Evolutionary Conservation in the Biogenesis of β-Barrel Proteins

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät

der Eberhard Karls Universität Tübingen

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

vorgelegt von

Thomas Franz Ulrich

aus Waiblingen

Tübingen

2014

Tag der mündlichen Qualifikation:	10.02.2015
Dekan:	Prof. Dr. Wolfgang Rosenstiel
1. Berichterstatter:	Prof. Dr. Doron Rapaport
2. Berichterstatter:	Prof. Dr. Ralf-Peter Jansen

Contents

1 List of abbreviations

AAA	ATPases associated with diverse cellular activities
AAC	ADP-ATP carrier
ATP	adenosine triphosphate
BAM	β-barrel assembly machinery
DHFR	dihydrofolate reductase
GFP	green fluorescent protein
HA	hemagglutinin
IMS	intermembrane space
MOM	mitochondrial outer membrane
Oep24	outer envelope protein 24
Oep37	outer envelope protein 37
OM	outer membrane
OMP	outer membrane protein
Omp85	outer membrane protein 85
P_i	inorganic phosphate
POTRA	polypeptide-transport-associated
pSu9	presequence of subunit 9 of *Neurospora crassa* F_O-ATPase
RuBisCO	ribulose-1,5-bisphosphate carboxylase/oxygenase
SAM	sorting and assembly machinery
TAA	trimeric autotransporter adhesins
TAM	translocation and assembly module
TOB	topogenesis of outer-membrane β-barrel proteins
TOC	translocase of the outer chloroplast membrane
TOM	translocase of the outer mitochondrial membrane
VDAC	voltage-dependent anion channel
WT	wild-type
YadA	*Yersinia* adhesin A

2 Summary

The vast majority of outer membrane proteins (OMPs) in Gram-negative bacteria belong to the class of membrane-embedded β-barrel proteins. Besides Gram-negative bacteria, the presence of these proteins is restricted to the outer membranes (OM) of the eukaryotic organelles mitochondria and chloroplasts. This can be seen as evidence for the endosymbiotic theory, according to which these organelles derived from the engulfment of prokaryotic ancestors into a progenitor of the eukaryotic cell. The process of organellogenesis led to a major DNA transfer of genes encoding mitochondrial proteins to the host genome. As a consequence, more than 99% of the proteins in present-day mitochondria are encoded in the nucleus and thus have to contain all the information required for specific sorting to their target compartment within mitochondria. Although progress has been made in understanding the targeting signals in numerous mitochondrial proteins, the signal that ensures targeting of β-barrel proteins still remains elusive. It is remarkable that in all membranes harboring these proteins the assembly is facilitated by dedicated protein complexes that contain a highly conserved central β-barrel protein termed BamA/YaeT/Omp85 in Gram-negative bacteria and Tob55/Sam50 in mitochondria. In this context it is astonishing that in spite of a very long divergent evolution of pro- and eukaryotes, mitochondria retained the ability to recognize and assemble bacterial β-barrel proteins. Due to this evolutionary conservation, yeast mitochondria can provide us a useful model system to study the biogenesis of β-barrel proteins from different origin.

Currently, little is known about the signal that ensures specific targeting of β-barrel proteins to either mitochondria or chloroplasts. To shed light on this topic, I investigated the targeting of the chloroplast β-barrel proteins Oep24 and Oep37 upon their expression in yeast cells. We could demonstrate their exclusive localization to the mitochondrial outer membrane (MOM). Assembly of Oep37 partially complemented the growth phenotype of yeast cells lacking the general metabolite transporter Porin. Interestingly,

both proteins followed in yeast cells a pathway similar to the one undertaken by *bona fide* mitochondrial β-barrel proteins.

In another part of my studies I investigated targeting of the trimeric autotransporter protein *Yersinia* adhesin A (YadA). Specifically, I was interested in the mechanism by which precursors of such proteins cross the periplasm and assemble in the OM. To that goal, we took advantage of the evolutionary conservation in the biogenesis of β-barrel proteins between bacteria and mitochondria. Upon expression in yeast cells, both monomeric and trimeric forms of YadA were targeted to mitochondria, but solely the trimeric form was fully assembled into the MOM. Remarkably, the co-expression of YadA with a mitochondrially-targeted form of the bacterial periplasmic chaperone Skp, but not with SurA or SecB, resulted in elevated levels of YadA.

Taken together, the results presented in this thesis demonstrate that in the course of evolution mitochondria retained the ability to assemble a wide range of β-barrel proteins. This ability allows insights into the nature of the β-barrel targeting signal.

3 Zusammenfassung

Die überwiegende Mehrzahl der Aussenmembranproteine in Gram-negativen Bakterien gehört zur Klasse der membranständigen β-barrel Proteine. Das Vorkommen dieser Proteine ist neben Gram-negativen Bakterien auf die Aussenmembranen der eukaryotischen Organellen Mitochondrien und Chloroplasten beschränkt. Dies kann als Beleg für die Endosymbiontentheorie betrachtet werden, gemäß welcher diese Organellen von der Aufnahme prokaryotischer Vorfahren in einen Vorläufer der eukaryotischen Zelle abstammen. Der Verlauf der Organellenevolution führte zu einem bedeutenden DNA-Transfer von Genen, die für mitochondriale Proteine kodieren, in das Wirtsgenom. Als Folge dessen sind in heutigen Mitochondrien mehr als 99% der Proteine im Kern kodiert und müssen alle Informationen enthalten, die für einen spezifischen Transport zum mitochondrialen Zielkompartiment benötigt werden. Obwohl Fortschritte im Verständnis der Transportsignale zahlreicher mitochondrialer Proteine gemacht werden konnten, ist das Signal, das den Transport und die Erkennung von β-barrel Proteinen sicherstellt noch immer unbekannt. Es ist bemerkenswert, dass die Assemblierung dieser Proteine in allen Membranen von speziell dafür vorgesehenen Proteinkomplexen ermöglicht wird. Diese Komplexe enthalten ein zentrales, hochkonserviertes β-barrel Protein, das in Gram-negativen Bakterien als BamA/YaeT/Omp85 und in Mitochondrien als Tob55/Sam50 bezeichnet wird. In diesem Zusammenhang ist es erstaunlich, dass Mitochondrien trotz der sehr langen divergenten Evolution von Pro- und Eukaryoten, die Fähigkeit bewahrt haben, bakterielle β-barrel Proteine zu Erkennen und zu Assemblieren. Aufgrund dieser evolutionären Konservierung, liefern uns Mitochondrien ein nützliches Modellsystem zur Untersuchung der Biogenese von β-barrel Proteinen unterschiedlichen Ursprungs.

Bislang ist wenig über das Erkennungssignal bekannt, das den Transport von β-barrel Proteinen zu Mitochondrien oder Chloroplasten sicherstellt. Um Erkenntnisse über dieses zu gewinnen, habe ich den Transport und die Erkennung der plastidären β-barrel Proteine Oep24 und Oep37 in Hefezellen untersucht. Wir konnten zeigen, dass diese

ausschließlich in die mitochondriale Aussenmembran eingebaut wurden. Die Insertion von Oep37 konnte den Wachstumsphänotyp von Hefezellen, denen der Metabolittransporter Porin fehlt, teilweise komplementieren. Interessanterweise folgten beide Proteine in Hefezellen einem Biogeneseweg, der dem endogener mitochondrialer β-barrel Proteine ähnlich war.

In einem anderen Teil meiner Arbeit untersuchte ich die Erkennung und den Transport des trimeren Autotransporterproteins *Yersinia* adhesin A (YadA). Dabei interessierte ich mich im Speziellen für den Mechanismus durch den Vorläufer dieser Proteine das Periplasma durchqueren und sich in die Aussenmembran assemblieren. Zu diesem Zweck haben wir uns die evolutionäre Konservierung in der Biogenese von β-barrel Proteinen zwischen Bakterien und Mitochondrien zu Nutze gemacht. Nach der Expression in Hefezellen wurden sowohl monomeres als auch trimeres YadA in Mitochondrien detektiert, wobei nur die trimere Form komplett in die mitochondriale Aussenmembrane eingebaut wurde. Bemerkenswerterweise führte die gemeinsame Expression von YadA und einer im Intermembranraum lokalisierten Version des baktierellen periplasmatischen Chaperones Skp zu erhöhten Mengen beider Formen von YadA.

Zusammengenommen zeigen die Ergebnisse dieser Dissertation, dass Mitochondrien im Laufe der Evolution die Fähigkeit erhalten haben, eine Vielzahl von β-barrel Proteinen zu assemblieren. Diese Fähigkeit erlaubt Einblicke in die Beschaffenheit des Erkennungssignals in β-barrel Proteinen.

4 List of publications contained in this thesis

1. Ulrich, T.*, L.E. Gross*, M.S. Sommer, E. Schleiff and D. Rapaport. 2012. Chloroplast β-barrel proteins are assembled into the mitochondrial outer membrane in a process that depends on the TOM and TOB complexes. *J. Biol. Chem.* 287:27467-27479.

   * both authors contributed equally to this work.

2. Ulrich, T., P. Oberhettinger, M. Schütz, K. Holzer, A.S. Ramms, D. Linke, I.B. Autenrieth and D. Rapaport. 2014. Evolutionary conservation in biogenesis of β-barrel proteins allows mitochondria to assemble a functional bacterial trimeric autotransporter protein. *J. Biol. Chem.* 289:29457-29470.

3. Ulrich, T., P. Oberhettinger, I.B. Autenrieth and D. Rapaport. 2015. Yeast mitochondria as a model system to study the biogenesis of bacterial β-barrel proteins. *Methods in Molecular Biology – The BAM complex: Methods and protocols*. Buchanan S. and Noinaj N., editors. Humana Press. Accepted for publication.

4. Ulrich, T. and D. Rapaport. 2015. Biogenesis of β-barrel proteins in evolutionary context. *Intl. J. Med. Microbiol.* Accepted for publication.

5 Personal contribution to the publications contained in this thesis

1. Ulrich, T., L.E. Gross, M.S. Sommer, E. Schleiff and D. Rapaport. 2012. Chloroplast β-barrel proteins are assembled into the mitochondrial outer membrane in a process that depends on the TOM and TOB complexes. *J. Biol. Chem.* 287:27467-27479.

I performed the subcellular fractionations of yeast cells expressing Oep37 (Fig. 5A) and executed carbonate extractions as well as proteinase protection assays with different proteases (Fig. 5B and Fig 5C). Moreover, I tested the ability of Oep37 to complement the growth phenotype of yeast cells deleted for *POR1* (Fig. 6). To decipher the biogenesis pathways of Oep37 and Oep24, I imported *in vitro* ^{35}S-labeled precursor proteins into mitochondria from various deletion strains (Fig. 7A and B, Fig. 9A, B and C, Fig 10A). In addition, I also compared the steady-state levels of Oep37 and Oep24 in mutant strains to those in the corresponding WT-strains (Fig. 7C and D, Fig. 9D, Fig. 10B). Furthermore, I tested the dependency of the *in vitro* import of ^{35}S-labeled Oep37, Oep24 and Porin on the TOM complex (Fig. 8). I participated in writing the manuscript.

Experiments shown in Fig. 5A and B, Fig. 6, Fig. 7C, Fig. 9D (left panel: *tim8/13Δ*) and Fig. 10B, C of the article were performed during my diploma studies and are already included in my diploma thesis "The chloroplast outer envelope protein Oep37 shares the import pathway with endogenous mitochondrial β-barrel proteins in *S. cerevisiae*".

2. Ulrich, T., P. Oberhettinger, M. Schütz, K. Holzer, A.S. Ramms, D. Linke, I.B. Autenrieth and D. Rapaport. 2014. Evolutionary conservation in biogenesis of β-barrel proteins allows mitochondria to assemble a functional bacterial trimeric autotransporter protein. *J. Biol. Chem.* 289:29457-29470.

In this report I performed all experiments described in Figures 1-6. For the immunofluorescence microscopy (Fig. 7A) and the interleukin-8 assays (Fig 7B) I isolated mitochondria from the indicated strains and handed them over to our cooperation partner (P. Oberhettinger), who performed the experiments. I was involved in the preparation of the manuscript.

3. Ulrich, T., P. Oberhettinger, I.B. Autenrieth and D. Rapaport. 2015. Yeast mitochondria as a model system to study the biogenesis of bacterial β-barrel proteins. In *Methods in Molecular Biology – The BAM complex: Methods and protocols*. Buchanan S. and Noinaj N., editors. Humana Press. Accepted for publication.

I wrote this chapter with the exception of paragraphs 2.3.5, 3.3 and 3.4, which were contributed by P. Oberhettinger. Moreover, I created Figures 1 and 2 of the manuscript.

4. Ulrich, T. and D. Rapaport. 2015. Biogenesis of β-barrel proteins in evolutionary context. *Intl. J. Med. Microbiol.* Accepted for publication.

I wrote the minireview and provided Figure 1.

6 Introduction

6.1 Membrane-embedded β-barrel proteins

Proteins belonging to the class of membrane-embedded β-barrel proteins can exclusively be found in the outer membranes (OM) of Gram-negative bacteria, mitochondria and chloroplasts. This restricted occurrence nicely supports the endosymbiotic theory, according to which the eukaryotic organelles mitochondria and chloroplasts originated from the engulfment of prokaryotic ancestors into the antecedent of the eukaryotic cell. Whereas comprehensive analyses of mitochondrial DNA (mtDNA) support the origin of extant mitochondria from a subgroup of α-proteobacteria, the plastids are thought to descend from the inclusion of an ancestral cyanobacterium about one billion years ago (Gray, 2011; Gray et al., 1999; McFadden, 2001).

Despite considerable functional diversity ranging from passive and active metabolite transporters, through enzymes and receptors to toxins and translocation machineries, all members belonging to the class of β-barrel proteins share a characteristic basic structure. They span the OM with 8-26 amphipathic anti-parallel β-sheets that are arranged in a cylindrical shape. Thereby the hydrophilic residues are orientated towards the interior of the barrel, whilst the hydrophobic side chains are facing towards the phospholipids of the membrane. The stability of the barrel structure is predominantly based on the hydrogen bonds between the individual β-strands (Qiao et al., 2014; Wimley, 2003). It is thought that duplication events of an ancestral ββ-hairpin gave rise to the broad variety of β-barrel proteins. Accordingly, the structures of all bacterial β-barrel proteins solved so far display a consistent composition of an even number of β-strands (Remmert et al., 2010). In contrast to the situation in bacteria, structure determinations of mitochondrial voltage-dependent anion channel 1 (VDAC1) revealed an unexpected architecture of 19 β-stands (Bayrhuber et al., 2008; Hiller et al., 2008; Ujwal et al., 2008). This arrangement was later on also suggested for Tom40, the pore forming component of the translocase of

the mitochondrial outer membrane (TOM complex) (Gessmann et al., 2011; Lackey et al., 2014; Zeth, 2010).

Although evolution gave rise to a wide range of β-barrel proteins, the basic principles of assembly into the corresponding membrane seem to be conserved from bacteria to humans. In each case a dedicated protein complex that contains a highly conserved β-barrel protein from the Omp85 superfamily facilitates the integration of the precursor protein into the lipid bilayer of the OM.

6.2 Biogenesis of β-barrel proteins in Gram-negative bacteria

The vast majority of outer membrane proteins (OMP) in Gram-negative bacteria belongs to the class of β-barrel proteins and is synthesized on cytoplasmic ribosomes with an N-terminal signal peptide, which ensure translocation across the bacterial inner membrane (Driessen and Nouwen, 2008; Papanikou et al., 2007). In contrast to inner membrane α-helical proteins, precursors of β-barrel proteins are guided to the Sec-machinery of the inner membrane in a post-translational manner. The cytoplasmic trigger factor (TF) recognizes the nascent polypeptide chain as it emerges at the exit channel of the ribosome (Ferbitz et al., 2004; Hagan et al., 2011; Valent et al., 1995). Subsequently, the tetrameric cytoplasmic chaperone SecB binds the precursor protein and escorts it to the Sec translocon (Fig. 1) (Bechtluft et al., 2010; Randall and Hardy, 2002; Ullers et al., 2004). Whereas hydrophobic transmembrane segments in α-helical proteins destined to the inner membrane trigger the lateral opening of the SecYEG translocon, precursors of β-barrel proteins are translocated across the inner membrane in a process depending on the hydrolysis of ATP and the proton-motive force ($\Delta\mu H^+$) (Papanikou et al., 2007; Park and Rapoport, 2012; Xie et al., 2007; Zimmer et al., 2008).

After reaching the periplasmic side of the inner membrane, the signal sequence is cleaved off by the signal peptidase (Paetzel, 2013). Periplasmic chaperones are involved in the biogenesis of β-barrel proteins in order to keep the precursor protein in an unfolded

state and to avoid misfolding and aggregation (Fig. 1). One of these is SurA, which contains two peptidyl-prolyl isomerase (PPIase) domains and was previously shown to chaperone the bulk of OM proteins in *Escherichia coli*. Furthermore, recent data demonstrated interaction of SurA with the dedicated insertion machinery of the OM (Bennion et al., 2010; Lazar and Kolter, 1996; Rouviere and Gross, 1996; Volokhina et al., 2011). A second parallel pathway involves the periplasmic chaperone Skp that can either act alone or together with the chaperone/protease DegP. In the periplasm Skp exists as a trimer and was shown to bind OMPs within its hydrophobic cavity that is generated between the α-helical tentacles (Walton and Sousa, 2004). In contrast to *E. coli*, where this second pathway was suggested to have a minor role in OMP biogenesis, deletion studies in *Neisseria meningitidis* proposed a major role for Skp in the biogenesis of the OMPs PorA and PorB, whilst deletion of SurA did not show a comparable phenotype (Sklar et al., 2007b; Vertommen et al., 2009; Volokhina et al., 2011). These apparent contradictory results suggest that the precise role of the periplasmic chaperones seems to depend on the substrate and organism.

Once having reached the inner surface of the OM, the β-barrel proteins are assembled into the lipid bilayer with the help of a dedicated protein assembly machinery termed β-barrel assembly machinery (BAM) complex (Fig. 1). Since its discovery, remarkable progress has been made in identifying and characterizing the roles of the individual BAM subunits in the biogenesis pathway of β-barrel proteins. In *E. coli*, the complex is composed of the central β-barrel protein BamA (YaeT) and the four lipoproteins BamB, BamC, BamD and BamE (formerly YfgL, NlpB, YfiO and SmpA, respectively)(Hagan et al., 2011; Voulhoux et al., 2003; Wu et al., 2005). As a member of the Omp85 superfamily BamA displays a characteristic architecture composed of five N-terminal polypeptide-transport-associated (POTRA) domains and a C-terminal 16-stranded β-barrel domain. As their name implies, the POTRA domains in the different Omp85 homologs are thought to function in the recognition and transport of incoming substrate

proteins (Sanchez-Pulido et al., 2003; Simmerman et al., 2014). Among the proteins of the BAM complex solely BamA and BamD are essential for viability (Malinverni et al., 2006; Wu et al., 2005). BamD was previously shown to bind to POTRA domain 5 of BamA and to scaffold the interaction of BamC, BamE and BamA. Furthermore its N-terminal domain was suggested to interact with incoming precursor proteins (Gatsos et al., 2008; Hagan et al., 2011; Sklar et al., 2007a; Vuong et al., 2008). Although the lipoproteins BamB, BamC and BamE are not essential for cell viability and are less conserved among different bacterial species, deletion of each one of them results in a decrease of the steady-state levels of β-barrel proteins (Hagan et al., 2011; Sklar et al., 2007a; Wu et al., 2005).

Despite the recent progress, the exact mechanism by which precursor proteins are finally assembled into the lipid bilayer is still ill-defined. Recently, the structure of the central component of the BAM complex, BamA from *Neisseria gonorrhoeae* and *Haemophilus ducreyi* has been solved (Noinaj et al., 2013). Analysis of the structural features revealed a reduction in the hydrophobicity on one side of the barrel, which leads to a local destabilization of the OM. Furthermore it was shown that due to a relatively weak interaction between the first and the last β-strand the barrel might undergo lateral opening, thus allowing the release of the precursor protein into the lipid bilayer. Accordingly, prevention of this lateral opening by formation of disulfide bridges between β-strands 1 and 16 resulted in a loss of BamA function (Noinaj et al., 2014).

