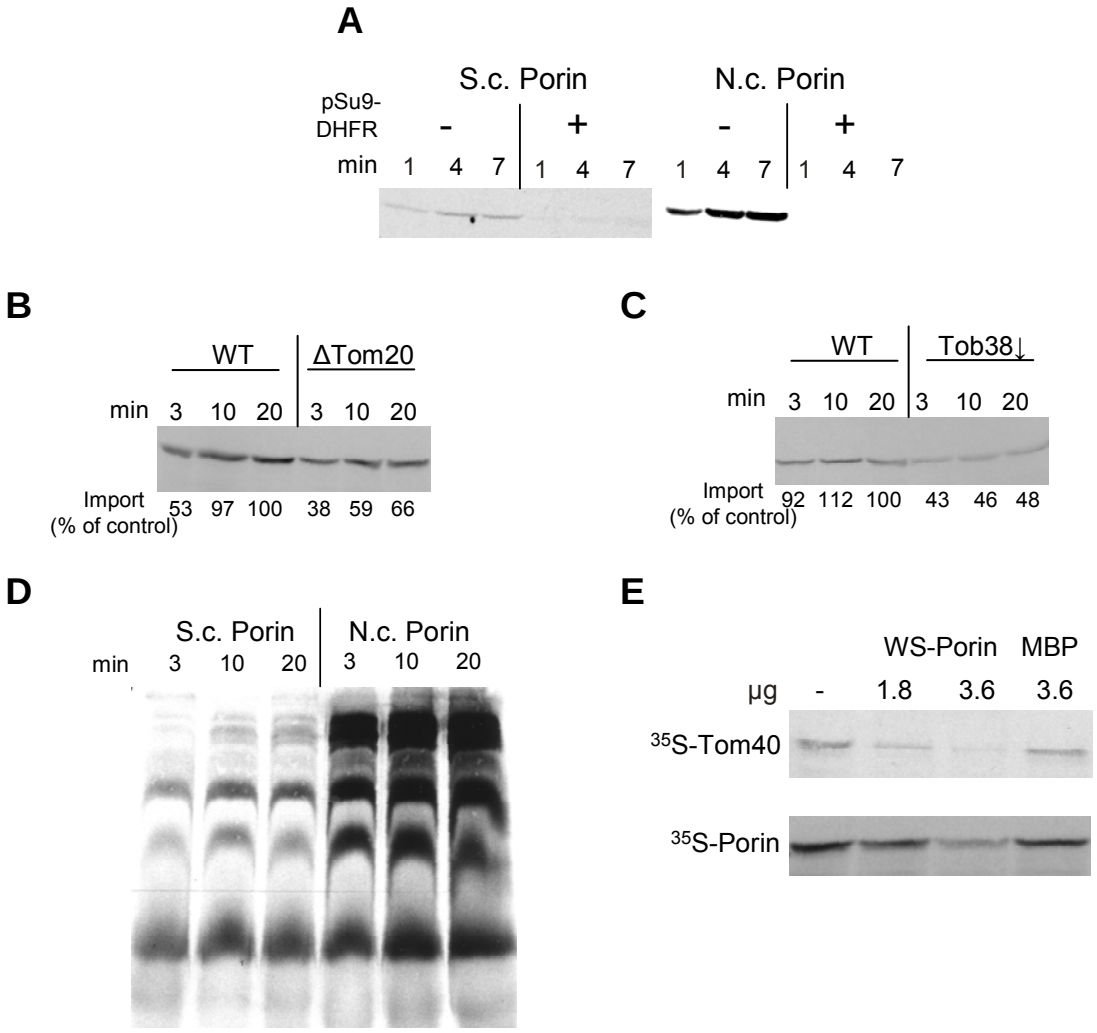

Figure 26. Precursor of *N. crassa* porin is correctly inserted *in vitro* into yeast mitochondrial outer membrane. A. Radiolabeled precursors of porin from either yeast or *N. crassa* were incubated with yeast mitochondria at 25°C for the indicated time periods in the presence or absence of excess recombinant matrix-destined precursor, pSu9-DHFR. At the end of the import reactions, PK (50 μg/ml) was added and inserted porin was analyzed by SDS-PAGE and autoradiography. **B.** Efficient insertion of *N. crassa* porin is dependent on Tom20. Radiolabeled precursors of *N. crassa* porin were incubated for the indicated time periods with mitochondria from either wild type strain or from strain lacking Tom20. Further treatment was as described in A. **C.** Efficient insertion of *N. crassa* porin is dependent on Tob38. Mitochondria isolated from either wild type or Tob55 depleted cells (Tob38↓) were incubated with radiolabeled precursors at 25°C for various time periods. Further treatment was as described in A. **D.** 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 and assembled porin was analyzed by BNGE and autoradiography. **E.** Water soluble 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 for 10 min at 0°C. Mitochondria were then added and the mixture was incubated for another 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 re-isolated and inserted proteins were analyzed by SDS-PAGE and autoradiography.

The overlay method described in Voulhoux *et al.* (Voulhoux and Tommassen, 2004) was used to evaluate the binding of water-soluble porin with the N-terminal domain of Tob55. Under semi-native gel electrophoresis, MBP-Tob55 migrated as two dominant bands (Fig. 27A). Various amounts of MBP-Tob55 and two control proteins were subjected to semi-native SDS-PAGE and then they were transferred onto a nitrocellulose membrane. Water-soluble porin was incubated with this membrane and the bound protein was detected by immunodecoration. The N-terminal domain of Tob55 was found to specifically bind water-soluble porin in a concentration-dependent manner (Fig. 27B). In conclusion, these results demonstrate that the N-terminal domain of Tob55 is able to interact with precursors of β-barrel proteins.