6.3 Biogenesis of β-barrel proteins in mitochondria

Compared to Gram-negative bacteria, mitochondria harbor a rather low number of β-barrel proteins in their OM. So far, only five members of this class have been identified in yeast. These are the two essential proteins Tom40 and Tob55, two isoforms of Porin (also known as VDAC, voltage-dependent anion channel) and Mdm10 (Paschen et al., 2005; Walther et al., 2009b). According to the endosymbiotic theory, mitochondria were

derived by the engulfment of an α-proteobacterium into an antecedent of the eukaryotic cell (Gray, 2011; Gray et al., 1999). In the process of organelle evolution more than 99% of the proteins of extant mitochondria have undergone DNA transfer to the host genome. As a consequence, the vast majority of mitochondrial proteins is synthesized on cytosolic ribosomes and thus precursor proteins have to contain all the information required to ensure a specific and efficient sorting to their final destination within mitochondria. Since precursors of mitochondrial β-barrel proteins do no longer have to cross the bacterial inner membrane, the requirement for N-terminal signal sequences got lost in the transformation from bacteria to a semi-autonomous eukaryotic organelle. Thus, newly synthesized mitochondrial β-barrel proteins are devoid of any cleavable targeting signal.

Upon their synthesis on cytosolic ribosomes precursor proteins of mitochondrial β-barrel proteins are initially recognized on the mitochondrial surface by import receptors of the TOM complex (Chacinska et al., 2009; Endo and Yamano, 2009; Paschen et al., 2005; Pfanner et al., 2004). Next, they are relayed to Tom40 (Fig. 1), the central unit of the TOM complex, which is a β-barrel protein itself and provides the general entry gate for most of the mitochondrial precursor proteins. It is thought that the stepwise increasing affinity of precursor proteins to components of the TOM complex is the driving force for the protein translocation across the mitochondrial OM (Komiya et al., 1998; Nargang et al., 1998; Rapaport et al., 1998a; Rapaport et al., 1998b; Schatz, 1997).

Upon the appearance of the β-barrel precursor proteins at the intermembrane space (IMS), they are protected from misfolding and aggregation by the hexameric small chaperone complexes Tim8/13 and Tim9/10 (Fig. 1). Albeit not being homologous, the hexameric arrangement in these complexes displays remarkable structural similarity to the bacterial periplasmic chaperone Skp and archaeal or eukaryotic prefoldin (Beverly et al., 2008; Habib et al., 2005; Hoppins and Nargang, 2004; Walton and Sousa, 2004; Webb et al., 2006; Wiedemann et al., 2004b).

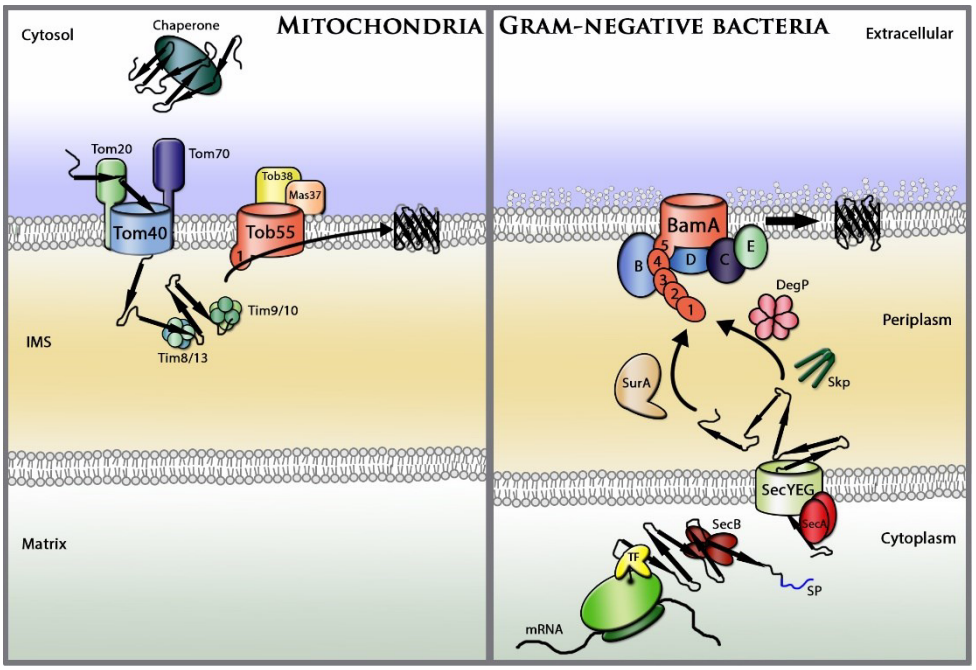


Figure 1. Comparison of the β-barrel biogenesis pathways in mitochondria and Gram-negative bacteria. Mitochondrial β-barrel proteins (left) are initially recognized at the mitochondrial surface by receptors of the TOM complex. Upon translocation across the mitochondrial OM through the Tom40 pore, they are escorted by the hexameric chaperone complexes Tim8/13 and Tim9/10. The assembly of precursor proteins into the mitochondrial OM is facilitated by the TOB complex, which is composed of the β-barrel Tob55 and the peripherally associated proteins Mas37 and Tob38. Precursors of bacterial β-barrel proteins (right) are synthesized in the cytoplasm with an N-terminal signal sequences (SP) which guides them to the Sec translocon where they are translocated across the inner membrane. In the periplasm the precursor proteins are protected from misfolding and aggregation by the chaperones SurA, Skp and the chaperone/protease DegP. Finally the precursor proteins are inserted into the lipid bilayer by the BAM complex, which is build up by the central component BamA and the four lipoproteins BamB-E.

The final step of protein assembly into the lipid bilayer of the OM is facilitated by a protein complex termed topogenesis of outer-membrane β-barrel proteins (TOB) complex also called sorting and assembly machinery (SAM) complex (Fig. 1) (Gentle et al., 2004; Paschen et al., 2003; Wiedemann et al., 2003). The complex is composed of the β-barrel protein Tob55 (Sam50), which is homologous to BamA, and the two auxiliary subunits Tob38 (Sam35, Tom38) and Mas37 (Sam37, Tom37), which are peripherally associated to the cytosolic side of the OM. Of these three components that form the core structure of the TOB complex, solely Tob55 and Tob38 were shown to be essential for cell viability

(Ishikawa et al., 2004; Milenkovic et al., 2004; Waizenegger et al., 2004). Whereas Tob38 was previously reported to be involved in the intramitochondrial recognition of β-barrel precursor proteins through a C-terminal recognition sequence termed β-signal, the second subunit Mas37 was suggested to act at a later stage of β-barrel assembly with a putative role in the release of the precursor into the lipid bilayer (Chan and Lithgow, 2008; Kutik et al., 2008; Waizenegger et al., 2004). Since in mitochondria the assembly of precursor proteins into the lipid bilayer occurs from the IMS side, it remains elusive how Tob38 can act in the recognition of precursor proteins. One possible explanation is that a cavity in the proteinaceous components of the complex allows Tob38 a direct contact with the substrate proteins (Kutik et al., 2008).

6.4 Biogenesis of β-barrel proteins in chloroplasts

Chloroplasts or plastids in general are the second organelles harboring membrane-embedded β-barrel proteins in the OM. According to the endosymbiotic theory, the plastids originated by the incorporation of an endosymbiotic prokaryote into a progenitor of the eukaryotic cell. Whereas mitochondria are thought to descend from a subgroup of α-proteobacteria, it is believed that the engulfment of an ancestral cyanobacterium was the initial step for the organellogenesis of plastids (Gray et al., 1999; McFadden, 2001). Of the approximately 2500-3500 proteins in chloroplasts only about 80-100 genes are still encoded on the plastome, mainly encoding ribosomal RNAs and proteins, tRNAs, the large subunit of the RuBisCO and proteins required for photosynthesis (Joyard et al., 2009). The remaining 95% of the chloroplast proteins are encoded by the nuclear genome and thus have to be imported into the chloroplasts. Although progress has been made in understanding the biogenesis of chloroplast proteins containing cleavable targeting sequences, currently very little is known about how chloroplast β-barrel proteins are assembled into the outer envelope. Like in mitochondria, most chloroplast precursors of β-barrel proteins are predicted to be devoid of N-terminal targeting signals. An

exceptional case, however, is the central component of the translocase of the outer chloroplast membrane (TOC) complex, Toc75-III. It is a highly abundant protein in the OM that is synthesized in the cytoplasm with an N-terminal bipartite transit peptide (Cline et al., 1981; Tranel and Keegstra, 1996). Interestingly, the first half guides the precursor protein to the chloroplast stroma, where it is cleaved by the stromal processing peptidase (SPP) (Inoue et al., 2005; Inoue et al., 2001; Tranel and Keegstra, 1996). The second half of the signal contains a stop-transfer segment which is thought to prevent full translocation across the inner membrane. Before the precursor protein is finally assembled into the chloroplast OM, the second part of the transit peptide is removed by the plastidic type I signal peptidase (Plsp1) (Inoue et al., 2005). Despite this exceptional biogenesis pathway, the precursor translocation across the OM and the insertion into the lipid bilayer from the inner face of the membrane resemble the biogenesis pathway of β-barrel proteins in mitochondria. In sharp contrast to the situation in mitochondria, several Omp85 homologs have been identified in chloroplasts. Among these is Toc75-V, also known as outer envelope protein 80 (Oep80), which was shown to be essential for viability in *Arabidopsis thaliana* (Patel et al., 2008). Phylogenetic analyses suggest that the two essential proteins Toc75-III and Toc75-V evolved from an ancestral cyanoOmp85 by gene duplication. Whilst sequence alignments and comparison of structural predictions imply that Toc75-V might have retained its ancestral function in the assembly of β-barrel proteins, Toc75-III evolved to form the general entry gate for chloroplast-destined proteins that are synthesized in the cytosol and have to be imported into chloroplasts (Day et al., 2014; Soll and Schleiff, 2004). However, so far there is no experimental support for the putative function of Toc75-V in the biogenesis of β-barrel proteins.

6.5 Commonalities and disparities in the biogenesis of β-barrel proteins

Three common characteristics are shared in the biogenesis pathways of β-barrel proteins between bacteria and mitochondria: (I) insertion into the lipid bilayer from the internal side of the membrane, (II) participation of soluble chaperones in the periplasm or IMS, and (III) homology in the central subunit of the assembly complexes (Fig. 1). Homologs of BamA, the central component of the BAM complex can be found in all Gram-negative bacteria and in the OM of the eukaryotic organelles mitochondria and chloroplasts. The most striking similarity among all members of the Omp85 superfamily is their unique structural organization. It was predicted that the structure contains a varying number of N-terminal POTRA domains followed by a 16-stranded C-terminal β-barrel pore. Indeed, the recent structure determination of BamA from *Neisseria gonorrhoeae* and *Haemophilus ducreyi* confirmed this assumption (Noinaj et al., 2013).

Despite such apparent similarity, comparison of several members of the Omp85 superfamily reveals that the number of POTRA domains varies from one in the mitochondrial Tob55 to three in the chloroplast homologs Toc75-III and Toc75-V, whereas up to seven POTRA domains were predicted for the Omp85 of *Myxococcus xanthus* (Arnold et al., 2010; Sanchez-Pulido et al., 2003). Regardless of the number of POTRA domains, a comparative study of 567 POTRA domains from all kingdoms of life identified the most C-terminal POTRA domain as the best conserved one, followed by the most N-terminal one (Arnold et al., 2010; Simmerman et al., 2014). Accordingly, it can be speculated that in the process of organelle evolution the mitochondrial Omp85 homolog Tob55 evolved in a way that it retained the most C-terminal POTRA domain as a minimal motif for β-barrel assembly in mitochondria. This assumption is in line with the discovery that POTRA domains 1-4 are dispensable for viability in *N. meningitidis* and their deletion results in merely mild assembly defects of OM proteins (Bos et al., 2007). However, in sharp contrast to the situation in *N. meningitidis*, POTRA domains

3-5 were shown to be essential in *E. coli* (Kim et al., 2007). Interestingly, the accessory lipoproteins of the BAM complex, BamC, BamD and BamE were reported to bind to POTRA domain 5, whereas the binding of BamB, which is not present in *N. meningitidis*, to the BAM complex was affected in a strain deleted for POTRA domains 2-5 (Kim et al., 2007).

In respect of these differences in the necessity of POTRA domains in the different organisms, it is also conceivable that Omp85 homologs with multiple POTRA domains evolved from an ordinary ancestral Omp85 harboring only one POTRA domain. It is still controversially discussed how the variable number of POTRA domains in the different Omp85 homologs might be explained. Conspicuously, the number of POTRA domains seems to correlate with the predicted number of β-barrel proteins in Gram-negative bacteria, mitochondria and chloroplasts (Habib et al., 2007; Schleiff et al., 2003; Wimley, 2003). On the other hand, the deletion of POTRA domains 1-4 in *N. meningitidis* had less effect on the assembly of the 8-stranded β-barrel protein NspA than on that of a 22-stranded β-barrel protein. This difference might also reflect the requirement for multiple POTRA domains in order to facilitate an efficient assembly of larger and more complex substrates (Bos et al., 2007).

Besides the varying number of POTRA domains that can be found among distinct Omp85 homologs, a crucial discrepancy persists regarding the orientation of the POTRA domains. Here, an exceptional position is held by chloroplast homologs Toc75-III and Toc75-V. Whilst the POTRA domains in all other Omp85 homologs protrude into the periplasm in bacteria or the IMS in mitochondria, a recent study using a split-GFP assay and electron cryotomography demonstrated that the N-terminal POTRA domains of Toc75-III/Toc75-V are exposed towards the cytosol (Sommer et al., 2011). This however, would exclude the putative involvement of the POTRA domains as a receptor or scaffold in the biogenesis of β-barrel proteins from the inner side of the membrane as it was previously shown for bacteria and mitochondria. Therefore it remains elusive whether

the cytoplasmic orientation of the POTRA domains in the chloroplast homologs allows for the recognition and assembly of incoming β-barrel precursor proteins without even entering chloroplasts through the TOC complex.

Even though structural data of the POTRA domains in different Omp85 homologs are available, very little is known about the exact mechanism by which the individual POTRA domains assist in the assembly of β-barrel precursor proteins. One possible mechanism that is often discussed is β-augmentation, which describes the interaction of β-strands of two different proteins by extending the β-sheet motif (Bennion et al., 2010; Harrison, 1996; Kim et al., 2007; Knowles et al., 2008; Koenig et al., 2010). In this regard the characteristic β1α1α2β2β3 secondary structure organization in POTRA domains might allow for the pairing of the exposed β-sheets with β-sheets in precursor proteins, thus facilitating a sequential substrate sliding to the core of the corresponding Omp85 homolog. Such mechanism would allow for the recognition and handling of a broad variety of OM proteins thereby being rather selective for alternating hydrophobic and polar patches in β-barrel proteins without the demand for a specific linear sequence information (Gatzeva-Topalova et al., 2008; Knowles et al., 2008).

Apart from the evolutionary conservation in the function and structure among the members of the Omp85 superfamily, the β-barrel assembly machineries in bacteria and mitochondria differ regarding their distinct accessory proteins. Although facilitating a basically similar process, the BAM and the TOB complexes display considerable disparity regarding the number and the orientation of their accessory proteins. In *E. coli*, the lipoproteins BamB, BamC, BamD and BamE are localized on the inner side of the OM and hence are able to directly interact with the incoming precursor proteins. The two mitochondrial subunits Tob38 and Mas37, however, are peripherally associated at the cytosolic side of the MOM and do not show any homology with their bacterial counterparts.

6.6 Heterologous expression of β-barrel proteins

In accordance with the aforementioned evolutionary relation between bacteria and mitochondria, previous studies revealed that their dedicated import machineries can recognize signals in heterologous bacterial and mitochondrial β-barrel proteins (Müller et al., 2011; Walther et al., 2010; Walther et al., 2009a). Yet, it is unclear whether this link also allows mitochondria to recognize and assemble β-barrel proteins of chloroplastic origin. In this thesis I took advantage of the evolutionary conservation in the biogenesis of β-barrel proteins and used yeast mitochondria as a model system to study the targeting of the bacterial trimeric autotransporter adhesin *Yersinia* adhesin A (YadA) and the two chloroplast β-barrel proteins Oep24 and Oep37.

6.6.1 The trimeric autotransporter *Yersinia* adhesin A (YadA)

An interesting exception of the consistent β-barrel architecture is constituted by the trimeric autotransporter adhesins (TAA) of the type Vc secretion system. These proteins are prevalent among pathogenic Gram-negative bacteria and mediate bacterial adherence to host cells or extracellular matrix (Grijpstra et al., 2013; Linke et al., 2006). TAAs form a distinctive lollipop structure on the bacterial surface with a characteristic tripartite molecular architecture. The N-terminal head domain is connected via a highly repetitive coiled-coil stalk domain to the C-terminal β-domain (Grijpstra et al., 2013; Hoiczyk et al., 2000; Linke et al., 2006). In contrast to ordinary β-barrel proteins, the TAAs assemble into the OM as homotrimers, in which each monomer contributes four β-stands to the 12-stranded β-barrel structure, which anchors the adhesins in the bacterial OM (Fig. 2). This architecture is unique among the class of β-barrel proteins and cannot be found in eukaryotic cells.

Yersinia adhesin A (YadA) from enteropathogenic *Yersinia enterocolitica* and *Yersinia pseudotuperculosis* strains is the best characterized trimeric adhesin and was

previously shown to bind laminin, collagen and fibronectin via its N-terminal head domain (Linke et al., 2006; Tahir et al., 2000; Tamm et al., 1993; Tertti et al., 1992). With a molecular size of 45 kDa YadA represents a relatively short member of the trimeric autotransporter adhesins. Upon binding to host cells, YadA triggers interleukin-8 (IL8) secretion from these cells and was furthermore reported to be the major factor in serum resistance of *Y. enterocolitica* by inhibiting activation of the complement system (Balligand et al., 1985; Linke et al., 2006; Schindler et al., 2012; Schmid et al., 2004).

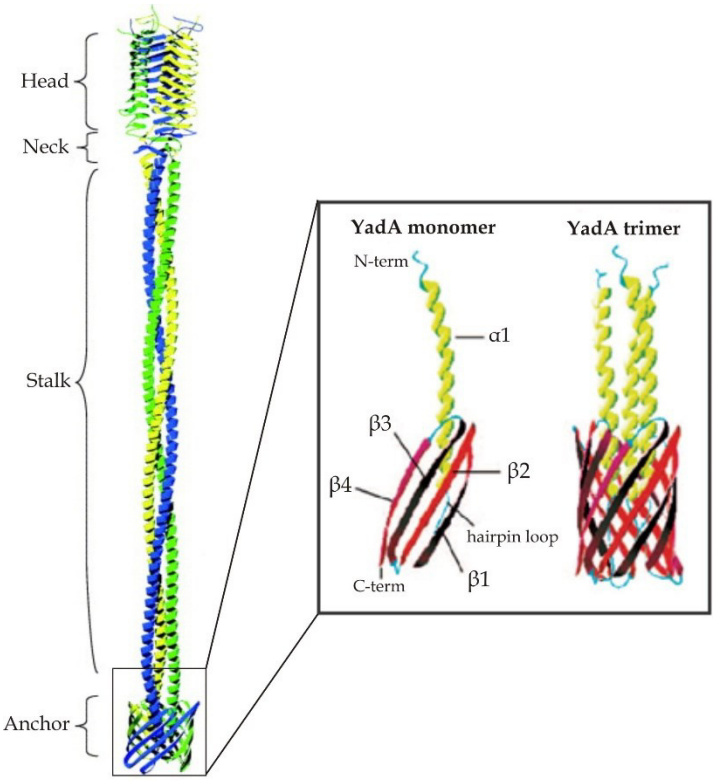


Figure 2. Model structure of YadA. The oligomeric structure of full-length YadA (left) displays the typical TAA architecture composed of an N-terminal head domain, the connecting coiled-coil stalk and the C-terminal membrane anchor. The individual monomers are shown in blue, yellow and green. Each of the monomers contributes four β-strands to the trimeric 12-stranded β-barrel anchor (right). Figure adopted and modified from (Linke et al., 2006) and (Ackermann et al., 2008).

6.6.2 Oep24 and Oep37

The chloroplast outer envelope proteins 24 and 37 (Oep24 and Oep37, respectively) form voltage-dependent high conductance β-barrel pores in the chloroplast OM (Goetze et al., 2006; Pohlmeyer et al., 1998). Both were predicted to harbor a common 12-stranded β-barrel conformation, which however differs severely in the size of the soluble loops. According to the predicted model, Oep37 forms relatively large loops facing both, the cytosol and the IMS, whereas the individual β-strands in Oep24 seem to be connected by comparatively short loops (Schleiff et al., 2003). Oep24 was shown to allow the passage of triose phosphates, dicarboxylic acids, sugars, positively and negatively charged amino acids, ATP and P_i across the outer membrane, thus representing a rather non-selective pore (Pohlmeyer et al., 1998). This goes in line with the finding that heterologous expression of Oep24 in yeast can complement the growth phenotype of a deletion of *POR1*, encoding the general solute channel in the MOM (Rohl et al., 1999). However, apart from these findings, the biogenesis pathway of chloroplast β-barrel proteins remains obscure.