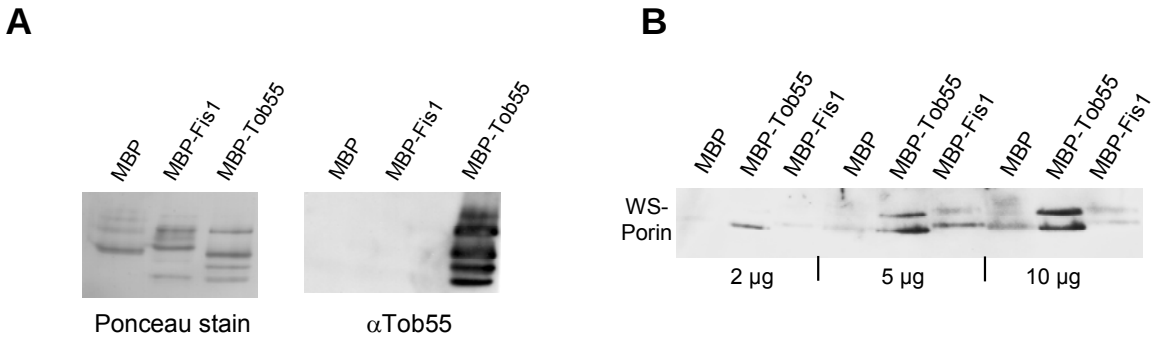

Figure 27. The N-terminal domain of Tob55 can recognize water-soluble porin. A. MBP-Tob55 migrates on semi-native gel as multiple bands. MBP-fusion proteins (10 µg each) were analyzed by semi-native SDS-PAGE and further stained with Ponceau (left panel) or immunodecorated with antibodies against Tob55 (right panel). **B.** The N-terminal domain of Tob55 recognizes water-soluble porin. The indicated amounts of MBP-fusion proteins were analyzed by semi-native gel and blotted onto nitrocellulose membrane. Water-soluble porin (ws-Porin) isolated from *N. crassa* mitochondria (11 µg/ml) was incubated with this membrane for 2 h at 4°C, the membrane was washed and immunodecorated with antibodies against porin.

To further substantiate the capacity of the N-terminal domain to bind water-soluble porin and to obtain more quantitative information on this binding, the interaction was examined in solution. To that end, water-soluble porin was radiolabeled with ^{14}C-formaldehyde by reductive methylation using cyanoborohydride ($NaBH_3CN$) as a reducing agent (Pfaller et al., 1985). Increasing amounts of ^{14}C-ws-porin were added to 2 µg of either MBP or MBP-Tob55 bound to amylose beads. The binding was performed at low temperature (4°C) and in the presence of BSA and salt. Under these conditions, the tendency of ws-porin to adhere to surfaces and thus to cause unspecific binding was reduced. The beads were washed and bound ^{14}C-ws-porin was eluted and analyzed (Fig. 28A). To quantify the binding, increasing amounts of ^{14}C -ws-porin were loaded directly on a gel to serve as loading standards. Much

higher levels of binding were observed with MBP-Tob55 than with the control protein. Saturable binding for ^{14}C-ws-porin was not obtained since, under the experimental conditions mentioned above, ^{14}C-ws-porin tends to aggregate at higher concentrations. As shown in Fig. 28A, the binding was concentration-dependent at concentrations ranging from 1 to 152 nM. After subtracting of the unspecific binding to MBP from the specific binding and analyzing the resulting data through a Scatchard plot (Fig. 28B), a binding parameter of K_d = 12 nM was obtained. Of note, this value is in the same range as the K_d of the binding of the presequence-containing precursor protein and the TOM complex (Stan et al., 2000).