7 Research objectives

In this thesis I intended to investigate the molecular mechanisms underlying the biogenesis of β-barrel proteins. To that end, I addressed two major questions: (I) Is the previously shown evolutionary conservation in the biogenesis of β-barrel proteins between Gram-negative bacteria and mitochondria also applicable to β-barrel proteins of chloroplastic origin? (II) Does the recognition and assembly of β-barrel proteins require the complete β-barrel sequence or does a fragment of a β-barrel contain enough targeting information?

To address the first question we chose Oep24 and Oep37 as examples for β-barrel proteins of the chloroplast outer envelope and investigated the sorting of these proteins upon expression in yeast cells. Here, we were also interested whether these proteins would share the biogenesis pathway of the endogenous mitochondrial β-barrel proteins or utilize an alternative one.

The second topic was investigated by taking advantage of the archetype trimeric autotransporter protein *Yersinia* adhesin A (YadA). We wanted to know whether the mitochondrial Omp85-homolog Tob55 retained the ability to recognize and assemble such proteins into the MOM. Furthermore, we intended to gain insight into the yet unidentified biogenesis pathway of YadA using yeast mitochondria as a model system.

As previous studies suggested an involvement of bacterial chaperones in the biogenesis pathway of trimeric autotransporter proteins in bacteria, we wondered whether we could employ yeast mitochondria to study the roles of the bacterial chaperones SecB, SurA and Skp in the biogenesis of YadA.

8 Summary of the results

8.1 "Chloroplast β-barrel proteins are assembled into the mitochondrial outer membrane in a process that depends on the TOM and TOB complexes"

Previous studies showed that heterologous expression of bacterial β-barrel proteins in yeast cells results in the proper assembly of those proteins into the MOM (Müller et al., 2011; Walther et al., 2009a). In a reciprocal approach the mitochondrial β-barrel protein Porin1 (VDAC1) from *Neurospora crassa* was assembled into the bacterial OM upon expression in *E. coli* (Walther et al., 2010). These findings revealed that the signals in β-barrel proteins can be processed by both prokaryotic and eukaryotic assembly machineries. Accordingly, also the chloroplast β-barrel protein Oep24 was reported to be assembled into the mitochondrial OM upon its expression in yeast (Rohl et al., 1999). However, since this study did not address the mechanism of such assembly, it remains obscure whether chloroplast β-barrel proteins share the biogenesis pathway with their mitochondrial counterparts.

Using *in vitro* import into either isolated chloroplasts or mitochondria or by employing a dual import system, in which radiolabeled precursor proteins were incubated with both isolated chloroplasts and mitochondria, Oep24 and Oep37 were found in both membranes. In contrast, the control protein VDAC1 was preferentially integrated into the MOM (Fig. 3 and 4).

In order to shed light on the biogenesis of the chloroplast β-barrel proteins, we expressed Oep24 and Oep37 in *S. cerevisiae* and examined their intracellular targeting. Subcellular fractionation revealed that Oep37 is exclusively targeted to mitochondria (Fig. 5A). To further demonstrate that Oep37 was integrated into the MOM rather than simply attached to the surface of the organelle we performed carbonate extraction, an established method to distinguish between membrane-embedded and soluble proteins. We found Oep37 in the pellet fraction together with the mitochondrial membrane

proteins Tom70 and Porin (Fig. 5B). Accordingly, resistance against externally added proteinase confirmed that Oep37 was embedded into the lipid bilayer of the MOM (Fig. 5B and C). To exclude targeting of Oep37 to the mitochondrial inner membrane, we further performed protease protection assays with high concentrations of trypsin. The formation of proteolytic fragments under these conditions demonstrated cleavage of loops exposed to the cytosol and thereby OM localization of Oep37 (Fig 5C).

Next, we wondered whether the expression of Oep37 would interfere with crucial mitochondrial functions. To address this issue we compared the growth rate of cells expressing Oep37 to cell harboring an empty plasmid and investigated the mitochondrial morphology in these cells. However, under all conditions tested, we could not observe an effect on crucial cellular and mitochondrial processes (Fig 6A). Given that Oep37 was previously shown to form a voltage-dependent high conductance channel in artificial membranes, we asked whether it can complement the absence of the general solute transporter of the mitochondrial outer membrane, Porin1. Indeed, *por1*Δ cells expressing Oep37 partially regained the ability to grow on a nonfermentable carbon source (Fig. 6B), indicating that Oep37 forms pores in the MOM, as it was previously reported for Oep24 (Rohl et al., 1999).

The endogenous mitochondrial β-barrel proteins are initially recognized on the mitochondrial surface via Tom20 and Tom70, the import receptors of the TOM complex (Krimmer et al., 2001; Rapaport and Neupert, 1999; Yamano et al., 2008). To address the dependency of chloroplast β-barrel proteins on these receptors, we employed an *in vitro* import assay of radiolabeled Oep24 and Oep37. Proteolytic removal of the exposed domains of receptors prior to the import reaction affected the membrane integration of both Oep24 and Oep37 (Fig. 7A). In order to verify this effect, we also performed *in vitro* imports into mitochondria isolated from strains deleted for either *TOM20* or *TOM70/71*. Under these conditions, the import of both chloroplast proteins was severely reduced (Fig. 7B). To investigate the *in vivo* situation, we compared the steady-state

levels of Oep24 and Oep37 expressed in WT cells to those in cells lacking either Tom20 or Tom70/71. Interestingly, mitochondria isolated from cells lacking Tom20 displayed strongly reduced amounts of Oep37, whereas the same deletion had no effect on the steady-state levels of Oep24. In contrast, deletion of *TOM70/71* did not influence the amounts of chloroplast β-barrel proteins in mitochondria (Fig. 7C and D).

Since precursors of β-barrel proteins are translocated across the outer membrane via the Tom40 pore (Endo and Yamano, 2009; Pfanner et al., 2004; Walther et al., 2009b), we asked whether this is also true for Oep24 and Oep37. To that end we imported radiolabeled precursors of the chloroplast proteins in the presence of increasing amounts of the matrix-destined precursor pSu9(1-69)-DHFR. Addition of high concentrations of this protein blocked the Tom40 pore and thus precluded TOM-dependent import of ^{35}S-Oep24, ^{35}S-Oep37 and ^{35}S-Porin (Fig. 8).

Following translocation across the Tom40 pore, precursors of β-barrel proteins are protected from misfolding and degradation in the IMS by the hexameric small Tim complexes (Hoppins and Nargang, 2004; Wiedemann et al., 2004a). Therefore we examined the role of the small Tim chaperones in the biogenesis of Oep24 and Oep37. To that end, we first imported ^{35}S-Oep37 and ^{35}S-AAC into mitochondria, of which the OM was ruptured by osmotic swelling to release the small Tim chaperones from the IMS. As expected, such treatment caused a reduction in the assembly of inner membrane ADP-ATP carrier (AAC), whereas the assembly of ^{35}S-Oep37 was not compromised in the ruptured organelles (Fig. 9A). In agreement with these findings, comparable results were obtained upon import of ^{35}S-Oep24 and ^{35}S-Oep37 into mitochondria isolated from either control cells or from cells lacking the Tim8/13 complex (Fig. 9B) or alternatively harboring a temperature sensitive allele of *TIM10* (Fig. 9C). To verify these results, we further expressed Oep24 and Oep37 in these mutant cells and compared their steady-state levels in crude mitochondria to those in the corresponding parental strains (Fig. 9D). With the exception of Oep24, where the steady-state levels were slightly reduced in

*tim8/13*Δ cells, the results nicely agree with those obtained by the *in vitro* import. Hence, the small Tim complexes seem to play a minor role, if at all, in the biogenesis of chloroplast β-barrel proteins.

As the membrane integration of mitochondrial β-barrel proteins heavily depends on the TOB complex, we further investigated whether the assembly of the chloroplast proteins Oep24 and Oep37 is facilitated in the same way. Therefore, we performed *in vitro* import of the radiolabeled precursor proteins into mitochondria lacking Mas37, a peripherally associated subunit of the TOB complex. As expected, import into these mitochondria was significantly hampered in comparison to import into control organelles (Fig. 10A). A similar effect could be observed upon expression of Oep24 and Oep37 in yeast cells. The steady-state levels of both proteins were strongly reduced in *mas37*Δ cells (Fig. 10B). Due to the fact that in these strains also the levels of the essential Tom40 protein were reduced, we aimed to substantiate our findings by demonstrating direct dependence on the central component of the TOB complex. Since Tob55 is essential for viability in yeast, we coexpressed Oep37 in a strain where Tob55 is expressed under the control of an inducible *GAL* promoter. In these cells, a switch from galactose-containing medium to glucose as a carbon source results in a gradual reduction in the levels of Tob55. We observed that the steady-state levels of Oep37 and the mitochondrial β-barrel protein Porin dramatically dropped upon the depletion of Tob55, whereas the level of Tom40 was gradually decreased over a longer period of time (Fig. 10C).

Taken together, these new findings demonstrate that the chloroplast β-barrel proteins Oep24 and Oep37 are assembled into the MOM in a pathway that comprises recognition and translocation of precursor proteins by the TOM complex as well as integration into the lipid bilayer by the TOB complex.

8.2 "Evolutionary conservation in the biogenesis of β-barrel proteins allows mitochondria to assemble a functional bacterial trimeric autotransporter protein"

Yersinia adhesin A (YadA) belongs to the class of trimeric autotransporter proteins that mediate bacterial adhesion in numerous pathogenic Gram-negative bacteria. In their mature trimeric form a 12-stranded β-barrel domain anchors the proteins to the bacterial OM. So far, very little is known about the factors that are involved in the biogenesis of these proteins. We took advantage of the evolutionary link between bacteria and mitochondria and investigated the yet unknown biogenesis pathway of the trimeric autotransporter protein YadA.

To test whether yeast mitochondria can accomplish the recognition and assembly of the trimeric autotransporter adhesin, we performed subcellular fractionation of cells expressing an N-terminally HA-tagged version of full-length YadA. As we previously showed for the truncated membrane anchor of YadA (Müller et al., 2011), the full-length protein was exclusively found in the mitochondrial fraction, where it assembled into a SDS-stable trimeric structure (Fig. 1A). Interestingly, we were also able to detect a fraction of monomeric YadA, which might represent import intermediates that cannot be found in the bacterial system (Grosskinsky et al., 2007; Schütz et al., 2010).

To verify the membrane insertion of YadA, we subjected mitochondria isolated from cells expressing YadA to carbonate extraction. As expected, trimeric YadA was solely found in the pellet but we could also observe that a fraction of the monomer appeared in the supernatant with other soluble proteins (Fig. 1B). In order to examine the topology of trimeric YadA in the MOM and to investigate the localization of the soluble monomer, we incubated isolated mitochondria with increasing amounts of externally added protease. Whilst trimeric YadA was already degraded at low concentrations of proteinase K (PK), suggesting the exposure of the N-terminus to the cytosol, a portion of the monomer remained protected from digestion most probably as import intermediates in the IMS

(Fig. 1C). This hypothesis was then further tested by proteolytic treatment of organelles where the OM was either left intact or ruptured by hypoosmolaric swelling (Fig. 1D). As anticipated, the portion of monomeric YadA that was protected from digestion in intact mitochondria was almost completely degraded upon rupturing the MOM.

The initial step in the import of mitochondrial β-barrel proteins is the recognition of substrates by the import receptors of the TOM complex (Krimmer et al., 2001; Model et al., 2001; Rapaport and Neupert, 1999). This dependence on the Tom receptors is shared by different bacterial β-barrel proteins, but could not be observed in the biogenesis of the truncated membrane anchor of YadA (Müller et al., 2011; Walther et al., 2009a). However, it remains elusive whether the import receptors Tom20 and Tom70 participate in the biogenesis of full-length YadA. To that end, we expressed YadA in either *tom20*Δ or *tom70/71*Δ cells and compared its steady-state levels to those in the corresponding wild-type strains. Interestingly, the steady-state levels of the full-length protein in mitochondria lacking Tom20 or Tom70/71 were comparable to those in wild-type organelles (Fig. 2A and B). As the biogenesis of YadA is apparently independent of the Tom receptors, we wanted to verify that the precursors of the TAA are translocated across the OM via the Tom40 pore. To that end, we expressed YadA in a conditional Tom40 mutant strain which was previously reported to be hampered in the import of TOM-dependent precursor proteins (Wenz et al., 2014). Demonstrating the importance of the Tom40 pore, the levels of YadA were drastically reduced in this strain (Fig. 2C).

As hydrophobic proteins are prone to misfolding and aggregation in the aqueous environment of the IMS, we asked whether YadA interacts with the mitochondrial small Tim chaperone complexes Tim8/13 or Tim9/10. To that end, we transformed yeast cells either deleted for both *TIM8* and *TIM13* or harboring a temperature-sensitive allele of *TIM10* with a plasmid encoding YadA and monitored the levels of YadA in the crude mitochondrial fraction. Whereas the amount of YadA in *tim8/13*Δ cells was comparable to those in wild-type cells, a clear reduction could be observed in the *tim10ts*-cells (Fig.

2D and E). Hence, the biogenesis of YadA depends on the soluble Tim9/10 complex in the IMS.

In a previous report, the assembly of YadA into the bacterial OM was suggested to depend on BamA (Lehr et al., 2010). Since Tob55 is the mitochondrial homologue of BamA, we next analyzed the relevance of the TOB complex for the proper membrane assembly of YadA. To that end, we transformed cells lacking Mas37, a non-essential peripheral subunit of the TOB complex with a plasmid encoding YadA and compared the steady-state levels with the corresponding parental strain. Mitochondria isolated from those cells exhibited dramatically reduced amounts of YadA, displaying the dependency on a functional TOB complex (Fig. 2F). To substantiate this finding, we analyzed the levels of YadA in a strain where Tob55 is under control of an inducible *GAL* promoter. To gradually deplete Tob55 in those cells, we switched from galactose- to glucose-containing medium and monitored the steady-state levels of YadA after certain periods of time. Upon the disappearance of the Tob55 signal, also no trimeric YadA could be observed anymore (Fig. 2G). Of note, the depletion of Tob55 had only minor effect on the levels of monomeric YadA. Thus, a functional TOB complex is essential for the trimerization and thereby proper assembly of YadA into the MOM.

The biogenesis pathways of β-barrel proteins in bacteria and mitochondria share the involvement of soluble chaperones in the periplasm or the IMS. However, the knowledge about the roles of periplasmic chaperones in this process is mainly based on conflicting results in *E. coli* and *N. meningitidis*. A major drawback of the bacterial system in investigating chaperone function is that the deletion of a single chaperone might either lead to the compensation by another one or result in pleiotropic effects. Thus, we used yeast mitochondria as a model system to investigate the effects of the bacterial chaperones SecB, SurA and Skp on the biogenesis of YadA. To that end, we co-expressed YadA together with SecB or mitochondrially-targeted versions of SurA and Skp (mtSurA, mtSkp) in yeast cells. Next, we verified correct targeting of the chaperones to the desired

cellular and mitochondrial compartment (Fig. 3A, C and D). In order to estimate the relative amounts of SurA and Skp in the IMS we compared their levels in mitochondria to those in *E. coli* cells (Fig. 3A). Next, we examined the contribution of the individual chaperones to the biogenesis of YadA. Interestingly, the co-expression of YadA and mtSkp led to a drastic increase in the levels of both trimeric and monomeric YadA, whereas neither SecB nor mtSurA showed such an effect (Fig. 4A). A similar increase could also be observed for the bacterial β-barrel protein PhoE, although not as pronounced as in the case of YadA (Fig. 4C). Since the levels of the mitochondrial β-barrel proteins were not altered in the presence of Skp, we aimed to substantiate the specificity of the chaperone effect by using *in vitro* import into mitochondria harboring mtSkp. Reflecting the *in vivo* situation, the presence of mtSkp did not increase the import of the radiolabeled mitochondrial β-barrel proteins Porin and Tom40 (Fig. 4B). To examine a conceivable synergistic effect of the chaperones, we compared the strain harboring YadA and mtSkp to a strains simultaneously co-expressing YadA with all three chaperones (SecB, mtSurA and mtSkp). This latter strain displayed a marginal increase in the levels of YadA, which could however also result from slightly elevated levels of mtSkp (Fig. 4D). We further asked whether the enhanced levels of YadA also influence the oligomerization behavior of the protein. To that end, we performed protease protection assays and carbonate extraction. Although, the levels of monomer and trimer were drastically increased, both species behaved similar to the situation when YadA was expressed alone (Fig. 4E and F).

The aforementioned findings left open the question of how mtSkp causes such highly elevated levels of YadA. This could be due to either an improved biogenesis or the protection of precursor proteins from degradation. To test the second hypothesis, we blocked protein translation by addition of cycloheximide to the cell culture and monitored the steady-state levels of YadA after various periods of incubation. Such treatment led to almost complete degradation of the monomeric form already after 30 min. Similar

results could be observed for a strain co-expressing mtSurA. However, the presence of mtSkp resulted in a stabilization of monomeric YadA (Fig. 5A). Since the AAA-protease Yme1 is known to degrade mitochondrial proteins in the IMS (Leonhard et al., 1999), we repeated the cycloheximide treatment in a strain deleted for this protease. Indeed, the absence of Yme1 reduced the degradation of monomeric YadA by 2-3-fold, thus substantiating the role of Skp in stabilization of YadA precursors in the IMS (Fig. 5B). To further test the direct interaction of Skp and YadA, we solubilized mitochondria co-expressing YadA-HA and mtSkp and performed pull-down assays using anti-HA beads. Supporting the previous results, we could pull-down mtSkp together with YadA-HA (Fig. 5C). In a reciprocal approach using antibodies against Skp, we were able to co-immunoprecipitate YadA-HA together with mtSkp (Fig. 5D). Hence, the periplasmic chaperone Skp directly binds percursors of YadA in the IMS and protects them from degradation.

To figure out whether the observed stabilizing effect of Skp depends on the interaction with the passenger domain, we also investigated the effects of the different bacterial chaperones on the membrane-anchor domain of YadA (YadA-MA). In agreement with the results with the full-length protein, solely the co-expression with mtSkp resulted in highly elevated levels of YadA-MA in mitochondria (Fig. 6A). Accordingly, the presence of mtSkp led to the stabilization of monomeric YadA-MA, although not to the same extent as we observed for full-length YadA (Fig. 6B). Simultaneous expression of all the three chaperones with YadA-MA did not further increase the protein levels when compared to expression of YadA-MA and Skp alone (Fig. 6C). Cycloheximide treatment of *yme1*Δ-cells revealed that Skp also functions in stabilizing the membrane-anchor domain of YadA (Fig. 6D). In summary it appears that even though Skp can interact with the truncated version of YadA, the presence of the passenger domain enhances the interaction.

9 Discussion

Beta-barrel proteins probably evolved from duplication events of an ancestral ββ-hairpin motif. Such duplications apparently gave rise to the broad variety of β-barrel proteins that can be found in the outer membranes of Gram-negative bacteria, mitochondria and chloroplasts (Remmert et al., 2010). Their exclusive occurrence in these membranes can be explained by the endosymbiotic theory, according to which the eukaryotic organelles mitochondria and chloroplasts descend from the engulfment of bacterial ancestors into progenitors of the eukaryotic cell over one billion years ago (Gray, 2011; Gray et al., 1999; McFadden, 2001). It is remarkable that despite the long process of organelle evolution all membranes harboring β-barrel proteins retained a member of the Omp85 superfamily to facilitate the assembly of these proteins into the OM (Gentle et al., 2004; Simmerman et al., 2014; Walther et al., 2009b). Interestingly, heterologous expression revealed that this evolutionary link allows the β-barrel assembly machineries in bacteria and mitochondria to recognize and process signals in prokaryotic and eukaryotic β-barrel proteins (Kozjak-Pavlovic et al., 2011; Müller et al., 2011; Walther et al., 2010; Walther et al., 2009a).

So far, it remains unclear whether the evolutionary conservation in the biogenesis of these proteins between bacteria and mitochondria can also be extended to β-barrel proteins of chloroplastic origin. To address this question, we expressed the chloroplast β-barrel proteins Oep24 and Oep37 in yeast cells. We demonstrated that the proteins were exclusively targeted to mitochondria, where they got assembled into the MOM in a process that includes recognition by the Tom receptors, translocation across the OM through the Tom40 pore and finally insertion into the lipid bilayer by the TOB complex. Collectively, they follow a similar biogenesis pathway as the endogenous mitochondrial β-barrel proteins and thus have to contain all the information required for their recognition as substrates by the mitochondrial import machineries. This also suggests that the yet unidentified chloroplast β-barrel assembly machinery uses similar signals as

the mitochondrial TOB complex. In this respect our results substantiate the assumption that such information can be rather found in structural elements than in a specific mitochondrial sequence motif.