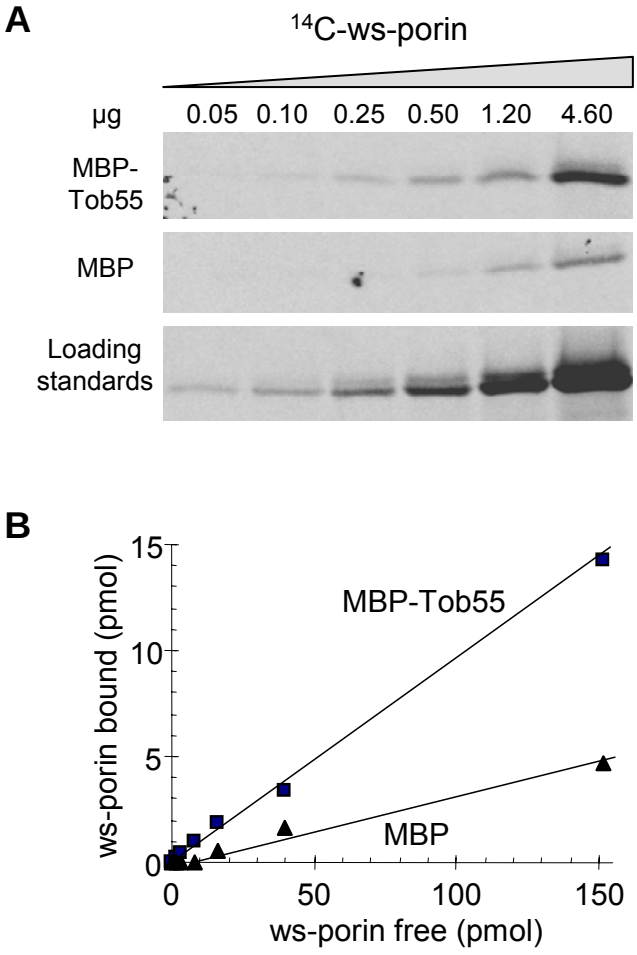

Figure. 28. Interaction of the N-terminal domain of Tob55 with water-soluble porin. A. Increasing amounts of ^{14}C-ws-porin were added to 2 µg of either MBP-Tob55 (upper panel) or MBP (middle panel) bound to amylose beads or were loaded directly on a gel (lower panel). After incubation at 4°C for 35 min the resins were washed as described in Materials and Methods and bound proteins were eluted and analyzed by SDS-PAGE and autoradiography. **B.** The bands in part 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.

3.4.6 Translocation of porin precursor across the TOM complex is required for its efficient insertion into the outer membrane

Is the translocation of β-barrel precursors through the TOM complex a prerequisite for their subsequent recognition by The TOB complex? The observations on the import of β-barrel precursors into mitochondria containing truncated Tob55 variants suggest that functional Tob55 is required for translocation of the β-barrel precursor across the outer membrane. This would in turn indicate that the passage across the TOM complex is tightly coupled to interaction with the TOB complex. To further investigate this proposal the general import pore of the TOM complex was blocked by an excess of matrix-destined precursor that reduce both membrane insertion of β-barrel proteins and the import of matrix-destined precursor proteins (Figs. 12A, 19C and (Hwang and Schatz, 1989; Krimmer et al., 2001; Rapaport and Neupert, 1999; Stan et al., 2003)). As expected, rupturing the outer membrane resulted in significant reduction in the insertion efficiency of porin precursors (Smith et al., 1994). Surprisingly, rupturing the outer membrane and blocking the TOM complex strongly impaired the membrane insertion of porin precursors (Fig. 29). This observation is different from that of matrix-destined precursors, where rupturing the outer membrane can overcome such blockage of the TOM channel (Hwang and Schatz, 1989). Accordingly, it seems that efficient recognition and translocation of β-barrel precursors by the TOB complex require a preceding translocation by the TOM complex.

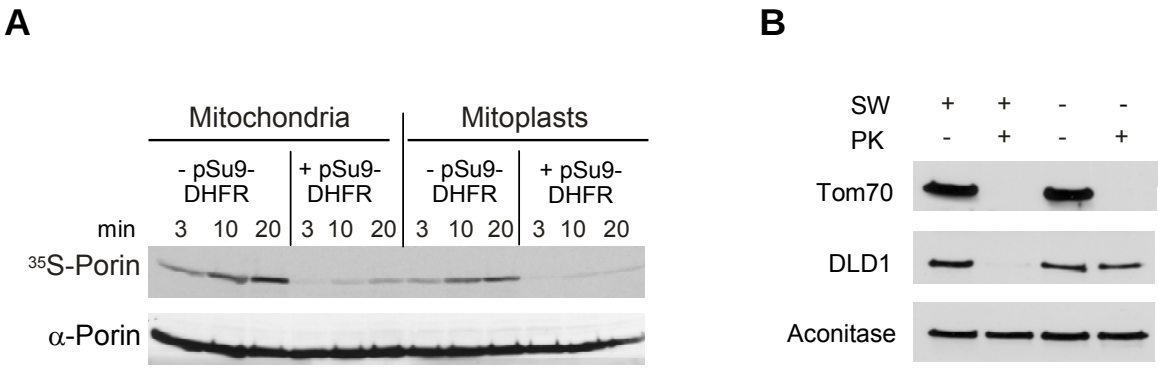

Figure 29. Translocation of porin precursor across the TOM complex is required for its efficient insertion into the outer membrane. **A.** Radiolabeled precursor of porin was incubated for the indicated time periods with isolated intact mitochondria or with mitochondria that had been subjected to osmotic 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 blotted to nitrocellulose membrane. Radiolabeled porin was detected by autoradiography whereas endogenous porin was detected by immunodecoration (α-Porin). **B.** To control the efficiency of 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 (an outer membrane protein with large cytosolic domain), DLD1 (an IMS protein) and aconitase (matrix protein).

4 Discussion

Proteins residing in the mitochondrial outer membrane fulfil a variety of essential functions for the biogenesis of the organelle and thereby for cell life. As of today, little is known about their molecular structures, topogenesis and the mechanism by which these proteins function. Here, I addressed several questions regarding the biogenesis of the mitochondrial outer membrane proteins. At the inception of this study I investigated the different roles that tail-anchored domains may play in the function of the proteins. In the second and the major part, I studied in detail the biogenesis of the TOB complex, which mediates the membrane insertion of β-barrel proteins. Several aspects pertaining to this machinery were elucidated including the identification of the essential component Tob38, and the exploration of the import routes of Tob55 and Mas37 and their assembly into the outer membrane. Furthermore, analyzing the function of the N-terminal domain of Tob55 enabled me to gain new insights into the mechanism by which the TOB complex may recognize and mediate the insertions of β-barrel proteins. In the following section I will discuss these results together with new data gained from other studies.