The fact that both Oep24 and Oep37 were imported *in vitro* into chloroplasts and mitochondria isolated from plant cells raises the question of how these cells avoid mistargeting of mitochondrial β-barrel proteins to chloroplasts and *vice versa*. One way to explain such specific targeting is to assume that differences between plant mitochondria and their yeast counterparts allow the former to discriminate between the substrates. Here, it is important to take into consideration that despite the conservation of the pore forming components in the mitochondrial inner and outer membranes across all eukaryotic kingdoms, conspicuous divergence exists in the mitochondrial import receptors between plants and fungi (Duncan et al., 2012). Whilst in plant mitochondria a homologue of Tom20 is missing, the equivalent protein of similar size and function displays remarkable difference in domain organization. A truncated version of Tom22 that can be found in plant mitochondria is even lacking the whole cytosolic domain (Jänsch et al., 1998; Macasev et al., 2000; Perry et al., 2008). Moreover, the absence of Tom70 from the plant TOM complex is thought to be compensated by the unrelated import receptor OM64 (Chew et al., 2004; Duncan et al., 2012). However, apart from these differences, the import receptors of plant and yeast mitochondria seem to recognize a similar set of substrate proteins and thus the distinction of β-barrel proteins on the basis of these receptors seems unlikely.

Another possible explanation is based on the fact that chloroplasts are predominant in plant cells and thus expose a much larger surface to newly synthesized precursor proteins than mitochondria. Assuming free diffusion of these proteins in the cytosol, distribution of β-barrel protein based on the number of potential binding sites on the surface of the organelles would demand some kind of bouncing-off mechanism for mitochondrial β-barrel proteins in chloroplasts.

However, these mechanisms would probably not meet the necessary requirements to ensure the efficient and specific targeting to the right organelle. Considering that, it would be rather beneficial for the plant cell to avoid any contact of mitochondrial β-barrel proteins with chloroplasts and *vice versa*. It is assumed that plant cells evolved by the incorporation of a cyanobacterium into an ancestral eukaryotic cells that already contained mitochondria. Therefore it appears conceivable that whilst mitochondrial β-barrel proteins followed a default pathway, the recently affiliated chloroplasts had to adapt in order to avoid this destination. A solution to this problem would be specific mRNA localization to the organelle and cotranslational import of certain chloroplast proteins. Although, so far only scant attention has been given to this issue, fluorescence *in situ* hybridization (FISH) and immunofluorescence microscopy (IF) revealed that mRNA encoding a subunit of the light harvesting complex II (LHCII) and 80S ribosomes colocalize at distinct regions of the chloroplast (Uniacke and Zerges, 2009; Weis et al., 2013). However, experimental evidence if such targeting pathway also exists for chloroplast β-barrel proteins is still lacking.

The discrimination between the two pools of β-barrel proteins could also be achieved by utilization of a distinct subset of cytosolic factors that guide chloroplast precursors to their particular target organelle. A challenge in future studies will be the identification of these putative factors. In case such mechanism exists, it remains to be explored how these factors recognize specifically chloroplast β-barrel proteins.

We further demonstrated that the evolutionary link between bacteria and mitochondria allows the latter to assemble trimeric prokaryotic β-barrel proteins in their functional form although such proteins are completely absent in eukaryotes. Taking advantage of this surprising capacity, yeast mitochondria provide a useful model system to shed light on the biogenesis of trimeric autotransporter adhesins. In addition to a detailed dissection of the biogenesis pathway this further allowed us to investigate the

potential contribution of single chaperones to the overall biogenesis of YadA. In this way we could show that upon expression in yeast cells YadA is targeted to mitochondria where it assembles into the OM in its native functional trimeric form. This assembly was highly dependent on the TOB complex. In contrast to the bacterial system, we were able to detect a partially soluble monomeric form of YadA in the IMS. This monomer was rather unstable and eventually degraded by mitochondrial proteases. Interestingly, co-expression of the periplasmic chaperone Skp with full-length YadA or with the membrane anchor of YadA resulted in significantly elevated levels of both the trimeric and monomeric species (Fig. 3). We could demonstrate that Skp stabilizes the precursor proteins in the IMS and therefore protects them from degradation by mitochondrial proteases. Similar effects were not observed for co-expression with other bacterial chaperones like SecB or SurA.

Of note, contradictory results exist about the different roles of bacterial chaperones in the biogenesis of OMPs and little is known about their importance in the biogenesis of TAAs (Grijpstra et al., 2013; Hagan et al., 2011; Volokhina et al., 2011). Our findings provide, for the first time, an indication for the involvement of the periplasmic chaperone Skp in the biogenesis of these proteins. However, future studies have to confirm whether these results also hold true in the bacterial system.

In bacteria, Skp was reported to assist the biogenesis of OMPs by specific binding to unfolded precursor forms and thereby preventing their unproductive aggregation or degradation in the periplasm (Grijpstra et al., 2013; Walton et al., 2009). The biogenesis of only certain *E. coli* OMPs was suggested to depend on Skp and the chaperone was assumed to mainly sequester precursors that have fallen off the favored SurA route. In contrast to this, a recent study in *N. meningitidis* has revealed an important role for Skp but not for SurA or DegP in the biogenesis of OMPs (Hagan et al., 2011; Schwalm et al., 2013; Volokhina et al., 2011). The capacity of Skp to stabilize the monomeric form of YadA suggests a similar role in the biogenesis of YadA. These results are in line with the

recent findings that Skp plays an important role in the early stages of the periplasmic transit of the autotransporter EspP (Ieva et al., 2011). In contrast to the effect of Skp on the stability of YadA in the IMS, we could not observe such an effect upon co-expression of the periplasmic chaperone SurA. Recent studies described the interaction of SurA with POTRA domain 1 of BamA (Simmerman et al., 2014; Sklar et al., 2007b; Vuong et al., 2008). As the single POTRA domain of the mitochondrial homolog Tob55 is thought to correspond to POTRA5 of BamA, it might also be conceivable that in the yeast model system SurA was simply unable to bind to the TOB complex (Fig. 3).

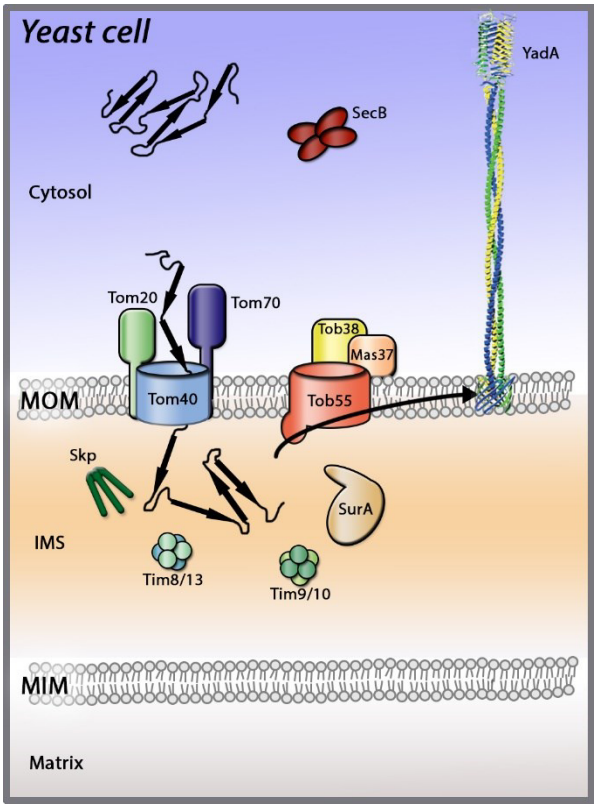


Figure 3. Yeast mitochondria as a model system to study the biogenesis of YadA

Upon transit across the periplasm, the BAM machinery is crucial for recognition of autotransporters at the OM and for the subsequent assembly of the membrane-anchor domain into the lipid bilayer. Although a recent study identified the translocation and assembly module (TAM) to be responsible for the membrane integration of certain autotransporter proteins, it is currently unknown whether this system is also involved in

the biogenesis of TAAs (Selkrig et al., 2012). At least the heterologous expression of YadA in *E. coli* argues against this idea, since its membrane assembly was shown to depend on BamA (Lehr et al., 2010). Little is known about how the BAM complex facilitates the translocation of the passenger domain across the OM and in which way the BAM subunits are involved in this process (Leo et al., 2012; Leyton et al., 2012; Pavlova et al., 2013; Sauri et al., 2011). Interestingly, our results demonstrated that the presence of the mitochondrial BamA homolog Tob55 can facilitate the targeting and assembly of YadA in its native functional conformation, although the BAM and TOB complexes display rather limited similarity in the accessory proteins. Accordingly, the BAM subunits BamB-E seem not to be absolutely necessary for these biogenesis steps. Most probably the presence of these components results in comparatively better kinetics in substrate assembly, since in the bacterial system intermediate forms of YadA could not be detected. Considering this, it is remarkable that the evolutionary conservation in the central components that facilitate the assembly of β-barrel proteins in bacteria and eukaryotes provides yeast mitochondria with the capacity to correctly fold and integrate TAAs into the MOM, although such exceptional proteins are completely absent from eukaryotes.

For a long time it has been under debate at which stage the individual subunits of TAAs come together in order to form the 12-stranded β-domain. The models range from early oligomerization in the periplasm to full trimerization only upon membrane integration of each of the three monomers. Our inability to detect trimeric YadA which is both soluble and protected from externally-added protease suggests that the trimerization process occurs only at the membrane, probably upon interaction with the TOB complex. This would be in line with the recent finding that precursors of mitochondrial β-barrel proteins do not acquire their β-barrel structure before they interact with the TOB complex (Qiu et al., 2013).

Collectively, our findings suggest that the common ancestry of bacteria and mitochondria allows the latter to recognize and process prokaryote-specific substrates. The evolutionary conservation in the biogenesis of β-barrel proteins allows the usage of yeast mitochondria as a model system to investigate the biogenesis pathways of TAAs and to gain insight into the potential involvement of periplasmic chaperones in these processes.

10 References

Ackermann, N., Tiller, M., Anding, G., Roggenkamp, A., Heesemann, J., 2008. Contribution of trimeric autotransporter C-terminal domains of oligomeric coiled-coil adhesin (Oca) family members YadA, UspA1, EibA, and Hia to translocation of the YadA passenger domain and virulence of Yersinia enterocolitica. J. Bacteriol. 190, 5031-5043.

Arnold, T., Zeth, K., Linke, D., 2010. Omp85 from the thermophilic cyanobacterium Thermosynechococcus elongatus differs from proteobacterial Omp85 in structure and domain composition. J. Biol. Chem. 285, 18003-18015.

Balligand, G., Laroche, Y., Cornelis, G., 1985. Genetic analysis of virulence plasmid from a serogroup 9 Yersinia enterocolitica strain: role of outer membrane protein P1 in resistance to human serum and autoagglutination. Infect. Immun. 48, 782-786.

Bayrhuber, M., Meins, T., Habeck, M., Becker, S., Giller, K., Villinger, S., Vonrhein, C., Griesinger, C., Zweckstetter, M., Zeth, K., 2008. Structure of the human voltage-dependent anion channel. Proc. Natl. Acad. Sci. U. S. A. 105, 15370-15375.

Bechtluft, P., Nouwen, N., Tans, S.J., Driessen, A.J., 2010. SecB—a chaperone dedicated to protein translocation. Mol. Biosyst. 6, 620-627.

Bennion, D., Charlson, E.S., Coon, E., Misra, R., 2010. Dissection of beta-barrel outer membrane protein assembly pathways through characterizing BamA POTRA 1 mutants of Escherichia coli. Mol. Microbiol. 77, 1153-1171.

Beverly, K.N., Sawaya, M.R., Schmid, E., Koehler, C.M., 2008. The Tim8-Tim13 complex has multiple substrate binding sites and binds cooperatively to Tim23. J. Mol. Biol. 382, 1144-1156.

Bos, M.P., Robert, V., Tommassen, J., 2007. Functioning of outer membrane protein assembly factor Omp85 requires a single POTRA domain. EMBO Rep. 8, 1149-1154.

Chacinska, A., Koehler, C.M., Milenkovic, D., Lithgow, T., Pfanner, N., 2009. Importing mitochondrial proteins: machineries and mechanisms. Cell 138, 628-644.

Chan, N.C., Lithgow, T., 2008. The Peripheral Membrane Subunits of the SAM Complex Function Codependently in Mitochondrial Outer Membrane Biogenesis. Mol. Biol. Cell 19, 126-136.

Chew, O., Lister, R., Qbadou, S., Heazlewood, J.L., Soll, J., Schleiff, E., Millar, A.H., Whelan, J., 2004. A plant outer mitochondrial membrane protein with high amino acid sequence identity to a chloroplast protein import receptor. FEBS letters 557, 109-114.

Cline, K., Andrews, J., Mersey, B., Newcomb, E.H., Keegstra, K., 1981. Separation and characterization of inner and outer envelope membranes of pea chloroplasts. Proc. Natl. Acad. Sci. U. S. A. 78, 3595-3599.

Day, P.M., Potter, D., Inoue, K., 2014. Evolution and targeting of Omp85 homologs in the chloroplast outer envelope membrane. Front. Plant. Sci. 5, 535.

Driessen, A.J., Nouwen, N., 2008. Protein translocation across the bacterial cytoplasmic membrane. Annu. Rev. Biochem. 77, 643-667.

Duncan, O., Murcha, M.W., Whelan, J., 2012. Unique components of the plant mitochondrial protein import apparatus. Biochim. Biophys. Acta 1833(2):304-313.

Endo, T., Yamano, K., 2009. Multiple pathways for mitochondrial protein traffic. Biol. Chem. 390, 723-730.

Ferbitz, L., Maier, T., Patzelt, H., Bukau, B., Deuerling, E., Ban, N., 2004. Trigger factor in complex with the ribosome forms a molecular cradle for nascent proteins. Nature 431, 590-596.

Gatsos, X., Perry, A.J., Anwari, K., Dolezal, P., Wolynec, P.P., Likic, V.A., Purcell, A.W., Buchanan, S.K., Lithgow, T., 2008. Protein secretion and outer membrane assembly in Alphaproteobacteria. FEMS Microbiol. Rev. 32, 995-1009.

Gatzeva-Topalova, P.Z., Walton, T.A., Sousa, M.C., 2008. Crystal structure of YaeT: conformational flexibility and substrate recognition. Structure 16, 1873-1881.

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

Gessmann, D., Flinner, N., Pfannstiel, J., Schlosinger, A., Schleiff, E., Nussberger, S., Mirus, O., 2011. Structural elements of the mitochondrial preprotein-conducting channel Tom40 dissolved by bioinformatics and mass spectrometry. Biochim. Biophys. Acta 1807(12):1647-1657.

Goetze, T.A., Philippar, K., Ilkavets, I., Soll, J., Wagner, R., 2006. OEP37 is a new member of the chloroplast outer membrane ion channels. J. Biol. Chem. 281, 17989-17998.

Gray, M.W., 2011. The incredible shrinking organelle. EMBO Rep. 12, 873.

Gray, M.W., Burger, G., Lang, B.F., 1999. Mitochondrial evolution. Science 283, 1476-1481.

Grijpstra, J., Arenas, J., Rutten, L., Tommassen, J., 2013. Autotransporter secretion: varying on a theme. Res. Microbiol. 164, 562-582.

Grosskinsky, U., Schutz, M., Fritz, M., Schmid, Y., Lamparter, M.C., Szczesny, P., Lupas, A.N., Autenrieth, I.B., Linke, D., 2007. A conserved glycine residue of trimeric autotransporter domains plays a key role in Yersinia adhesin A autotransport. J. Bacteriol. 189, 9011-9019.

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

Habib, S.J., Waizenegger, T., Niewienda, A., Paschen, S.A., Neupert, W., Rapaport, D., 2007. The N-terminal domain of Tob55 has a receptor-like function in the biogenesis of mitochondrial beta-barrel proteins. J. Cell Biol. 176, 77-88.

Hagan, C.L., Silhavy, T.J., Kahne, D., 2011. beta-Barrel membrane protein assembly by the Bam complex. Annu. Rev. Biochem. 80, 189-210.

Harrison, S.C., 1996. Peptide-surface association: the case of PDZ and PTB domains. Cell 86, 341-343.

Hiller, S., Garces, R.G., Malia, T.J., Orekhov, V.Y., Colombini, M., Wagner, G., 2008. Solution structure of the integral human membrane protein VDAC-1 in detergent micelles. Science 321, 1206-1210.

Hoiczyk, E., Roggenkamp, A., Reichenbecher, M., Lupas, A., Heesemann, J., 2000. Structure and sequence analysis of Yersinia YadA and Moraxella UspAs reveal a novel class of adhesins. EMBO J. 19, 5989-5999.

Hoppins, S.C., Nargang, F.E., 2004. The Tim8-Tim13 complex of Neurospora crassa functions in the assembly of proteins into both mitochondrial membranes. J. Biol. Chem. 279, 12396-12405.

Ieva, R., Tian, P., Peterson, J.H., Bernstein, H.D., 2011. Sequential and spatially restricted interactions of assembly factors with an autotransporter beta domain. Proceedings of the Nat. Acad. Sci. U. S. A. 108, E383-391.

Inoue, K., Baldwin, A.J., Shipman, R.L., Matsui, K., Theg, S.M., Ohme-Takagi, M., 2005. Complete maturation of the plastid protein translocation channel requires a type I signal peptidase. J. Cell Biol. 171, 425-430.

Inoue, K., Demel, R., de Kruijff, B., Keegstra, K., 2001. The N-terminal portion of the preToc75 transit peptide interacts with membrane lipids and inhibits binding and import of precursor proteins into isolated chloroplasts. Eur. J. Biochem. 268, 4036-4043.

Ishikawa, D., Yamamoto, H., Tamura, Y., Moritoh, K., Endo, T., 2004. Two novel proteins in the mitochondrial outer membrane mediate {beta}-barrel protein assembly. J. Cell Biol. 166, 621-627.

Jänsch, L., Kruft, V., Schmitz, U.K., Braun, H.-P., 1998. Unique Composition of the Preprotein Translocase of the Outer Mitochondrial Membrane from Plants. J. Biol. Chem. 273, 17251-17257.

Joyard, J., Ferro, M., Masselon, C., Seigneurin-Berny, D., Salvi, D., Garin, J., Rolland, N., 2009. Chloroplast proteomics and the compartmentation of plastidial isoprenoid biosynthetic pathways. Mol. Plant 2, 1154-1180.

Kim, S., Malinverni, J.C., Sliz, P., Silhavy, T.J., Harrison, S.C., Kahne, D., 2007. Structure and function of an essential component of the outer membrane protein assembly machine. Science 317, 961-964.

Knowles, T.J., Jeeves, M., Bobat, S., Dancea, F., McClelland, D., Palmer, T., Overduin, M., Henderson, I.R., 2008. Fold and function of polypeptide transport-associated domains responsible for delivering unfolded proteins to membranes. Mol. Microbiol. 68, 1216-1227.

Koenig, P., Mirus, O., Haarmann, R., Sommer, M.S., Sinning, I., Schleiff, E., Tews, I., 2010. Conserved properties of polypeptide transport-associated (POTRA) domains derived from cyanobacterial Omp85. J. Biol. Chem. 285, 18016-18024.

Komiya, T., Rospert, S., Koehler, C., Looser, R., Schatz, G., Mihara, K., 1998. Interaction of Mitochondrial Targeting Signals with Acidic Receptor Domains along the Protein Import Pathway: Evidence for the "Acidic Chain" hypothesis. EMBO J. 17, 3886-3898.

Kozjak-Pavlovic, V., Ott, C., Gotz, M., Rudel, T., 2011. Neisserial omp85 protein is selectively recognized and assembled into functional complexes in the outer membrane of human mitochondria. J. Biol. Chem. 286, 27019-27026.

Krimmer, T., Rapaport, D., Ryan, M.T., Meisinger, C., Kassenbrock, C.K., Blachly-Dyson, E., Forte, M., Douglas, M.G., Neupert, W., Nargang, F.E., Pfanner, N., 2001. Biogenesis of the major mitochondrial outer membrane protein porin involves a complex import pathway via receptors and the general import pore. J. Cell Biol. 152, 289-300.

Kutik, S., Stojanovski, D., Becker, L., Becker, T., Meinecke, M., Kruger, V., Prinz, C., Meisinger, C., Guiard, B., Wagner, R., Pfanner, N., Wiedemann, N., 2008. Dissecting membrane insertion of mitochondrial beta-barrel proteins. Cell 132, 1011-1024.

Lackey, S.W., Taylor, R.D., Go, N.E., Wong, A., Sherman, E.L., Nargang, F.E., 2014. Evidence supporting the 19 beta-strand model for Tom40 from cysteine scanning and protease site accessibility studies. J. Biol. Chem. 289, 21640-21650.