4.1 Multiple functions of tail-anchor domains of mitochondrial outer membrane

Tail-anchored proteins constitute a class of integral membrane proteins that are held in the phospholipid bilayer by a single stretch of hydrophobic amino acids close to the carboxy terminus, while the entire amino-terminal portion faces the cytosol. Examples of this class in the mitochondrial outer membrane include: Fis1, a protein of the mitochondrial fission machinery, Tom5, Tom6 and Tom7, components of the TOM complex. The results of my investigation suggest multiple functions of tail-anchor domains. Initially, the domain is required for targeting of the respective protein to the outer membrane of mitochondria. The importance of positive net charges in the C-terminal segment for correct mitochondrial targeting has been demonstrated before in mammalian mitochondria, but was questioned regarding targeting to yeast mitochondria (Horie et al., 2002). An example in mammalian cells for the importance of positive charges at the C-terminal segment is provided by cytochrome *b5* and VAMP-1. Both of these proteins exist in two homologous isoforms that are localized specifically either to the mitochondrial outer membrane or to the endoplasmic reticulum. In both cases it has been shown that the mitochondrial localization requires a lack of net negative charge at the C-terminal segment (Borgese et al., 2003; Isenmann et al., 1998; Kuroda et al., 1998).

In the case of Fis1, I could show that such positive charges are crucial for targeting the protein to mitochondria in yeast cells. During this part of the study Horie *et al* analyzed in detail the targeting signal of Tom5 in yeast(Horie et al., 2003). They found that correct targeting and proper assembly require the following: (1) an appropriate length of the TMD rather than hydrophobicity (2) a proline residue located at the correct position (proline 39) in the TMD and specific residues near the proline In particular, residues localizing in the C-terminal vicinity of proline 39 are mainly important for targeting efficiency, whereas those in the N-terminal vicinity are important for the functionality of the protein. They further observed that correct targeting and functional integration of Tom5 into the yeast TOM complex do not require the basic C-segment. However, and in agreement with the results in this thesis, proteins dispersed in the outer membrane (Fis1 and the yeast Tom5 expressed in mammalian system (Horie et al., 2002)) do require the basic C-segment for proper targeting.

My observation that the tail-anchor domain of Fis1 is functionally interchangeable with that of Tom5 and Tom6 is quite surprising considering the lack of sequence similarity in these regions. Although the tail-anchor domain has a crucial role in topogenic signalling, it appears to play only a limited role in the specific function of Fis1. Tom5 and Tom6 are unique in the sense that the tail-anchor domain can fulfil the function of the protein even when the cytosolic domain is replaced by an unrelated passenger domain. In the case of Tom6 this might have been expected considering the proposed function of the protein in linking membrane-embedded Tom components (Alconada et al., 1995; Dekker et al., 1998; Dembowski et al., 2001). For Tom5, however, the ability of the tail-anchor domain to largely fulfil the function of the whole protein is quite surprising. This interesting observation was also demonstrated by Horie *et al* (Horie et al., 2003). In the latter study, they constructed green fluorescence protein fusions with a C-terminal TMD in which a deduced sequence containing proline residue (minimum Ser-Por-Met) was inserted at an appropriate positions within artificial Leu-Ala repeats. These fusion proteins were not only targeted to mitochondria but also complemented the *ts* phenotype of *Δtom5* strain. The cytosolic domain of Tom5 has a net negative charge and was suggested to be part of an 'acid chain' that guides the sequential transport of positively charged mitochondrial presequences (Dietmeier et al., 1997; Schatz, 1997). As Tom5 was found to be in the vicinity of preproteins *in transit* (Dietmeier et al., 1997), it is tempting to speculate that the tail-anchor domain of Tom5 builds part of the preprotein translocation pore. A recent study has demonstrated that the

deletion of Tom5 in *N. crassa* did not exhibit any growth defects and did not result in any effect on proteins import in *Neurospora* mitochondria (Schmitt et al., 2005b). It was demonstrated that yeast Tom5, but not *N. crassa* Tom5, is required for the stability of the TOM complex. In agreement with the notion mentioned above, Schmitt *et al* also suggest a structural role rather than a receptor–like function of Tom5.

In summary, this part of the study identified four distinct functions of the tail-anchor domain. First, the domain mediates the targeting to mitochondria in a process that probably requires a net positive charge at the C-terminally flanking segment. Second, tail-anchor domains facilitate the insertion into the mitochondrial outer membrane. Third, the tail-anchor is responsible for the assembly of the respective protein into functional multi-subunit complexes; and fourth, tail-anchor domains can stabilize such complexes.

The mechanism(s) by which tail anchored proteins are recognized at the surface of mitochondria and sequentially are inserted into the outer membrane is poorly understood. The main reason for this is the difficulty in distinguishing between non specific binding of precursor protein (due to the polar residues down stream the TMD) and proper integration into the membrane in an *in vitro* import assay (Borgese et al., 2003; Rapaport, 2003). This problem might be the source of conflicting data regarding the involvement of different elements, like the receptors, in the import pathway of tail-anchored proteins. For example, while the targeting of Bcl-2 and Cyt*b5* has been suggested to be independent of trypsin-sensitive components of the recipient membranes (Janiak et al., 1994), the membrane targeting of VAMP1B to mitochondria was found to be dependent on surface receptors (Lan et al., 2000). The import of the Bcl-2 precursor into yeast mitochondria was also investigated. Motz *et al* showed that in comparison with wild type organelles, the import of Bcl-2 precursor was reduced in mitochondria lacking Tom20 (Motz et al., 2002). Furthermore, their study suggests that neither Tom70 nor components of the TOM core complex are involved in the import pathway of Bcl-2.