Lazar, S.W., Kolter, R., 1996. SurA assists the folding of Escherichia coli outer membrane proteins. J. Bacteriol. 178, 1770-1773.

Lehr, U., Schutz, M., Oberhettinger, P., Ruiz-Perez, F., Donald, J.W., Palmer, T., Linke, D., Henderson, I.R., Autenrieth, I.B., 2010. C-terminal amino acid residues of the trimeric autotransporter adhesin YadA of Yersinia enterocolitica are decisive for its recognition and assembly by BamA. Mol. Microbiol. 78, 932-946.

Leo, J.C., Grin, I., Linke, D., 2012. Type V secretion: mechanism(s) of autotransport through the bacterial outer membrane. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 367, 1088-1101.

Leonhard, K., Stiegler, A., Neupert, W., Langer, T., 1999. Chaperone-like activity of the AAA domain of the yeast Yme1 AAA protease. Nature 398, 348-351.

Leyton, D.L., Rossiter, A.E., Henderson, I.R., 2012. From self sufficiency to dependence: mechanisms and factors important for autotransporter biogenesis. Nat. Rev. Microbiol. 10, 213-225.

Linke, D., Riess, T., Autenrieth, I.B., Lupas, A., Kempf, V.A., 2006. Trimeric autotransporter adhesins: variable structure, common function. Trends Microbiol. 14, 264-270.

Macasev, D., Newbigin, E., Whelan, J., Lithgow, T., 2000. How Do Plant Nitochondria Avoid Importing Chloroplast Proteins? Components of the Import Apparatus Tom20 and Tom22 from Arabidopsis Differ from Their Fungal Counterparts. Plant Physiol. 123, 811-816.

Malinverni, J.C., Werner, J., Kim, S., Sklar, J.G., Kahne, D., Misra, R., Silhavy, T.J., 2006. YfiO stabilizes the YaeT complex and is essential for outer membrane protein assembly in Escherichia coli. Mol. Microbiol. 61, 151-164.

McFadden, G.I., 2001. Chloroplast origin and integration. Plant Physiol. 125, 50-53.

Milenkovic, D., Kozjak, V., Wiedemann, N., Lohaus, C., Meyer, H.E., Guiard, B., Pfanner, N., Meisinger, C., 2004. Sam35 of the mitochondrial protein sorting and assembly machinery is a peripheral outer membrane protein essential for cell viability. J. Biol. Chem. 279, 22781-22785.

Model, K., Meisinger, C., Prinz, T., Wiedemann, N., Truscott, K.N., Pfanner, N., Ryan, M.T., 2001. Multistep assembly of the protein import channel of the mitochondrial outer membrane. Nat. Struct. Biol. 8, 361-370.

Müller, J.E., Papic, D., Ulrich, T., Grin, I., Schutz, M., Oberhettinger, P., Tommassen, J., Linke, D., Dimmer, K.S., Autenrieth, I.B., Rapaport, D., 2011. Mitochondria can recognize and assemble fragments of a beta-barrel structure. Mol. Biol. Cell 22, 1638-1647.

Nargang, F.E., Rapaport, D., Ritzel, R.G., Neupert, W., Lill, R., 1998. Role of the negative charges in the cytosolic domain of TOM22 in the import of precursor proteins into mitochondria. Mol. Cell. Biol. 18, 3173-3181.

Noinaj, N., Kuszak, A.J., Balusek, C., Gumbart, J.C., Buchanan, S.K., 2014. Lateral Opening and Exit Pore Formation Are Required for BamA Function. Structure 22, 1055-1062.

Noinaj, N., Kuszak, A.J., Gumbart, J.C., Lukacik, P., Chang, H., Easley, N.C., Lithgow, T., Buchanan, S.K., 2013. Structural insight into the biogenesis of beta-barrel membrane proteins. Nature 501, 385-390.

Paetzel, M., 2014. Structure and mechanism of Escherichia coli type I signal peptidase. Biochim. Biophys. Acta 1843(8):1497-1508.

Papanikou, E., Karamanou, S., Economou, A., 2007. Bacterial protein secretion through the translocase nanomachine. Nat. Rev. Microbiol. 5, 839-851.

Park, E., Rapoport, T.A., 2012. Mechanisms of Sec61/SecY-mediated protein translocation across membranes. Annu. Rev. Biophys. 41, 21-40.

Paschen, S.A., Neupert, W., Rapaport, D., 2005. Biogenesis of beta-barrel membrane proteins of mitochondria. Trends Biochem. Sci. 30, 575-582.

Paschen, S.A., Waizenegger, T., Stan, T., Preuss, M., Cyrklaff, M., Hell, K., Rapaport, D., Neupert, W., 2003. Evolutionary conservation of biogenesis of b-barrel membrane proteins. Nature 426, 862-866.

Patel, R., Hsu, S.C., Bedard, J., Inoue, K., Jarvis, P., 2008. The Omp85-related chloroplast outer envelope protein OEP80 is essential for viability in Arabidopsis. Plant Physiol. 148, 235-245.

Pavlova, O., Peterson, J.H., Ieva, R., Bernstein, H.D., 2013. Mechanistic link between beta barrel assembly and the initiation of autotransporter secretion. Proc. Natl. Acad. Sci. U. S. A. 110, E938-947.

Perry, A.J., Rimmer, K.A., Mertens, H.D., Waller, R.F., Mulhern, T.D., Lithgow, T., Gooley, P.R., 2008. Structure, topology and function of the translocase of the outer membrane of mitochondria. Plant Physiol. Biochem. 46(3), 265-274.

Pfanner, N., Wiedemann, N., Meisinger, C., Lithgow, T., 2004. Assembling the mitochondrial outer membrane. Nat. Struct. Mol. Biol. 11, 1044-1048.

Pohlmeyer, K., Soll, J., Grimm, R., Hill, K., Wagner, R., 1998. A high-conductance solute channel in the chloroplastic outer envelope from Pea. Plant Cell 10, 1207-1216.

Qiao, S., Luo, Q., Zhao, Y., Zhang, X.C., Huang, Y., 2014. Structural basis for lipopolysaccharide insertion in the bacterial outer membrane. Nature 511, 108-111.

Qiu, J., Wenz, L.S., Zerbes, R.M., Oeljeklaus, S., Bohnert, M., Stroud, D.A., Wirth, C., Ellenrieder, L., Thornton, N., Kutik, S., Wiese, S., Schulze-Specking, A., Zufall, N., Chacinska, A., Guiard, B., Hunte, C., Warscheid, B., van der Laan, M., Pfanner, N., Wiedemann, N., Becker, T., 2013. Coupling of mitochondrial import and export translocases by receptor-mediated supercomplex formation. Cell 154, 596-608.

Randall, L.L., Hardy, S.J., 2002. SecB, one small chaperone in the complex milieu of the cell. Cell. Mol. Life Sci. 59, 1617-1623.

Rapaport, D., Künkele, K.-P., Dembowski, M., Ahting, U., Nargang, F.E., Neupert, W., Lill, R., 1998a. Dynamics of the TOM Complex of Mitochondria during Binding and Translocation of Preproteins. Mol. Cell. Biol. 18, 5256-5262.

Rapaport, D., Mayer, A., Neupert, W., Lill, R., 1998b. Cis and trans sites of the TOM complex in unfolding and initial translocation of preproteins. J. Biol. Chem. 273, 8806-8813.

Rapaport, D., Neupert, W., 1999. Biogenesis of Tom40, core component of the TOM complex of mitochondria. J. Cell Biol. 146, 321-331.

Remmert, M., Biegert, A., Linke, D., Lupas, A.N., Soding, J., 2010. Evolution of outer membrane beta-barrels from an ancestral beta beta hairpin. Mol. Biol. Evol. 27, 1348-1358.

Rohl, T., Motzkus, M., Soll, J., 1999. The outer envelope protein OEP24 from pea chloroplasts can functionally replace the mitochondrial VDAC in yeast. FEBS letters 460, 491-494.

Rouviere, P.E., Gross, C.A., 1996. SurA, a periplasmic protein with peptidyl-prolyl isomerase activity, participates in the assembly of outer membrane porins. Genes Dev. 10, 3170-3182.

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

Sauri, A., Oreshkova, N., Soprova, Z., Jong, W.S., Sani, M., Peters, P.J., Luirink, J., van Ulsen, P., 2011. Autotransporter beta-domains have a specific function in protein secretion beyond outer-membrane targeting. J. Mol. Biol. 412, 553-567.

Schatz, G., 1997. Just follow the acid chain. Nature 388, 121-122.

Schindler, M.K., Schutz, M.S., Muhlenkamp, M.C., Rooijakkers, S.H., Hallstrom, T., Zipfel, P.F., Autenrieth, I.B., 2012. Yersinia enterocolitica YadA mediates complement evasion by recruitment and inactivation of C3 products. J. Immunol. 189, 4900-4908.

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

Schmid, Y., Grassl, G.A., Buhler, O.T., Skurnik, M., Autenrieth, I.B., Bohn, E., 2004. Yersinia enterocolitica adhesin A induces production of interleukin-8 in epithelial cells. Infect. Immun. 72, 6780-6789.

Schütz, M., Weiss, E.M., Schindler, M., Hallstrom, T., Zipfel, P.F., Linke, D., Autenrieth, I.B., 2010. Trimer stability of YadA is critical for virulence of Yersinia enterocolitica. Infect. Immun. 78, 2677-2690.

Schwalm, J., Mahoney, T.F., Soltes, G.R., Silhavy, T.J., 2013. Role for Skp in LptD assembly in Escherichia coli. J. Bacteriol. 195, 3734-3742.

Selkrig, J., Mosbahi, K., Webb, C.T., Belousoff, M.J., Perry, A.J., Wells, T.J., Morris, F., Leyton, D.L., Totsika, M., Phan, M.D., Celik, N., Kelly, M., Oates, C., Hartland, E.L., Robins-Browne, R.M., Ramarathinam, S.H., Purcell, A.W., Schembri, M.A., Strugnell, R.A., Henderson, I.R., Walker, D., Lithgow, T., 2012. Discovery of an archetypal protein transport system in bacterial outer membranes. Nat. Struct. Mol. Biol. 19, 506-510, S501.

Simmerman, R.F., Dave, A.M., Bruce, B.D., 2014. Structure and function of POTRA domains of Omp85/TPS superfamily. Intl. Rev. Cell Mol. Biol. 308, 1-34.

Sklar, J.G., Wu, T., Gronenberg, L.S., Malinverni, J.C., Kahne, D., Silhavy, T.J., 2007a. Lipoprotein SmpA is a component of the YaeT complex that assembles outer membrane proteins in Escherichia coli. Proc. Natl. Acad. Sci. U. S. A. 104, 6400-6405.

Sklar, J.G., Wu, T., Kahne, D., Silhavy, T.J., 2007b. Defining the roles of the periplasmic chaperones SurA, Skp, and DegP in Escherichia coli. Genes Dev. 21, 2473-2484.

Soll, J., Schleiff, E., 2004. Protein import into chloroplasts. Nat. Rev. Mol. Cell Biol. 5, 198-208.

Sommer, M.S., Daum, B., Gross, L.E., Weis, B.L., Mirus, O., Abram, L., Maier, U.G., Kuhlbrandt, W., Schleiff, E., 2011. Chloroplast Omp85 proteins change orientation during evolution. Proc. Natl Acad. Sci. U. S. A. 108, 13841-13846.

Tahir, Y.E., Kuusela, P., Skurnik, M., 2000. Functional mapping of the Yersinia enterocolitica adhesin YadA. Identification Of eight NSVAIG - S motifs in the amino-terminal half of the protein involved in collagen binding. Mol. Microbiol. 37, 192-206.

Tamm, A., Tarkkanen, A.M., Korhonen, T.K., Kuusela, P., Toivanen, P., Skurnik, M., 1993. Hydrophobic domains affect the collagen-binding specificity and surface polymerization as well as the virulence potential of the YadA protein of Yersinia enterocolitica. Mol. Microbiol. 10, 995-1011.

Tertti, R., Skurnik, M., Vartio, T., Kuusela, P., 1992. Adhesion protein YadA of Yersinia species mediates binding of bacteria to fibronectin. Infect. Immun. 60, 3021-3024.

Tranel, P.J., Keegstra, K., 1996. A novel, bipartite transit peptide targets OEP75 to the outer membrane of the chloroplastic envelope. Plant Cell 8, 2093-2104.

Ujwal, R., Cascio, D., Colletier, J.P., Faham, S., Zhang, J., Toro, L., Ping, P., Abramson, J., 2008. The crystal structure of mouse VDAC1 at 2.3 A resolution reveals mechanistic insights into metabolite gating. Proc. Natl. Acad. Sci. U. S. A. 105, 17742-17747.

Ullers, R.S., Luirink, J., Harms, N., Schwager, F., Georgopoulos, C., Genevaux, P., 2004. SecB is a bona fide generalized chaperone in Escherichia coli. Proc. Natl. Acad. Sci. U. S. A. 101, 7583-7588.

Uniacke, J., Zerges, W., 2009. Chloroplast protein targeting involves localized translation in Chlamydomonas. Proc. Natl. Acad. Sci. U. S. A. 106, 1439-1444.

Valent, Q.A., Kendall, D.A., High, S., Kusters, R., Oudega, B., Luirink, J., 1995. Early events in preprotein recognition in E. coli: interaction of SRP and trigger factor with nascent polypeptides. EMBO J. 14, 5494-5505.

Vertommen, D., Ruiz, N., Leverrier, P., Silhavy, T.J., Collet, J.F., 2009. Characterization of the role of the Escherichia coli periplasmic chaperone SurA using differential proteomics. Proteomics 9, 2432-2443.

Volokhina, E.B., Grijpstra, J., Stork, M., Schilders, I., Tommassen, J., Bos, M.P., 2011. Role of the periplasmic chaperones Skp, SurA, and DegQ in outer membrane protein biogenesis in Neisseria meningitidis. J. Bacteriol. 193, 1612-1621.

Voulhoux, R., Bos, M.P., Geurtsen, J., Mols, M., Tommassen, J., 2003. Role of a highly conserved bacterial protein in outer membrane protein assembly. Science 299, 262-265.

Vuong, P., Bennion, D., Mantei, J., Frost, D., Misra, R., 2008. Analysis of YfgL and YaeT interactions through bioinformatics, mutagenesis, and biochemistry. J. Bacteriol. 190, 1507-1517.

Waizenegger, T., Habib, S.J., Lech, M., Mokranjac, D., Paschen, S.A., Hell, K., Neupert, W., Rapaport, D., 2004. Tob38, a novel essential component in the biogenesis of beta-barrel proteins of mitochondria. EMBO Rep. 5, 704-709.

Walther, D.M., Bos, M.P., Rapaport, D., Tommassen, J., 2010. The mitochondrial porin, VDAC, has retained the ability to be assembled in the bacterial outer membrane. Mol. Biol. Evol. 27, 887-895.

Walther, D.M., Papic, D., Bos, M.P., Tommassen, J., Rapaport, D., 2009a. Signals in bacterial b-barrel proteins are functional in eukaryotic cells for targeting to and assembly in mitochondria. Proc. Natl. Acad. Sci. USA 106, 2531-2536.

Walther, D.M., Rapaport, D., Tommassen, J., 2009b. Biogenesis of beta-barrel membrane proteins in bacteria and eukaryotes: evolutionary conservation and divergence. Cell. Mol. Life Sci. 66, 2789-2804.

Walton, T.A., Sandoval, C.M., Fowler, C.A., Pardi, A., Sousa, M.C., 2009. The cavity-chaperone Skp protects its substrate from aggregation but allows independent folding of substrate domains. Proc. Natl. Acad. Sci. U. S. A. 106, 1772-1777.

Walton, T.A., Sousa, M.C., 2004. Crystal structure of Skp, a prefoldin-like chaperone that protects soluble and membrane proteins from aggregation. Mol.Cell 15, 367-374.

Webb, C.T., Gorman, M.A., Lazarou, M., Ryan, M.T., Gulbis, J.M., 2006. Crystal structure of the mitochondrial chaperone TIM9.10 reveals a six-bladed alpha-propeller. Mol. Cell 21, 123-133.

Weis, B.L., Schleiff, E., Zerges, W., 2013. Protein targeting to subcellular organelles via MRNA localization. Biochim. Biophys. Acta 1833, 260-273.

Wenz, L.S., Opalinski, L., Schuler, M.H., Ellenrieder, L., Ieva, R., Bottinger, L., Qiu, J., van der Laan, M., Wiedemann, N., Guiard, B., Pfanner, N., Becker, T., 2014. The presequence pathway is involved in protein sorting to the mitochondrial outer membrane. EMBO Rep. 15, 678-685.

Wiedemann, N., Frazier, A.E., Pfanner, N., 2004a. The protein import machinery of mitochondria. J. Biol. Chem. 279, 14473-14476.

Wiedemann, N., Kozjak, V., Chacinska, A., Schönfish, B., Rospert, S., Ryan, M.T., Pfanner, N., Meisinger, C., 2003. Machinery for protein sorting and assembly in the mitochondrial outer membrane. Nature 424, 565-571.

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

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

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

Xie, K., Hessa, T., Seppala, S., Rapp, M., von Heijne, G., Dalbey, R.E., 2007. Features of transmembrane segments that promote the lateral release from the translocase into the lipid phase. Biochemistry 46, 15153-15161.

Yamano, K., Yatsukawa, Y., Esaki, M., Hobbs, A.E., Jensen, R.E., Endo, T., 2008. Tom20 and Tom22 share the common signal recognition pathway in mitochondrial protein import. J. Biol. Chem. 283, 3799-3807.

Zeth, K., 2010. Structure and evolution of mitochondrial outer membrane proteins of beta-barrel topology. Biochim. Biophys. Acta 1797, 1292-1299.

Zimmer, J., Nam, Y., Rapoport, T.A., 2008. Structure of a complex of the ATPase SecA and the protein-translocation channel. Nature 455, 936-943.

11 Acknowledgements

First of all I want to articulate my special thanks to Prof. Doron Rapaport for providing me the opportunity to work on these interesting projects in his lab. For his indomitable optimism whenever something went wrong and for his extensive support in all respects.

I am deeply grateful to Dr. Kai Stefan Dimmer and Dr. Dražen Papić for always having an open ear for my questions, for all their suggestions and advices and for critically reading the manuscript.

I also want to express my gratitude to all current and former lab members for fruitful discussions, helpful suggestions and for a pleasant working atmosphere:
Anja Kosnopfel, Benjamin Schumann, Bogdan Cichocki, Daniela Vitali, Diana Antunes, Desiree Sperl, Elisa Merklinger, Dr. Gertraud Engel, Dr. Hiroki Kato, Hoda Hoseini, Jonas Müller, Dr. Jovana Đukanović, Dr. Katrin Krumpe, Monika Sinzel, Ravi Singhal, Sophia Koerdt, Dr. Tan Tao and Tobias Jores.

Additionally, I want to acknowledge Elena Kracker, Katharina Rehn and Caroline Schoenfeld for their excellent technical assistance as well as AK Jansen and AK Dodt for sharing materials and equipment in times of need.

For their unconditional support through all phases of my life my deepest and sincere thanks go to my parents.

Anja, thanks for your patience and sympathy, for always being there for me and for your love.

12 Appendix

1. Ulrich, T., L.E. Gross, M.S. Sommer, E. Schleiff and D. Rapaport. 2012. Chloroplast β-barrel proteins are assembled into the mitochondrial outer membrane in a process that depends on the TOM and TOB complexes. *J. Biol. Chem.* 287:27467-27479.

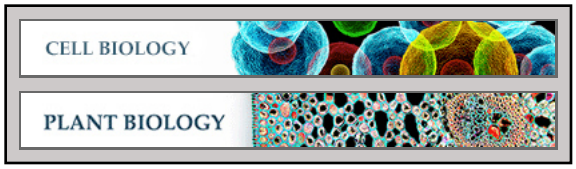
2. Ulrich, T., P. Oberhettinger, M. Schütz, K. Holzer, A.S. Ramms, D. Linke, I.B. Autenrieth and D. Rapaport. 2014. Evolutionary conservation in biogenesis of β-barrel proteins allows mitochondria to assemble a functional bacterial trimeric autotransporter protein. *J. Biol. Chem.* 289:29457-29470.

3. Ulrich, T., P. Oberhettinger, I.B. Autenrieth and D. Rapaport. 2015. Yeast mitochondria as a model system to study the biogenesis of bacterial β-barrel proteins. In *Methods in Molecular Biology – The BAM complex: Methods and protocols*. Buchanan S. and Noinaj N., editors. Humana Press. Accepted for publication.

4. Ulrich, T. and D. Rapaport. 2015. Biogenesis of β-barrel proteins in evolutionary context. *Intl. J. Med. Microbiol.* Accepted for publication.

Cell Biology:

Chloroplast β-Barrel Proteins Are Assembled into the Mitochondrial Outer Membrane in a Process That Depends on the TOM and TOB Complexes



Thomas Ulrich, Lucia E. Gross, Maik S. Sommer, Enrico Schleiff and Doron Rapaport

J. Biol. Chem. 2012, 287:27467-27479.
doi: 10.1074/jbc.M112.382093 originally published online June 28, 2012

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 33, pp. 27467–27479, August 10, 2012

Chloroplast β-Barrel Proteins Are Assembled into the Mitochondrial Outer Membrane in a Process That Depends on the TOM and TOB Complexes*

Received for publication, May 15, 2012, and in revised form, June 27, 2012 Published, JBC Papers in Press, June 28, 2012, DOI 10.1074/jbc.M112.382093

Thomas Ulrich[‡1], **Lucia E. Gross**[§1], **Maik S. Sommer**[§], **Enrico Schleiff**[§], **and Doron Rapaport**[‡2]

From the [‡]*Interfaculty Institute of Biochemistry, University of Tübingen, 72076 Tübingen and the* [§]*Centre of Membrane Proteomics and Cluster of Excellence Frankfurt, Department of Biosciences, Molecular Cell Biology of Plants, Goethe University, D-60438 Frankfurt, Germany*

Background: The signal that ensures the specific targeting of β-barrel proteins to either mitochondria or chloroplasts is ill-defined.
Results: Chloroplast β-barrel proteins can be assembled *in vitro* into the mitochondrial outer membrane.
Conclusion: The mitochondrial import machinery can recognize and process chloroplast β-barrel proteins as substrates.
Significance: Dedicated targeting factors had to evolve in plant cells to prevent mis-sorting of chloroplast β-barrel proteins to mitochondria.

Membrane-embedded β-barrel proteins are found in the outer membranes (OM) of Gram-negative bacteria, mitochondria and chloroplasts. In eukaryotic cells, precursors of these proteins are synthesized in the cytosol and have to be sorted to their corresponding organelle. Currently, the signal that ensures their specific targeting to either mitochondria or chloroplasts is ill-defined. To address this issue, we studied targeting of the chloroplast β-barrel proteins Oep37 and Oep24. We found that both proteins can be integrated *in vitro* into isolated plant mitochondria. Furthermore, upon their expression in yeast cells Oep37 and Oep24 were exclusively located in the mitochondrial OM. Oep37 partially complemented the growth phenotype of yeast cells lacking Porin, the general metabolite transporter of this membrane. Similarly to mitochondrial β-barrel proteins, Oep37 and Oep24 expressed in yeast cells were assembled into the mitochondrial OM in a pathway dependent on the TOM and TOB complexes. Taken together, this study demonstrates that the central mitochondrial components that mediate the import of yeast β-barrel proteins can deal with precursors of chloroplast β-barrel proteins. This implies that the mitochondrial import machinery does not recognize signals that are unique to mitochondrial β-barrel proteins. Our results further suggest that dedicated targeting factors had to evolve in plant cells to prevent mis-sorting of chloroplast β-barrel proteins to mitochondria.

In addition to the outer membrane (OM)[3] of Gram-negative bacteria, membrane-embedded β-barrel proteins are found also in the OM of the endosymbiotic organelles, mitochondria and chloroplasts. Compared with the diversity of β-barrel membrane proteins in prokaryotes, the number of organellar OM proteins confirmed to have this structural type is rather limited. In *Saccharomyces cerevisiae,* bakers' yeast, only five members have been identified: Tom40, Tob55/Sam50, two isoforms of Porin/VDAC, and Mdm10. The number of characterized β-barrel proteins in chloroplast OM is not much higher, although sequence analysis predicted the presence of many such proteins in this membrane (1). Some of those (for example, outer envelope proteins OEP21, OEP24, and OEP37) were proposed to function as pores for small metabolites, and their distinct substrate specificities may point to discrete roles in various metabolic processes (2, 3). The chloroplast OM harbors also Toc75, a β-barrel protein with several isoforms in *Arabidopsis thaliana* (4). Toc75 (Toc75-III in *A. thaliana*) forms the protein-conducting pore of the translocase of the OM of chloroplasts (TOC complex) (5, 6).

Newly synthesized mitochondrial β-barrel precursor proteins are devoid of canonical N-terminal presequences or any other characterized linear targeting signal. They are initially recognized by the Tom20 and Tom22 receptor components of the translocase of the outer mitochondrial membrane (TOM complex) before their translocation across the OM via the import pore of this complex. Next, these proteins are relayed to the dedicated complex for topogenesis of outer membrane β-barrel proteins (TOB complex, also known as sorting and assembly machinery), which mediates their assembly into the OM. The known components of the TOB core complex are Tob55/Sam50/Omp85, Tob38/Sam35, and Mas37/Tom37/Sam37. On their way from the TOM to the TOB complex, the β-barrel precursors are exposed to the intermembrane space (IMS) where they interact with the small Tim chaperones (for detailed reviews see Refs. 7–9).

In contrast to our detailed picture on the biogenesis of β-barrel proteins in mitochondria, relatively little is known about their assembly pathways in chloroplasts. Specific signals for tar-

* This work was supported by the Deutsche Forschungsgemeinschaft Grants RA 1048/4-1 and SFB766/TP B11 (to D. R.) and SFB807/P17 (to E. S.).
[1] Both authors contributed equally to this work.
[2] To whom correspondence should be addressed: Interfaculty Institute of Biochemistry, University of Tübingen, Hoppe-Seyler-Str. 4, 72076 Tübingen, Germany. Tel.: 49-7071-2974184; Fax: 49-7071-294016; E-mail: doron.rapaport@uni-tuebingen.de.
[3] The abbreviations used are: OM, outer membrane; IMS, intermembrane space; sa, self-assembly; AAC, ADP-ATP carrier; ALDH, aldehyde dehydrogenase.

geting of most β-barrel proteins to the chloroplast have not yet been identified. An interesting exception is provided by the unique biogenesis pathway of the precursor of Toc75-III. This precursor protein is synthesized with an N-terminal extension, which functions as a bipartite transit peptide and is processed during maturation (10, 11). The first portion of the targeting signal directs the precursor protein to the chloroplast stroma where it is cleaved by a stromal processing peptidase (10). The second portion functions probably as a stop-transfer segment and was found to be processed by a type I signal peptidase (12). The overall import pathway of Toc75-III seems to support the idea that sorting of β-barrel membrane proteins of chloroplasts occurs in a manner similar to that of mitochondria. The Toc75-III precursor is first completely translocated across the outer envelope by the TOC complex and thus is likely inserted from the inner face into the lipid phase of the OM. Another Toc75 isoform (Toc75-V/OEP80 in *A. thaliana*) was speculated to be involved in the membrane integration of chloroplast OM β-barrel proteins; however, experimental evidence to support this proposal is still lacking. In any case, this isoform is not a component of the TOC complex (13). Surprisingly, the N-terminal region of Toc75-V is not essential for the targeting, biogenesis, or functionality of the protein suggesting that Toc75-III and Toc75-V do not follow the same targeting pathway (14). Thus, currently Toc75-III is the only known protein in the OM of chloroplasts or mitochondria with a cleavable targeting sequence. Hence, the question regarding how the vast majority of the β-barrel proteins of chloroplasts and all those of mitochondria is targeted to their respective organelle is still an open one.

Bacterial β-barrel proteins can be targeted to mitochondria when expressed in eukaryotic cells suggesting that signals in these proteins are functional in eukaryotic cells for targeting to and assembly in mitochondria (15–18). In addition, previous studies failed to identify a linear sequence that functions as an intracellular targeting signal for mitochondrial β-barrel proteins (9). These findings indicate that the signal for targeting of β-barrel precursors to mitochondria is probably not confined to a single linear sequence but rather involves structural elements unique to membrane-embedded β-barrel proteins. Supporting this assumption is a report that the chloroplast β-barrel protein Oep24 was integrated into the mitochondrial OM upon its expression in yeast cells (19). However, the mechanism of such assembly was not studied, and therefore, it is not clear whether a chloroplast β-barrel can be recognized and processed by the same elements that mediate the biogenesis of mitochondrial β-barrel proteins.

To better understand the specific targeting of these proteins, we expressed the chloroplast β-barrel proteins Oep37 and Oep24 in yeast cells and studied their biogenesis in this system. The proteins were located exclusively in mitochondria where they assembled into the OM. *In vitro* experiments revealed that both Oep24 and Oep37 were first translocated across the OM by the TOM complex and then integrated into the outer membrane by the TOB complex. Collectively, our results suggest that chloroplast β-barrel proteins can be imported into the OM in a similar pathway to that undertaken by the *bona fide* mitochondrial β-barrel proteins. Thus, these findings imply the following: (i) the mitochondrial import machinery does not recognize signals that are unique to mitochondrial β-barrel proteins, and (ii) dedicated targeting factors had to evolve in plant cells to avoid mis-targeting of these proteins to mitochondria.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Methods—Standard genetic techniques were used for growth and manipulation of yeast strains. The wild-type strains YPH499 and W303α were employed. The *tom20*Δ, *mas37*Δ, and *tim8*Δ/*tim13*Δ strains were described before (Refs. 17, 20, 21, respectively). The *tom70*Δ/*tom71*Δ double-deletion strain is a kind gift of Dr. K. Okamoto (22). For drop-dilution assays, yeast cells were grown in synthetic medium to an A_{600} of 1.0 and diluted in 5-fold increments, and then 5 μl of each dilution were spotted onto solid media, and growth was monitored for a few days.

Recombinant DNA Techniques—Oep37 and Oep24 were amplified from pea cDNA using standard PCR techniques and subsequently cloned into pGEM4 for cell-free transcription and translation. In addition, Oep37- and Oep24-encoding sequences were cloned into the yeast expression vectors pYX242 or pYX142, respectively. VDAC1 was amplified from *A. thaliana* cDNA and cloned into pGEM4 for *in vitro* transcription and translation. ALDH and OE33 constructs for *in vitro* import experiments were previously described (23, 24).

The constructs for the self-assembly GFP assays were amplified from *A. thaliana* cDNA using standard PCR techniques and subsequently cloned into the pAVA plasmid (25) containing the fragments for saGFP11 (N-/C-terminal) or saGFP(1–10). Templates for the saGFP(1–10) and saGFP11 fragments were obtained from Dr. G. S. Waldo (Los Alamos, NM).

Mitochondria Isolation and Subcellular Fractionations of Yeast Cells—Mitochondria were isolated from yeast cells grown on galactose-containing medium by differential centrifugation as described (26). For isolation of mitochondria from temperature-sensitive mutants and their parental strains, cells were grown at 24 °C, unless otherwise stated. For highly pure mitochondria, a Percoll gradient purification was performed. For that goal, isolated mitochondria were layered on top of a self-forming gradient (25% Percoll in an SEM buffer (250 mM sucrose, 1 mM EDTA and 10 mM MOPS, pH 7.2)) and centrifuged (80,000 × *g*, 45 min, 4 °C) (16). Mitochondrial fraction from the lower third of the gradient was collected and resuspended in 30 ml of SEM buffer and reisolated by centrifugation (15,000 × *g*, 15 min, 4 °C). Microsomes were isolated from yeast cells by differential centrifugation. After obtaining the first mitochondrial pellet, the supernatant was centrifuged (15,000 × *g*, 15 min, 4 °C) again to avoid contaminations by mitochondrial elements. The post-mitochondrial fraction was subjected to a centrifugation (100,000 × *g*, 1 h, 4 °C), and the pelleted microsomes were resuspended in SEM buffer.

Biochemical Procedures—Radiolabeled precursor proteins were synthesized in rabbit reticulocyte lysate in the presence of [^{35}S]methionine (PerkinElmer Life Sciences) after *in vitro* transcription by SP6 polymerase from pGEM4 vector (Promega). Radiolabeled precursor proteins were incubated at 25 °C with isolated yeast mitochondria in an import buffer (250 mM

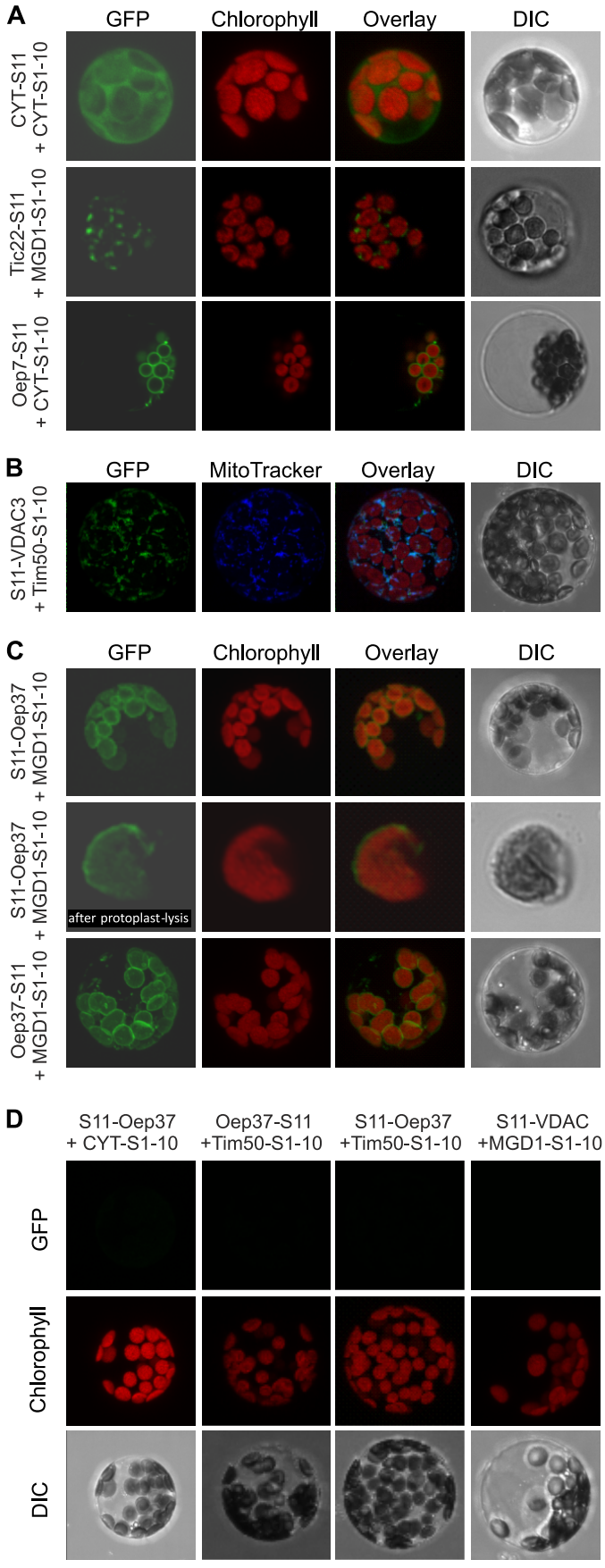


FIGURE 1. **Chloroplast β-barrel protein Oep37 is exclusively targeted *in vivo* to chloroplast outer envelopes and possesses an N_{in}-C_{in} topology.** The indicated constructs were co-transfected into *A. thaliana* protoplasts that were subsequently analyzed by confocal fluorescence microscopy. The GFP fluorescence (*GFP*), the autofluorescence of chlorophyll, the overlay of all fluorescence signals, and the differential interference contrast image (*DIC*) are shown for a representative example. *A,* two saGFP fragments were targeted to the cytoplasm (CYT-S11 and CYT(1–10), *top panel*), to the chloroplasts IMS (Tic22-S11 and Mgd1(S1–10), *middle panel*), or to the outer envelope of chloroplasts (Oep7-S11 and CYT(S1–10), *bottom panel*). *B,* two saGFP fragments were targeted to the mitochondrial IMS (S11-VDAC3 and Tim50(S1–10)). A staining of mitochondria with MitoTracker is shown. *C,* Oep37 either N- or C-terminally fused to saGFP11 (S11-Oep37 or Oep37-S11, respectively) was co-expressed with the chloroplast IMS-located Mgd1(S1–10). The *middle panel* shows an isolated chloroplast after osmolysis of protoplasts. *D,* S11-Oep37 fusion proteins were co-expressed with either cytosolically localized S1–10 (CYT(1–10)) or with S1–10 located in the mitochondrial IMS (Tim50(S1–10)). As another control, VDAC3 fused to saGFP11 (S11-VDAC3) was co-expressed with the chloroplast IMS-located Mgd1(S1–10).

sucrose, 0.25 mg/ml BSA, 80 mM KCl, 5 mM $MgCl_2$, 10 mM MOPS-KOH, 2 mM NADH, 2 mM ATP, pH 7.2). Organelles isolated from *mas37*Δ and *TIM10-1* strains or from their corresponding parental strains were incubated at 37 °C for 15 min before initiating the import reaction.

Plant Organelle Isolation and Biochemical Assays Employing These Organelles—Import of the translated ^{35}S-labeled precursor proteins into isolated pea chloroplasts was performed as described before (27). Pea mitochondria for single import assays were isolated according to published procedure (28), and single *in vitro* import experiments were done as described previously (29). After import (30 min, RT), the organelles were reisolated and subsequently treated with 0.1 M Na_2CO_3, pH 11.5, with or without the addition of Triton X-100 (1%) and incubated for 30 min on ice. Subsequently, they were centrifuged (100,000 × *g*, 20 min, 4 °C), and the pellet fractions were analyzed by SDS-PAGE and autoradiography.

Isolation of pea chloroplasts and mitochondria for dual *in vitro* import was performed according to Rödiger *et al.* (30). Dual import reactions were done for 30 min at 25 °C as described (31). At the end of the import reactions, the organelles were treated with a final concentration of 120 μg/ml thermolysin or 5 μg/ml proteinase K in dual import buffer supplemented with 50 mM $CaCl_2$ and incubated on ice for 30 or 15 min, respectively. The proteolytic digestion was stopped by 10 mM EDTA, pH 8.0, for thermolysin and 10 mM PMSF for proteinase K. The organelles were repurified and analyzed by SDS-PAGE and autoradiography. For sodium carbonate and Triton X-100 treatment, the organelles were repurified directly after import, subsequently treated with sodium carbonate buffer (0.1 M Na_2CO_3, pH 11.5, 1 mM EDTA) in the presence or absence of 1% Triton X-100, and incubated on ice for 30 min. The samples were then centrifuged (100,000 × *g*, 30 min, 4 °C), and the pellet fractions were analyzed by SDS-PAGE and autoradiography. The purity of organelles after dual import was analyzed using one-half of each import reaction sample for immunodecoration with antibodies against *ps*Toc75 and *at*V-DAC1 (Agrisera).

Proteolytic digestion of pea chloroplast outer envelope vesicles (isolated according to Ref. 32) was performed as described before (33). The samples were incubated on ice with thermolysin or PK for 30 or 15 min, respectively. Envelope vesicles were recovered by centrifugation (100,000 × *g*, 30 min, 4 °C) and subsequently analyzed via SDS-PAGE and immunodecoration.

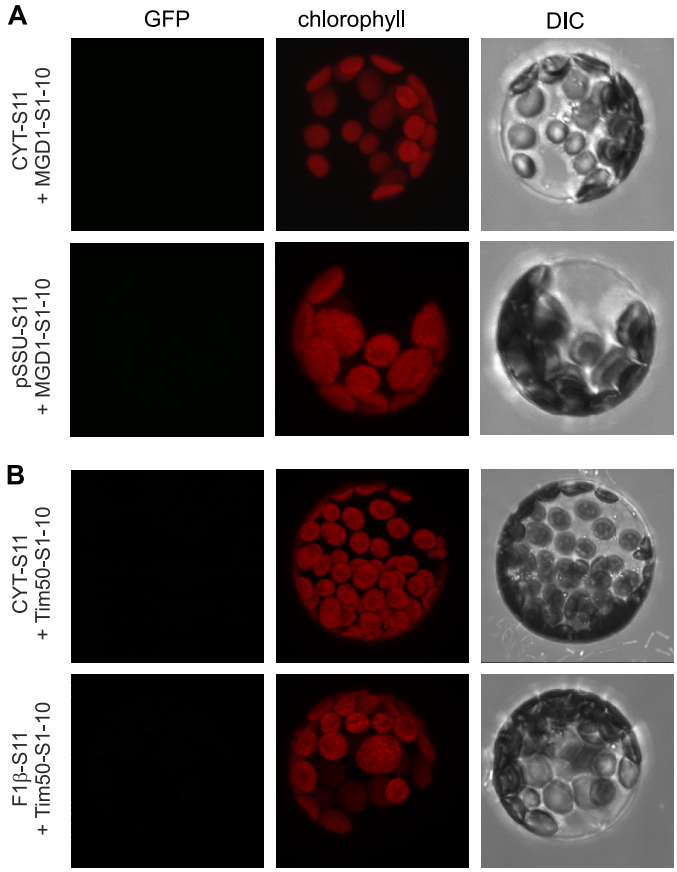


FIGURE 2. **GFP signal is not observed when the two fragments are not in the same compartment.** The indicated constructs were co-transfected into *A. thaliana* protoplasts that were subsequently analyzed by confocal fluorescence microscopy. The GFP fluorescence after GFP assembly (*GFP*), the autofluorescence of chlorophyll, and the differential interference contrast image (*DIC*) are shown for a representative example. *A,* large fragment was targeted to the chloroplast IMS (MGD1(S1–10)), whereas the small one was targeted to either the cytosol (*CYT-S11*) or the chloroplast stroma (*pSSU-S11*). *B,* large fragment was targeted to the mitochondrial IMS (Tim50(S1–10)), whereas the small one was targeted to either the cytosol (*CYT-S11*) or the mitochondrial matrix (*F1β-S11*).