Several studies on tail anchored proteins of the TOM core complex shed the light on possible components involved in the import pathway of theses proteins. Accordingly, membrane integration of full-length Tom22 has been found to be dependent on the receptor proteins Tom20 and Tom70 (Keil and Pfanner, 1993). Similarly, the insertion of Tom6 and Tom7 into the outer membrane of isolated mitochondria was inhibited by the proteolytic removal of the cytosolic domains of surface receptors (Dembowski et al., 2001).

The challenge for the future is to establish a reliable *in vitro* import assay as a tool to generate more biochemical information. These data together with genetic approaches will provide important insights into the molecular details of the mechanism by which tail-anchored proteins are inserted into the outer membrane and assembled into functional complexes.

4.2 Tob38, a novel component of the TOB complex

Until recently, the biogenesis of β-barrel membrane proteins was an unresolved process. An important breakthrough was achieved when a novel pathway for the membrane insertion of β-barrel proteins was identified in both mitochondria and Gram-negative bacteria. The key component of these pathways are Tob55 (Sam50) in mitochondria and Omp85 in bacteria (Gentle et al., 2004; Kozjak et al., 2003; Paschen et al., 2003; Voulhoux et al., 2003). Both proteins are embedded into the outer membrane and form β-barrels of themselves. Tob55 was found to be part of the hetero-oligomeric TOB (SAM) complex. The other known component of this complex is Mas37, a protein associated to the cytosolic surface of the membrane. We have identified Tob38 (also known as Sam35 (Milenkovic et al., 2004) or Tom38 (Ishikawa et al., 2004b)) as a new component with an essential role in the biogenesis of mitochondrial β-barrel proteins (Waizenegger et al., 2004). Tob38 appears to be present at the cytoplasmic surface of the mitochondria as a peripheral membrane protein. It is tightly bound to Tob55 and the levels of Tob38 are strongly reduced in Tob55-depleted cells. Thus, Tob38 is most probably anchored to mitochondria via Tob55. In the present study I have shown that depletion of Tob38 has a sever effect on the import of β-barrel precursors into isolated mitochondria, but not on other preproteins destined to other mitochondrial sub-compartments. Together with Tob55, Tob38 forms a functional TOB core complex even in the absence of Mas37. A subcomplex of Mas37 with Tob55 on depletion of Tob38 was not detected. Hence, Tob38 is required for the stability and assembly of the TOB complex. Due to the location of Tob38 on the cytosolic surface of mitochondria several additional functions could be suggested. Tob38 may promote interactions of the TOB complex with cytosolic chaperones, helping to keep the β-barrel precursors in an import-competent conformation before their interaction with the TOM complex. Tob38 could be involved in mediating a transient association between these two complexes, thereby facilitating the transfer of precursor proteins. Furthermore, as the pore structure formed by Tob55 is probably involved in the membrane insertion of precursor proteins, Tob38

could also function as a regulator of this pore. In order to shed the light on these possibilities, detailed dissection of the import pathway of β-barrel proteins is required.

4.3 Assembly of the TOB complex

The TOB complex belongs to those protein machineries that are composed of two different types of proteins, membrane embedded and membrane associated proteins. In this part of the project I investigated how these different classes of proteins are targeted to mitochondria and assembled into functional TOB complexes. I have shown that the import pathway of Tob55 shares in principle the conserved route of other β-barrel proteins. Initially, Tob55 is recognized by the mitochondrial surface receptors Tom70 and Tom20. Then, it is translocated via the general import pore of the TOM complex and transferred to the TOB complex. The small Tim complexes (Tim8/13 and Tim9/10) seem to be involved in this process. On the other hand, the assembly pathway of Tob55 differs from the assembly pathways of other β-barrel precursors in two major aspects. First, all β-barrel precursors are substrates of Tob38, but only Tob55 is an interacting partner of Tob38 in the functional TOB complex. This difference is reflected by the requirement of Tob38 for the stability of assembled Tob55. Although Tob38 is a peripheral membrane protein, its extraction destabilizes the structure of the membrane-embedded Tob55. A second difference in the assembly pathway of Tob55 in comparison with other β-barrel precursors is the role played by Mas37. Although Mas37 was reported to have an important role in the assembly of Tom40 and porin (Wiedemann et al., 2003), the deletion of Mas37 had only a moderate effect on the insertion of Tob55 into the outer membrane. These results are consistent with the previous observations that Tob55 and Tob38 form the TOB core complex in the absence of Mas37 and that the levels of expressed Tob55 are not affected by the deletion of Mas37 (Waizenegger et al., 2004).

The different roles that Mas37 plays in the topogenesis of various β-barrel proteins may point to a function downstream of Tob55-Tob38 core complex. All β-barrel precursors may interact first with Tob55-Tob38 before being transferred further to other elements of the outer membrane in a process that requires Mas37. Since the precursor of Tob55 does not have to leave the TOB complex, it may not require Mas37 for its assembly. Recent findings on the role of Mdm10 in the assembly of the Tom40 precursor support this proposal (Meisinger et al., 2004). It was suggested that Mdm10 is involved in the assembly pathway of Tom40 precursor in a step downstream of the TOB complex.