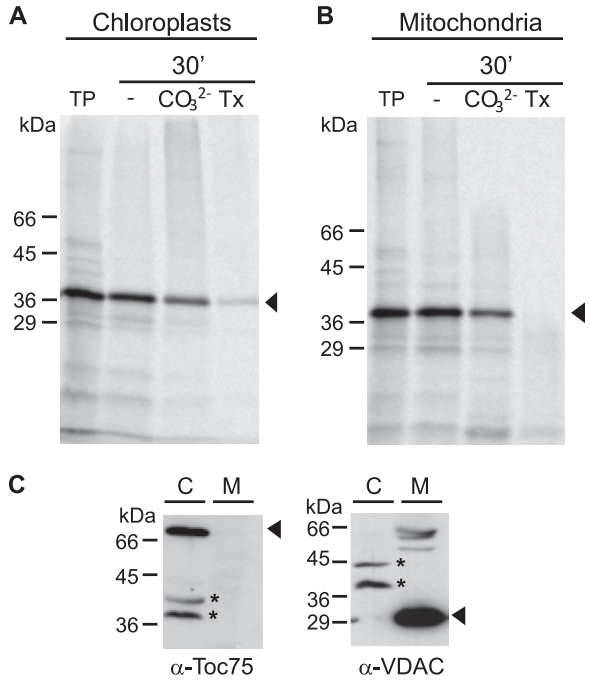


FIGURE 3. **Oep37 can be imported *in vitro* into chloroplasts and mitochondria.** Radiolabeled Oep37 was incubated for 30 min at RT with either isolated pea chloroplasts (*A*) or isolated mitochondria (*B*). Membrane insertion was assayed by the resistance to carbonate treatment (CO_3^{2-}) in the presence or absence of Triton X-100 (*Tx*). An aliquot (5%) of the translation product (*TP*) used for each import reaction was loaded as control. The bands corresponding to Oep37 are indicated with an *arrowhead*. *C,* purity of the isolated mitochondria (*M*) or chloroplasts (*C*) was monitored by analyzing 10 μg of total organellar protein by SDS-PAGE followed by immunodecoration, using organelle-specific antibodies (α-VDAC for mitochondria and α-Toc75 for chloroplasts). The bands corresponding to the marker proteins are indicated with an *arrowhead,* whereas unspecific bands resulting from cross-reactivity of the antibodies are marked with an *asterisk.*

Protoplast Isolation, Transfection, and saGFP Analysis—*A. thaliana* mesophyll protoplasts were isolated and transfected as described (33). GFP and chloroplast fluorescence was monitored by confocal laser scanning microscopy using a TCS SP5 microscope (Leica) with an HCX PL APO CS 40 × 1.25 NA 1.25 oil objective. Fluorescence was excited and detected as follows: GFP 488/505–525 nm, chlorophyll fluorescence 514/650–750 nm.

RESULTS

Chloroplast β-Barrel Protein Oep37 Is Assembled into the Mitochondrial OM—To understand how specific targeting of β-barrel proteins can be achieved in plant cells, we used the model protein Oep37. To study the location of this protein *in vivo*, we utilized the recently established sa-GFP system where the first 10 β-strands of GFP are fused to one protein while the complementing 11th β-strand is attached to another protein. Only if the two proteins, to which the GFP fragments are fused, are located in the same cellular compartment can a GFP signal be observed (34). As expected, if both fragments were in the cytosol or the IMS of chloroplasts was a GFP signal observed in the corresponding compartment (Fig. 1*A*). Similarly, a signal was obtained when both parts of the GFP were localized in the mitochondrial IMS (Fig. 1*B*). Next, strand 11 of GFP was fused to either the N or C terminus of Oep37, and the fusion protein was co-expressed with Mdg1(S1–10) that positions strands 1–10 in between the outer and inner membranes (33). Both combinations resulted in a distinct GFP staining of the chloroplasts (Fig. 1*C*). Of note, no signal was observed when the fragment containing strands 1–10 was located either in the cytosol or in mitochondria (Fig. 1*D*). As expected, also the control combination of Mdg1(S1–10) in chloroplasts and S11-VDAC3 in mitochondria did not result in a GFP signal (Fig. 1*D*). Further control experiments demonstrated that false-positive signal was not observed in other cases where both fragments were not in the same compartment (Fig. 2). Thus, Oep37 is located *in vivo* solely in chloroplast OM where its two termini are facing the intermembrane space. Interestingly, Oep37 was detected in isolated chloroplasts before (1, 2), but it was predicted to have converse topology (1). Thus, to validate our method, we fused the S11 fragment to the C terminus of the single-span membrane protein Oep7 that was reported to have a C_{out} orientation (35). As expected, co-expression of this protein with the cytosolic S1–10 fragment gave a GFP signal (Fig. 1*A*, *bottom panel*). Thus, the absence of a signal upon co-expression of Oep37-S11 and cytosolic S1–10 is not a technical problem of the used

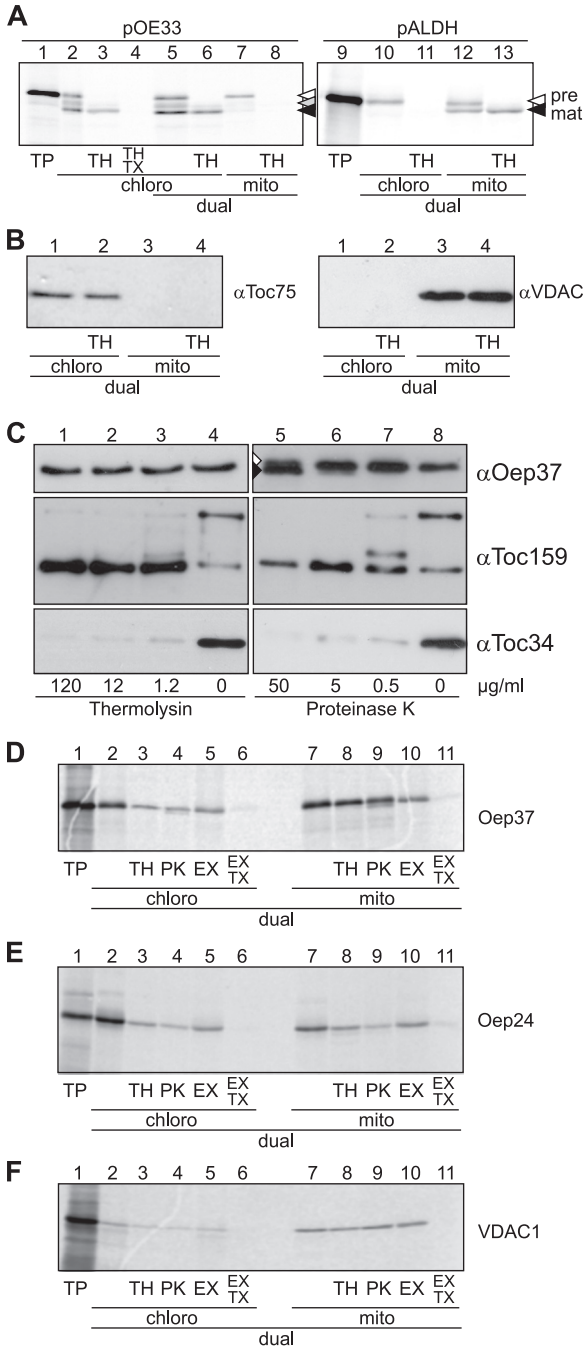


FIGURE 4. **Oep37 and Oep24 are targeted *in vitro* in a dual system to the OM of mitochondria and chloroplasts.** *A,* single (*lanes 2–4*) and dual (*lanes 5–13*) import of rabbit reticulocyte-translated pOE33 or pALDH into isolated pea chloroplasts (*chloro*) (*lanes 2–6, 10,* and *11*) and mitochondria (*mito*) (*lanes 7* and *8* and *12* and *13*). Translation products (*TP*, 10%) as input control are shown in *lanes 1* and *9*. Nonimported proteins were removed by thermolysin (*TH, lanes 3, 6, 8, 11,* and *13*). In addition, 1% (v/v) Triton X-100 was added to one sample (*TX, lane 4*). Precursor and mature forms of pOE33 and pALDH are indicated by *white* and *black arrowheads,* respectively. The stromal intermediate form of pOE33 is indicated with a *gray arrowhead*. *B,* purity of the chloroplasts (*lanes 1* and *2*) and mitochondrial (*lanes 3* and *4*) fractions after dual import and re-purification of the organelles was assayed by Western blotting with the indicated organelle-specific antibodies. *C,* chloroplast outer envelope membranes were treated with the indicated concentrations of either thermolysin (*lanes 1–4*) or proteinase K (*lanes 5–8*). The membranes were then assayed by Western blotting using the indicated organelle-specific antibodies. Oep37 was partially sensitive to high amounts of proteinase K, and in addition to full-length protein, a slightly smaller degradation product was also observed (*lane 5, white* and *black arrowheads,* respectively). *D–F,* dual import of radiolabeled Oep37 (*D*), Oep24 (*E*), and VDAC1 (*F*) into chloroplasts and mitochondria (*lanes 2–11*). Translation products (*TP*, 10%) as input control are shown in *lane 1*. Nonimported proteins were removed by either thermolysin (*TH, lanes 3* and *8*) or proteinase K (*PK, lanes 4* and *9*) treatment. Full integration into the membrane was assayed by carbonate extraction (*EX*) in the presence or absence of Triton X-100 (*TX, lanes 5, 6, 10,* and *11*).

method but rather results from the N_{in}/C_{in} topology of the protein.

To further study the biogenesis of Oep37, we used a cell-free import system where radiolabeled Oep37 was mixed with either chloroplasts or mitochondria isolated from pea cells. Integration of Oep37 into the target membrane was monitored by alkaline extraction. Surprisingly, the protein was imported into both compartments in a similar efficiency (Fig. 3, *A* and *B*). To make sure that the samples used in the *in vitro* experiments are not cross-contaminated, we analyzed them by immunodecoration with antibodies against marker proteins. Of note, signals corresponding to chloroplast Toc75 and mitochondrial VDAC were observed only in the chloroplasts or mitochondrial samples, respectively (Fig. 3*C*).

The aforementioned binding experiments were not performed under competitive conditions, and it was often reported that mitochondrial and chloroplast proteins can be imported *in vitro* into the wrong organelle (36, 37). Therefore, we wanted to exclude the possibility that the rather hydrophobic Oep37 molecules are inserted *in vitro* into mitochondria simply because this is the only membrane present in the binding reaction. To test the specificity of the binding, we asked whether Oep37 would insert into mitochondria also when competing chloroplasts are present. To this end, we employed a dual system where both isolated chloroplasts and mitochondria are present during the import reaction and are separated only at its end. To check whether the specificity of import is kept under these conditions, we incubated the organelle mixture with radiolabeled precursors of either the chloroplast thylakoid protein pOE33 or the mitochondrial protein pALDH. The results shown in Fig. 4*A* demonstrate that each precursor protein was imported into its corresponding organelle. Next, we controlled by Western blotting for the purity of the organelles after their separation and found that the chloroplast protein Toc75 was detected only in the chloroplasts fraction, whereas the mitochondrial protein VDAC was found exclusively in the mitochondrial fraction (Fig. 4*B*). These results validate the dual import system as a specific and reliable assay to monitor the import of precursor proteins.

We aimed to establish an assay to monitor the *in vitro* import of the chloroplast β-barrel protein Oep37. To this end, we investigated the protease resistance of the endogenous protein in isolated organelles. Oep37 was resistant against the used amounts of both PK and thermolysin. In contrast, as expected, the exposed receptors Toc159 and Toc34 were cleaved under these conditions (Fig. 4*C*). The protease resistance of Oep37 under these conditions provides further support for the notion that both termini of Oep37 are in the intermembrane space of chloroplasts. The earlier proposal that the N and C termini are exposed to the cytosol was based only on proteolysis of outer envelope membranes with very high concentrations of thermolysin (1). As contrast, our current model is based on improved methodology, namely *in vivo* data with intact cells and proteolytic assay with reduced proteases concentrations.

Using such proteolytic assays and carbonate extraction, we then analyzed the import of Oep37 in the dual import system. In agreement with our results in the single organelle system (Fig. 3), the protein became protected from proteases upon import into both organelles (Fig. 4*D*). Furthermore, Oep37 was found in the pellet fraction of an alkaline extraction as expected from membrane-embedded proteins. Triton X-100 at concentrations up to 2% is frequently used to wash protein from inclusion bodies that are aggregates of non-native proteins (38). Thus, we applied the previously established principle of Triton X-100 treatment (39) to test whether the protein was indeed inserted into the membrane or just co-sedimented as a non-membrane-inserted aggregate. Oep37 was not detected in the pellet when we performed the extraction in the presence of the detergent Triton X-100, excluding the possibility of aggregation as a cause for the appearance in the pellet (Fig. 4*D*). As Oep24 was previously reported to be targeted to mitochondria upon its expression in yeast cells (19), we also imported this protein in the dual system. Similarly to Oep37, Oep24 was integrated into the membrane of both organelles (Fig. 4*E*). Of note, under these conditions the mitochondrial β-barrel protein VDAC was efficiently imported into mitochondria but only sparsely into chloroplasts (Fig. 4*F*). The mitochondria and chloroplast fractions were also analyzed after their re-isolation by SDS-PAGE followed by Coomassie staining. This analysis revealed that similar amounts of proteins were contained in each fraction (data not shown), thus excluding the possibility that unequal amounts of proteins affect the import efficiencies. Taken together, our findings suggest that chloroplasts β-barrel proteins can be imported *in vitro* into isolated mitochondria.

Next, we were interested whether this mitochondrial targeting capacity can also be observed in an *in vivo* system. We therefore cloned Oep37 into a yeast expression vector and transformed the resulting plasmid into *S. cerevisiae* cells. Subcellular fractionation of the transformed cells revealed that Oep37 was located exclusively in the mitochondrial fraction (Fig. 5*A*). As a control for the specificity of the Oep37 antibody, we verified the absence of a signal in mitochondria isolated from a nontransformed strain (Fig. 5*A*, *left lane*). Hence, although several different membranes are available for the newly synthesized Oep37 molecules upon their translation in the yeast cytosol, the protein is targeted solely to mitochondria.

To demonstrate that Oep37 was indeed integrated into the outer membrane rather than simply attached on the surface of the organelle, mitochondria were subjected to alkaline extraction. Oep37 was found in the pellet fraction similarly to other membrane-embedded mitochondrial proteins like Tom70 or Porin (Fig. 5*B*). In contrast, soluble proteins like Hep1 and the IMS isoform of Mcr1 were found in the supernatant fraction (Fig. 5*B*). Because we observed that native Oep37 is resistant to PK treatment in intact chloroplasts (Fig. 4*C*), the membrane integration of Oep37 was further analyzed by a similar treatment of intact mitochondria. Similarly to mitochondrial β-barrel proteins like Porin and Tob55, Oep37 was unaffected by addition of external protease to intact mitochondria. Oep37 became accessible to proteinase K only when mitochondrial membranes were solubilized with the detergent, Triton X-100 (Fig. 5, *B* and *C*). Similarly, rupturing of the outer membrane by osmotic swelling caused exposure of loops in the IMS to proteinase K and disappearance of the protein signal (Fig. 5*C*, *left panel*). As the experiments described so far cannot exclude the

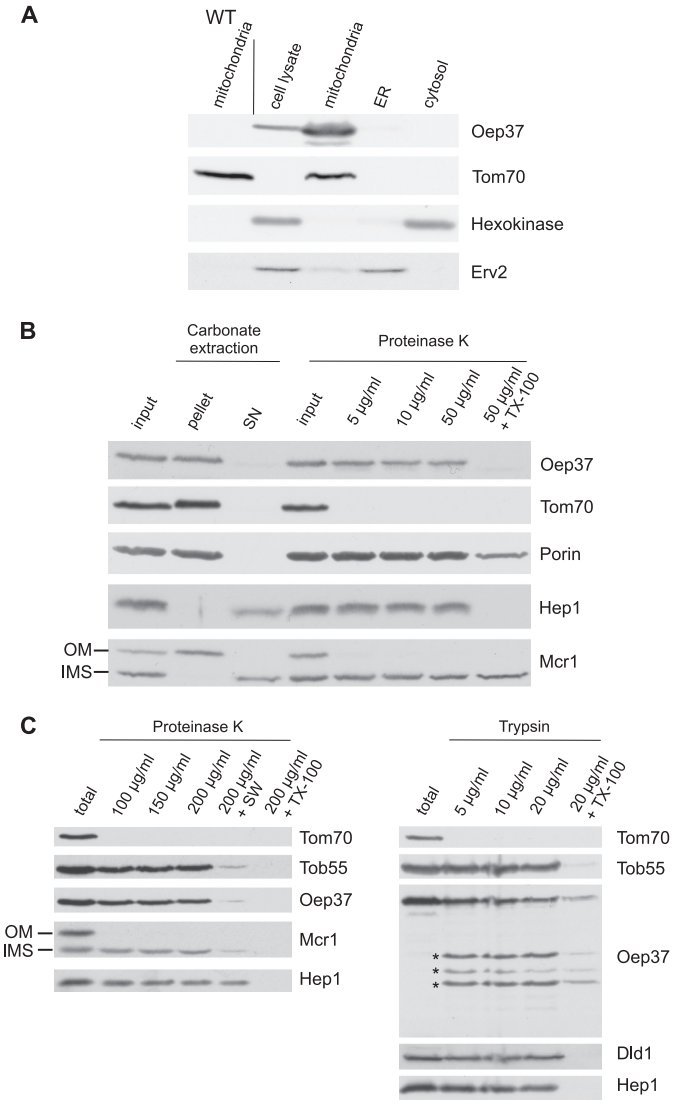


FIGURE 5. **Oep37 expressed in yeast cells is assembled into the mitochondrial OM in a native conformation.** *A,* Oep37 is located in mitochondria. Lysates of Oep37-expressing cells and fractions corresponding to mitochondria, endoplasmic reticulum (*ER*), and cytosol were analyzed by SDS-PAGE and immunodecoration with antibodies against Oep37, the mitochondrial protein Tom70, a marker protein for the cytosol (hexokinase), and the endoplasmic reticulum protein Erv2. Mitochondria isolated from untransformed WT cells were co-analyzed as a control. *B,* mitochondria isolated from cells expressing Oep37 were analyzed directly by SDS-PAGE (*input*) or were subjected first to carbonate extraction and then centrifuged to discriminate between membrane proteins in the pellet and soluble proteins in the supernatant (*SN*). Additional aliquots of mitochondria were left intact or were treated with the indicated amounts of proteinase K. Proteins were analyzed by SDS-PAGE and immunodecorated with antibodies against the indicated proteins as follows: Tom70, an OM protein exposed to the cytosol; Porin, a protein embedded in the OM; Hep1, a mitochondrial soluble matrix protein; Mcr1, a protein with two isoforms, a 34-kDa species exposed on the OM and a 32-kDa soluble one in the IMS. *C,* mitochondria isolated from cells expressing Oep37 were left intact (*total*) or were treated with the indicated amounts of proteinase K (*left panel*) or trypsin (*right panel*). In one sample, the mitochondria were swelled (+*SW*) before the treatment with PK. Proteins were analyzed by SDS-PAGE and immunodecorated with antibodies against the indicated proteins. *Tob55*, a protein embedded in the OM; *Dld1*, an inner membrane protein exposed to the IMS. Proteolytic fragments are indicated with an *asterisk*.