The reported interaction of Mdm10 with Mas37 is compatible with a role of Mas37 in mediating the release of β-barrel precursors from the TOB complex.

Up to date, the TOM complex was shown to be involved in the import of all mitochondrial proteins analyzed so far. On the other hand, the import pathway of proteins with similar topology to Mas37, namely membrane associated proteins, was not investigated. Despite the previous report on a possible interaction between Mas37 and the receptor Tom70 (Gratzer et al., 1995), the data provided here suggests that TOM complex is dispensable for the association of Mas37 with mitochondria. The data propose that Mas37 follows a unique association pathway, where it is directly recognized by and assembled into the TOB core complex. Notably, the levels of Mas37 in mitochondria depleted of either Tob38 or Tob55 were clearly reduced in comparison with those in mitochondria from wild-type cells (Ishikawa et al., 2004b; Waizenegger et al., 2004). The observed higher efficiencies of association of Mas37 precursor with mitochondria lacking endogenous Mas37 molecules are consistent with such a pathway. In the wild type organelles, the vast majority of the TOB core complex is in association with Mas37. In such a situation, a docking site for newly synthesized Mas37 is occupied by the preexisting molecules of Mas37. In the absence of endogenous Mas37, this site is free and more newly synthesized Mas37 can dock on and assemble with the TOB core complex. Further studies will have to analyze the targeting information within Mas37 and the way this information is decoded by the mitochondrial outer membrane.

Due to a lack of proper *in vitro* import assay, the import pathway and the assembly of Tob38 were not investigated. Notably, upon depletion of Tob55 form yeast cells, no accumulation of Tob38 was observed in the cytosolic fraction (data not shown). This observation might suggest that due to a lack of docking site on the outer membrane of mitochondria, Tob38 most likely was degraded. Since Tob38 is also peripherally attached to the TOB complex, it is assumable that Tob38 associates with the outer membrane in a similar pathway to Mas37.

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

Bacterial proteins from the family of Omp85/YaeT and eukaryotic proteins homologues to Tob55 share a putative functional domain in their N-terminal part which was named POTRA (for polypeptide-transport-associated domain) (Sanchez-Pulido et al., 2003b). This domain has been suggested to be involved in interaction with precursor proteins although experimental evidence for interaction of this domain with β-barrel precursors is still missing. According to the prediction program (http://www.pdg.cnb.uam.es/POTRA/cgCon.msf) the POTRA domain within Tob55 from yeast covers amino acid residues 29-108. This proposal is very appealing because most of the N-terminal domain of Tob55 is predicted not to be part of the β-barrel structure (http://www.imtech.res.in/cgibin/betatpred2/) and resides in the IMS (Paschen et al., 2003). Thus, the N-terminal domain is ideally positioned to interact with β-barrel precursors on their way between the TOM and the TOB complexes. This thesis presents evidence that the N-terminal domain of Tob55 is indeed involved in recognition of β-barrel precursors and thus in the transfer of precursors to the TOB complex. Notably, chloroplast Toc75, another protein belonging to the β-barrel-type pores, also has a POTRA-like region at its N-terminus. It was reported that the N-terminal domain of Toc75 functions as a unit for recognition and complex assembly (Ertel et al., 2005). In contrast to Tob55, Toc75 is involved in the translocation of precursor proteins with chloroplast targeting signals across the outer membrane. It seems currently unclear whether this protein is also involved in the insertion of β-barrel precursors into the chloroplast outer membrane.

The reduced growth of cells harboring Tob55 that lacks the N-terminal domain underscores the functional importance of this part of the protein. This domain seems to be required neither for targeting to mitochondria nor for assembly of the protein into TOB complexes. However, it might be that the N-terminal domain of Tob55 is also involved in the stability and/or the integrity of the TOB complex. Tob55 interacts with the other two components, Mas37 and Tob38, which are attached to the cytosolic side of the outer membrane (Waizenegger et al., 2004; Wiedemann et al., 2003). Due to the location of the N-terminal domain of Tob55 in the intermebrane space, an interaction with Mas37 and/or Tob38 seems to be unlikely.

Which information within the sequence of mitochondrial β-barrel preproteins is required for the recognition by the N-terminal domain of Tob55? The observation made

here with Tob55 and other reported results regarding porin and Tom40 (Court et al., 1996; Rapaport et al., 2001) demonstrate that such an information is not found in the N-terminal domain of these precursors. Currently, there are six putative β-barrel proteins in yeast mitochondria: 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 overall similar general structure these proteins present extensive divergence of their primary sequences. Hence, a linear consensus sequence as a recognition and/or sorting signal is unlikely.

The biogenesis of β-barrel proteins in gram-negative bacteria which are evolutionarily related to the mitochondrial system provides some possibilities for features within substrate sequence that can be used for selective substrate recognition. It was reported that the conserved C-terminal phenylalanine residue in PhoE of *E. coli* is (part of) a sorting signal, directing the protein efficiently to the outer membrane (de Cock et al., 1997; Jansen et al., 2000). However, it is currently not clear whether this signal plays a role in the insertion pathway mediated by Omp85/YaeT. Furthermore, it is unclear whether similar features are used in the mitochondrial system, while most of mitochondrial β-barrel proteins do not have Phe as their most C-terminal residues.

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.

One of the key proteins involved in the biogenesis of β-barrel proteins in bacteria is SurA. SurA is a perplasmic chaperone that interacts with non native periplasmic porins-folding intermediates and assists in their maturation from early to late outer membrane-associated steps. Recent peptide libraries screen of β-barrel substrate proteins of SurA has

revealed two features which are critical for peptide binding by this protein: specific patterns of aromatic residues and the orientation of their side chains (Hennecke et al., 2005). Homologues of SurA in yeast were not identified yet and the small TIM complexes which were suggested to be involved in biogenesis of β-barrel proteins were not required for the observed interaction in our *in vitro* assays. Although the small Tims might help to keep the β-barrel precursors in an insertion competent conformation, they are not crucial for recognition by Tob55. Future screens with peptides related to mitochondrial β-barrel proteins might shed light on the recognized pattern within these substrates.

The crucial function of the N-terminal domain of Tob55, and its ability to recognize β-barrel precursors raise interesting questions for future studies, such as: what is the molecular structure of the POTRA-like domain and which signals in the β-barrel precursors are recognized by this domain? Does the N-terminal domain of Tob55 play a role in coupling the precursors to TOB complex? Furthermore, it will be very important to understand how the precursor is passed on to the membrane, after being recognized by the intermembrane space domain, to be folded and integrated into the outer membrane. The answer for such questions will provide exciting insights into the molecular mechanism by which Tob55 and TOB complex mediate the insertion of β-barrel precursors.

At present, one can only speculate how Tob55 might function. Tob55 is predicted to be 12-stranded β-barrel with an N-terminal extension in the IMS that is suggested to have a chaperone-like qualities, namely the POTRA domain. The Tob55-POTRA domain is involved in recognition and in binding the unfolded β-barrel precursors merged from the TOM complex, whereupon folding could take place with the precursor still being accessible to different folding catalysts in the IMS. Electron microscopy studies of purified Tob55 complexes revealed the existence of channels with an inner diameter of 7 nm that should be large enough to accommodate a β-barrel of 16–22 β-strands. Hence, one possibility could be that the insertion of newly synthesized precursor into the pore and lateral release into the complex. However, such a mechanism would require major structural rearrangements of the β-barrel and disruption of many hydrogen bonds (Gabriel et al., 2001). The more likely mechanism suggests that the Tob55 molecules serve as a scaffold for the β-barrel precursors. In such a mechanism, assembly can follow a concerted partitioning of the bulky β-barrel into the membrane.

After the release of β-barrel precursors into the membrane, they adopt the proper folding and assemble into relevant complexes. Further components in the outer membrane might be involved in this process. For instance, it was recently demonstrated that the outer membrane protein, Mim1 (also named Tom13) is involved, in a step after the TOB complex, in the assembly of Tom40 (Ishikawa et al., 2004b; Waizenegger et al., 2005). Further studies will be required to investigate in detail the final folding and assembly of β-barrel proteins into the membrane and to identify the elements that assist theses processes. Studies on the bacterial OMP insertion machineries will also, by comparison, contribute to understanding the mitochondrial machineries. Theses studies will also provide new insights into the evolutionary process that have occurred since mitochondria developed from prokaryotic endosymbiont.

5 Summary

The mitochondrial outer membrane harbors different sub-classes of proteins that mediate numerous interactions between the mitochondrial metabolic and genetic system and the rest of the eukaryotic cell. Two classes of these proteins were investigated in this thesis. The first class, tail-anchored proteins, exposes a large domain to the cytosol and is anchored to the membrane by a single hydrophobic segment close to the C-terminus. This segment is usually flanked by positively–charged amino acids residues. In the first part of my study I could identify that the tail anchor domain fulfills four distinct functions: First, the tail anchor domain mediates the targeting to mitochondria in a process that probably requires the positive charges down stream the transmembrane segment. Second, the domain facilitates the insertion into the outer membrane. Third, the tail anchor domain is required for assembly of the proteins into the relevant complexes. Finally, it can stabilize such complexes.

In the second part of my study I investigated the biogenesis of β-barrel proteins. These membrane proteins are unique for the outer membrane of mitochondria, chloroplast and gram-negative bacteria. Recently a complex which mediates the biogenesis of β-barrel proteins was identified and termed TOB (SAM) complex. At the beginning of this work, the TOB complex was known to consist of the peripheral associated membrane protein Mas37 and the essential membrane embedded component Tob55. Initially, we could identify Tob38 as a new component of the TOB complex. Tob38 is encoded by an essential gene and the protein associates with the TOB complex at the cytosolic side of mitochondria. It interacts with Mas37 and Tob55 and is associated with Tob55 even in the absence of Mas37. The Tob38–Tob55 core complex binds precursors of β-barrel proteins and facilitates their insertion into the outer membrane. The import of β-barrel precursors into Tob38-depleted mitochondria was demonstrated here to be dramatically reduced in comparison to wild type organelles. Notably, such an effect was not observed for other precursors of the outer membrane proteins and precursors distained to the various sub-mitochondrial compartments. Taken together, we conclude that Tob38 has a crucial function in the biogenesis of β-barrel proteins of mitochondria.

Next, the import and the assembly pathways of Mas37 and Tob55 were analyzed in detail. Reduced insertion of the Tob55 precursor in the absence of Tom20 and Tom70 argues for initial recognition of the precursor of Tob55 by the import receptors. It is then transferred through the import channel formed by Tom40. Variants of the latter protein influenced the insertion of Tob55. Assembly of newly synthesized Tob55 into pre-

existing TOB complexes, as analyzed by blue native gel electrophoresis, depended on pre-existing Tob55 and Tob38 but to a less extent on Mas37. In contrast, both the association of Mas37 precursor with mitochondria and its assembly into the TOB complex were not affected by mutation in the TOM complex. My results suggest that Mas37 assembled directly with the TOB core complex. Hence, the biogenesis of Mas37 represents a novel import pathway of mitochondrial proteins.

Finally, the structure function relationships of Tob55 were investigated by combination of biochemical and genetic approaches. Tob55 exposes an N-terminal hydrophilic domain into the intermembrane space and is anchored in the outer membrane by its C-terminal β-barrel domain. Deletion of various lengths at the N-terminal domain did not affect the targeting of Tob55 precursor to mitochondria and its insertion into the outer membrane. Replacement of wild type Tob55 by these deletion variants resulted in reduced growth of cells. Mitochondria isolated from such cells contain reduced levels of β-barrel proteins and are impaired in their capacity to import newly synthesized β-barrel precursors. Finally, purified N-terminal domain of Tob55 was found to be able to bind β-barrel precursors in a specific manner. Taken together, these results demonstrate that the N-terminal domain of Tob55 has a function in recognizing precursors of β-barrel proteins. Furthermore, the receptor-like function of the N-terminal domain of Tob55 seems to have a role in coupling the translocation of β-barrel precursors across the TOM complex to their interaction with the TOB complex.

6 Abbreviation

AAC	ADP/ATP carrier
Ac	acetate
ADH	alcohol dehydrogenase
ADP	adenosine diphosphate
amp	ampicillin
APS	ammonium peroxodisulfate
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
β-ME	β-mercaptoethanol
BSA	bovine serum albumin
BNGE	blue native gel electrophoresis
Ci	Curie
CCCP	carbonyl cyanide m-chlorphenylhydrazone
CCHL	cytochrome *c* haem lyase
CV	column volume
DDM	n-dodecyl-β-maltopyranosid
DHFR	dihydrofolate reductase
DMSO	dimethylsulfoxid
dNTP	deoxyribonucleoside triphosphate
DNA	desoxyribonucleic acid
DTT	dithiotreitol
$\Delta\psi$	membrane potential
E. coli	*Escherichia coli*
EDTA	ethylendiamine tetraacetate
ER	endoplasmatic reticulum
GIP	general import pore
HEPES	N-2 hydroxyl piperazine-N`-2-ethane sulphonic acid 99
Hsp	heat shock protein
IgG	immunoglobulin G
IM	inner membrane
IMS	intermembrane space
IP	immunoprecipitation
IPTG	isopropyl-β,D-thiogalactopyranoside
KAN	kanamycin
kDa	kilodalton
LB	Luria Bertani
MBP	maltose-binding protein
MBS	m-maleimidobenzoyl-N-hydroxysuccinimide ester
MOPS	N-morpholinopropane sulphonic acid
MPP	mitochondrial processing peptidase
MSF	mitochondrial import stimulating factor
MTS	matrix targeting signal
MW	molecular weight
N-	amino-
N. crassa	*Neurospora crassa*
NADH	nicotine amide adenine dinucleotide
Ni-NTA	nickel-nitrilotriacetic acid
ODx	optical density at x nm

OM	outer membrane
OMVs	outer membrane vesicles
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
PIS	preimmune serum
PK	Proteinase K
PMSF	phenylmethylsulfonyfluoride
PVDF	polyvinylidene difluoride
RNase	ribonuclease
RNasin	ribonuclease inhibitor
RT	room temperature
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulfate
STI	soybean trypsin inhibitor
TBS	Tris-buffered saline
TCA	trichloroacetic acid
TCEP	Tris-(2-carboxyethyl) phopshine
TEMED	N,N,N‘,N‘-tetramethylene diamine
TIM	translocase of the inner mitochondrial membrane
TOM	translocase of the outer mitochondrial membrane
Tris	tris-(hydroxymethyl)-aminomethane
TX-100	Triton X-100
Vol.	volumes
v/v	volume per volume
w/v	weight per volume
ws-porin	water soluble porin
wt	wild type

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2. Vasiljev, A., U. Ahting, F.E. Nargang, N.E. Go, **S.J. Habib**, C. Kozany, V. Panneels, I. Sinning, H. Prokisch, W. Neupert, S. Nussberger and D. Rapaport (2004) Reconstituted TOM core complex and Tim9/Tim10 complex of mitochondria are sufficient for translocation of the ADP/ATP carrier across membranes. Mol. Biol. Cell, 15, 1445-1458.

3. 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 β-barrel proteins of mitochondria. EMBO Rep. 5(7), 407-709.

4. **Habib S.J**., T. Waizenegger, M. Lech, W. Neupert and D. Rapaport (2004) Assembly of the TOB complex of mitochondria. J.Biol.Chem 280(8):6434-40

5. **Habib S.J**., W. Neupert and D. Rapaport (2006) Analysis and prediction of mitochondrial targeting signals. Methods Cell Biol. (in press)

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