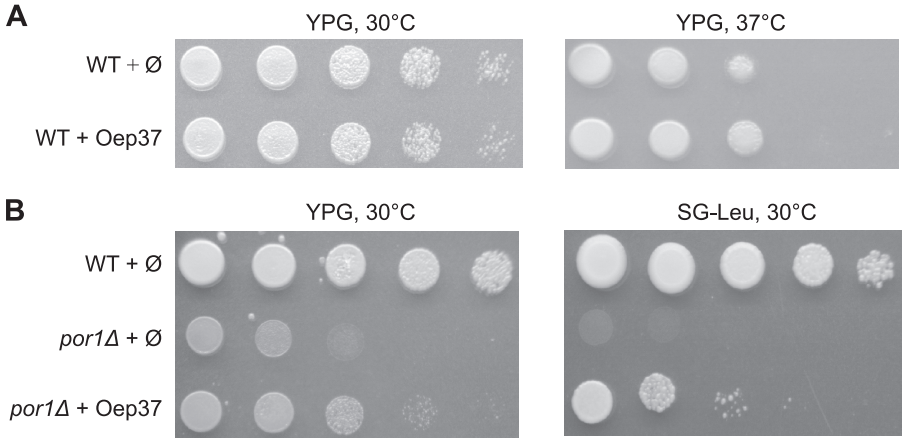


FIGURE 6. **Overexpression of Oep37 can partially complement the *por1*Δ phenotype.** *A,* expression of Oep37 does not interfere with growth on a nonfermentable carbon source. Cells harboring either a plasmid encoding Oep37 or an empty plasmid (Ø) as control were tested by drop dilution assay for their ability to grow on glycerol-containing medium (*YPG*) at 30 and 37 °C. *B,* wild-type cells harboring an empty plasmid (Ø) and cells deleted for *POR1* (*por1*Δ) that were transformed with either an empty plasmid (Ø) or a plasmid encoding Oep37 were tested by drop dilution assay for their ability to grow at 30 °C on rich (YPG) or synthetic glycerol-containing medium (*SG-Leu*).

possibility that Oep37 is integrated into the inner membrane, we treated intact organelles with higher concentrations of PK or with trypsin as the latter protease cleaves other β-barrel proteins like Tom40. Indeed, upon addition of such elevated amounts of PK or trypsin to intact organelles, we observed the formation of proteolytic fragments (Fig. 5*C*). Such fragments can be formed only if the protein is embedded into the OM and exposes loops toward the cytosol. The intactness of mitochondria under these conditions is reflected by the resistance of the IMS proteins DLD1 and the IMS isoform of Mcr1 as well as the matrix protein Hep1 to the proteases treatment (Fig. 5*C*). We conclude that Oep37 is assembled into the mitochondrial OM in a native-like conformation.

Oep37 Can Complement the Absence of Mitochondrial Porin—We further investigated whether the expression of Oep37 interferes with crucial functions of mitochondria. To that end, we compared the growth rate of yeast cells expressing the chloroplast protein to those bearing an empty plasmid. The growth of Oep37-expressing cells on a nonfermentable carbon source, where yeast cells require fully functional mitochondria, was comparable with that of control cells (Fig. 6*A*). Next, we verified that expressing Oep37 in yeast cells did not have any effect on the morphology of the organelle (data not shown). Collectively, it seems that the expression of Oep37 does not obstruct crucial cellular and mitochondrial processes.

Oep37 was reported to form a rectifying high conductance channel in artificial membranes (2). Thus, we asked whether this protein can complement the absence of the general solute transporter of the mitochondrial outer membrane, Porin (also called VDAC in higher eukaryotes). Cells lacking Porin hardly grow on a nonfermentable carbon source at elevated temperatures (40). We observed that *por1*Δ cells expressing Oep37 partially regained their capacity to grow under these conditions (Fig. 6*B*). This finding suggests that Oep37 can form pores in the mitochondrial outer membrane. Of note, another chloroplast OM protein, Oep24, was previously shown to partially complement Porin deficiency in yeast cells (19). Thus, our current findings indicate that although Oep24 and Oep37 probably have different substrate specificity in chloroplast membranes, they share the ability to complement the function of Porin that serves as the single and general solute transporter in the yeast mitochondrial OM (40).

Oep37 Assembly into Mitochondria Requires the TOM and TOB Complexes—Mitochondrial β-barrel proteins like Tom40, Porin, and Tob55 or bacterial β-barrel proteins expressed in yeast cells are initially recognized by the import receptors Tom20 and Tom70 (16, 17, 20, 41–43). Hence, we used an *in vitro* import assay to address the importance of these receptors for the mitochondrial assembly of Oep37 and Oep24. Membrane integration of the precursor molecules was analyzed by monitoring those molecules that are proteinase K-resistant. Initially, we observed that the removal of the exposed domains of import receptors by externally added trypsin affected the *in vitro* import of both proteins into isolated mitochondria (Fig. 7*A*). To verify the importance of the import receptors, we imported newly synthesized Oep37 or Oep24 molecules into mitochondria isolated from strains lacking either Tom20 or Tom70 and its low abundant paralog Tom71. The import of newly synthesized chloroplast β-barrels and the control mitochondrial β-barrel protein Porin into both types of mutated mitochondria was strongly compromised (Fig. 7*B*). To further study the importance of the receptors, we transformed plasmids encoding either Oep37 or Oep24 into cells lacking either Tom20 or Tom70/Tom71 and analyzed the steady-state levels in these mutated cells. Crude mitochondria isolated from *tom20*Δ cells had significantly reduced amounts of Oep37 but wild-type-like levels of Oep24 (Fig. 7, *C* and *D*). In contrast, the absence of Tom70/71 caused only a slight reduction or none at all in the observed levels of Oep37 and Oep24, respectively (Fig. 7, *C* and *D*). The reduced Tom20 dependence of the *in vivo* biogenesis of Oep24 might be related to its smaller size. We previously observed that upon their expression in yeast cells the biogenesis of small bacterial β-barrel proteins is Tom20-independent whereas that of their larger counterparts required the presence of this receptor (17). We assume that the higher dependence on Tom70/71 in the *in vitro* system is due to the use of the reticulocyte lysate in these experiments and the function of Tom70 as an anchor for chaperones present in this

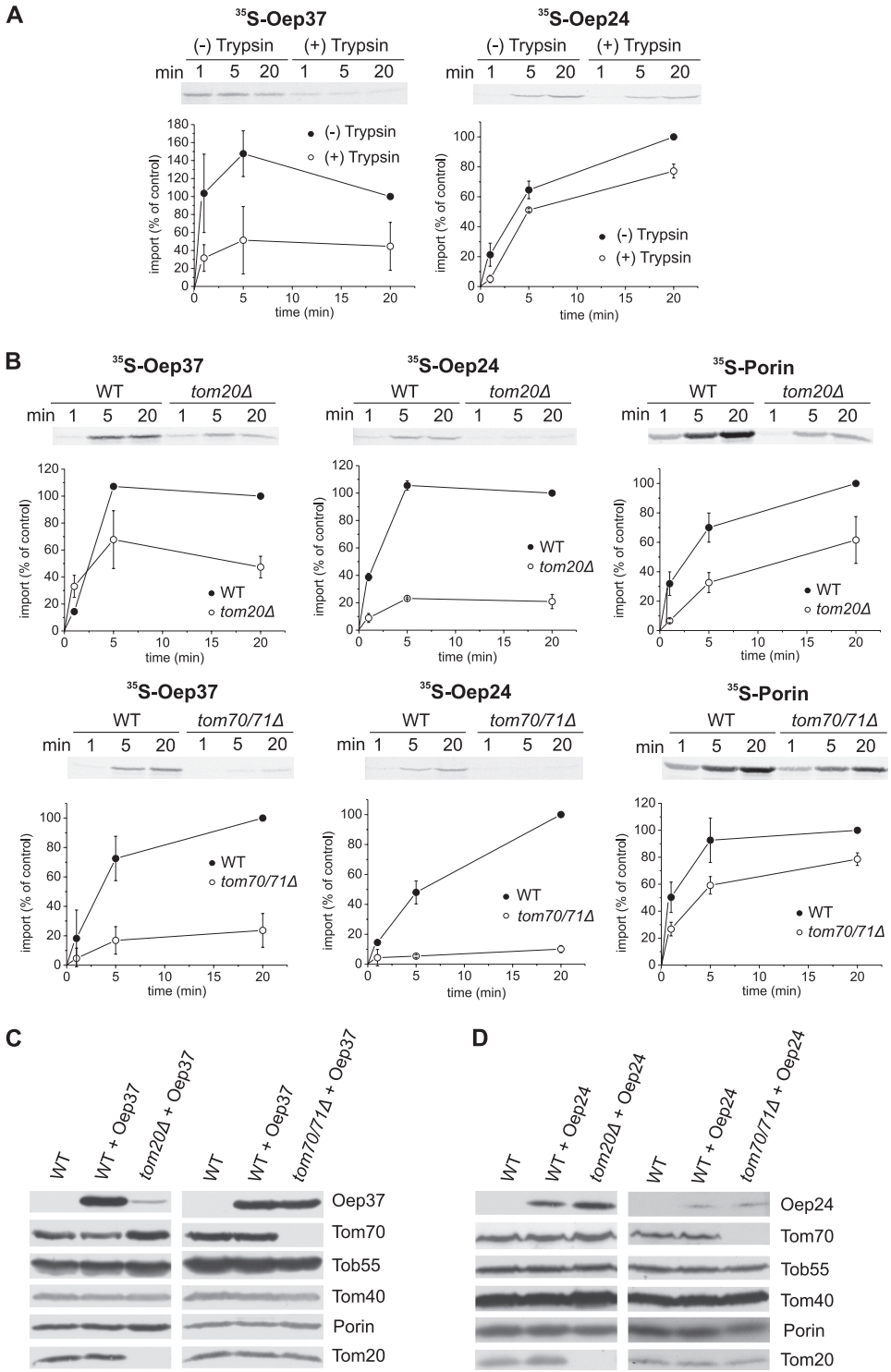


FIGURE 7. **Oep37 and Oep24 require the mitochondrial import receptors for their assembly into the OM.** *A,* isolated mitochondria were left intact or pretreated with trypsin followed by re-isolation of the organelles. Next, radiolabeled precursor of either Oep37 or Oep24 was incubated with the trypsin-treated or intact mitochondria for the indicated time periods. At the end of the import reactions, samples were treated with PK, and proteins were analyzed by SDS-PAGE and autoradiography. The insertion of the proteins was quantified by analyzing the PK-protected molecules. The amount of precursor proteins imported into intact mitochondria for 20 min was set to 100%. An autoradiographic representative of three independent repeats and quantification of three independent experiments is presented. *B,* radiolabeled precursors of Oep37, Oep24, and Porin (as a control) were imported into mitochondria isolated from either *tom20*Δ or *tom70/71*Δ and their corresponding WT strains. Imported proteins were analyzed and quantified as described in the legend to *A*. *C* and *D,* mitochondria isolated from nontransformed WT cells and those isolated from either *tom20*Δ or *tom70/71*Δ and their corresponding WT strains transformed with either Oep37 (*C*) or Oep24 (*D*) encoding plasmid were analyzed by SDS-PAGE and immunodecoration with antibodies against Oep37 or Oep24, respectively. In addition, immunodecoration with antibodies against the indicated mitochondrial proteins was performed.

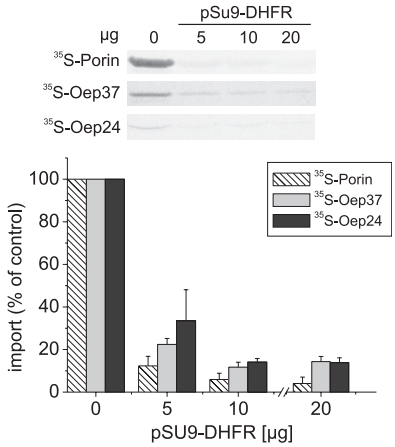


FIGURE 8. **Integration of Oep37 and Oep24 into the mitochondrial OM requires the TOM import pore.** Radiolabeled precursors of Oep37, Oep24, and Porin (as a control) were imported into mitochondria in the absence or presence of the indicated amounts of recombinant pSu9-DHFR. Imported proteins were analyzed and quantified as described in the legend to Fig. 7*A*. The amount of precursor proteins imported into mitochondria without added pSu9-DHFR was set to 100%.

lysate. Similar dependence on Tom70 was observed for the import of multispan proteins of the outer and inner mitochondrial membranes (44–47).

Mitochondrial β-barrel proteins are translocated through the import pore of the TOM complex before their insertion into the mitochondrial OM (7–9). Therefore, we asked whether Oep37 and Oep24 follow a similar pathway. To this end, we added to the *in vitro* import reaction excess amounts of recombinant matrix-destined precursor (pSu9(1–69)-DHFR), which can block the TOM pore and thus compete with import of other TOM-dependent precursor proteins. This addition resulted in a significant reduction in the membrane integration of Oep37, Oep24, and of Porin as a control (Fig. 8). Hence, it appears that the TOM import pore is used in the membrane-assembly pathway of Oep37 and Oep24.

Upon their translocation across the pore of the TOM complex, β-barrel proteins are chaperoned by the small Tim proteins residing in the IMS (17, 20, 48, 49). Therefore, we next investigated whether the chloroplast proteins require these small Tim chaperones for their assembly in the mitochondrial outer membrane. First, we imported Oep37 into mitochondria where the OM was ruptured by osmotic swelling. This treatment results in the release of the small Tims from the IMS that in turn causes a reduction in the assembly efficiency of mitochondrial β-barrel proteins and inner membrane carrier proteins like ADP-ATP carrier (AAC) (Fig. 9*A*) (20, 49–51). Of note, the membrane integration of Oep37, as monitored by resistance to alkaline extraction, was not compromised in the ruptured organelles, whereas that of AAC was significantly reduced (Fig. 9*A*). Next, we observed that the capacity of mitochondria isolated from a strain lacking both Tim8 and Tim13 or a strain harboring a temperature-sensitive allele of *TIM10* to import *in vitro* newly synthesized Oep37 molecules was not reduced as compared with that of organelles isolated from wild-type cells (Fig. 9, *B* and *C*). Furthermore, Oep37-encoding plasmid was transformed into these mutated cells. Crude mitochondria were isolated from these cells and subjected to SDS-PAGE and immunodecoration. Our results revealed that the observed levels of Oep37 in the mutated cells are very similar to those in the corresponding parental strains (Fig. 9*D*). The results with Oep24 were slightly different as its import into mitochondria lacking Tim8/13 complex and its levels in cells lacking these proteins were mildly hampered (Fig. 9, *B* and *D*). Collectively, these findings suggest that the small Tim proteins play only a minor role, if at all, in the import pathway of Oep24 and Oep37.

As the TOB complex is absolutely essential for the membrane insertion of mitochondrial β-barrel proteins, we investigated whether Oep37 and Oep24 share this TOB dependence. As anticipated, the *in vitro* integration of both proteins was heavily compromised in mitochondria lacking Mas37, a peripheral subunit of the TOB complex (Fig. 10*A*). We further investigated the Mas37 dependence by transforming cells deleted for Mas37 with an Oep37- or Oep24-encoding plasmid. Crude mitochondria were isolated from WT and the mutant cells, and their proteins were analyzed by SDS-PAGE and immunodecoration. In accordance with the *in vitro* results, this analysis revealed that the steady-state levels of both proteins are strongly reduced in *mas37*Δ cells (Fig. 10*B*). To substantiate the dependence on the TOB complex, we expressed Oep37 under the control of the *TPI* promoter in cells where the essential component Tob55 is under the control of the inducible *GAL* promoter (52). Growing these cells on glucose results in a gradual reduction in the levels of the essential protein Tob55, which in turn compromises growth of the cells (16, 52). Mitochondria from these Tob55-depleted cells were isolated after growth for various periods on glucose-containing medium, and the levels of mitochondrial proteins were monitored. We also observed in parallel to the gradual reduction of Tob55 a reduction in the other mitochondrial β-barrel protein Tom40 and Porin (Fig. 10*C*). Importantly, the amounts of Oep37 were also compromised upon the depletion of Tob55 (Fig. 10*C*). Taken together, our findings demonstrate the involvement of the TOB complex in the membrane assembly of Oep37 and Oep24.

DISCUSSION

β-Barrel proteins in modern endosymbiotic organelles evolved most probably from structurally similar proteins in their corresponding ancestors (53, 54). Furthermore, detailed studies on the biogenesis of these proteins in bacteria and mitochondria demonstrated that the central protein component and the basic mechanism in the biogenesis pathway are conserved (9, 55). Accordingly, we and others could previously show that signals in bacterial β-barrel proteins are functional in eukaryotic cells for targeting to and assembly in mitochondria (16, 18). However, it is not clear whether this similarity between two β-barrel containing systems, mitochondria and bacteria, can be extrapolated to the biogenesis of β-barrel proteins in the third membrane that contains such structures, namely the OM of chloroplasts. Thus, in this study we addressed the question whether signals in chloroplast β-barrel proteins can be recognized and processed by the mitochondrial import machinery.

To that goal, the chloroplast β-barrel proteins Oep37 and Oep24 were expressed in yeast cells. Our current results demonstrate that the proteins were assembled into the yeast mito-

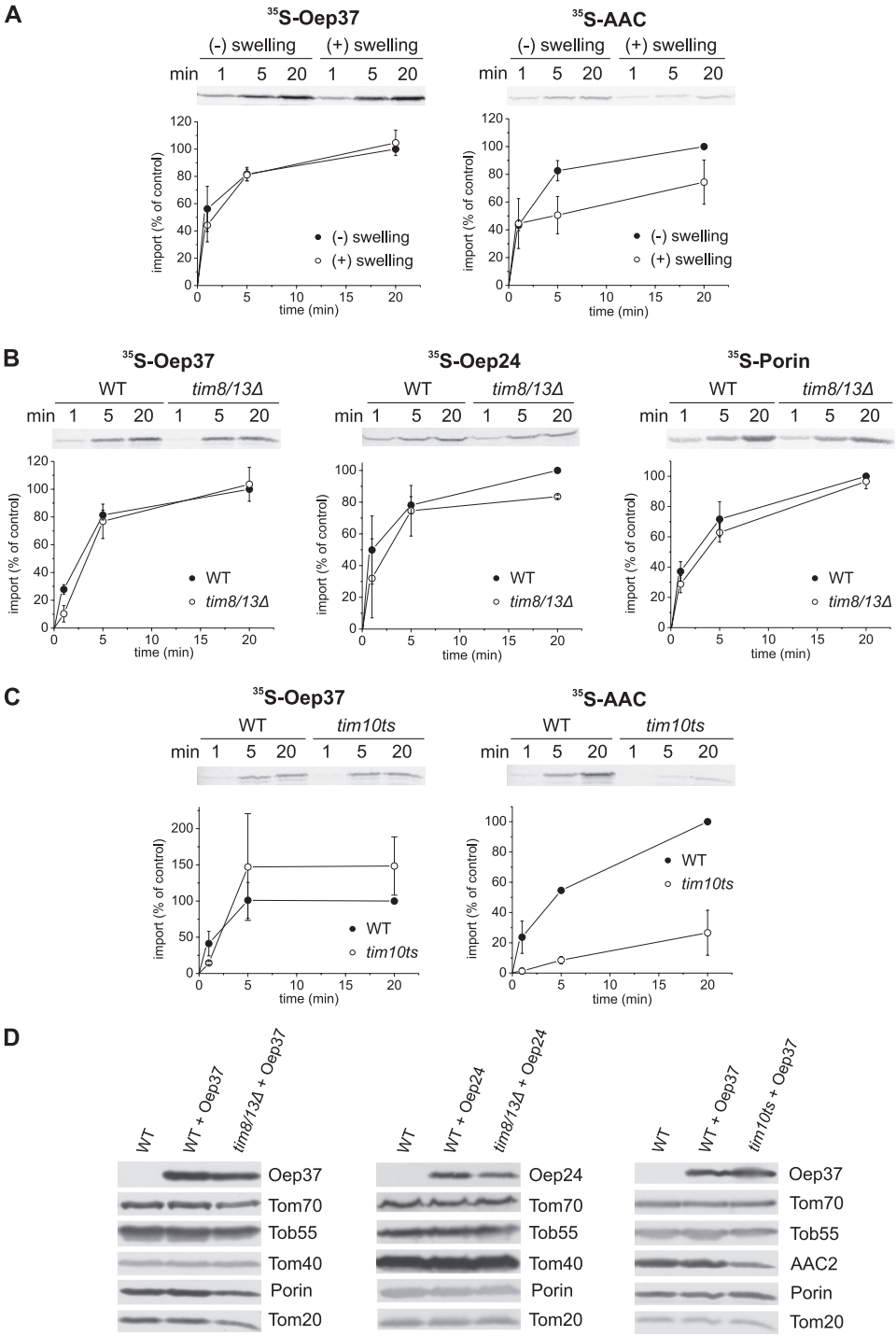


FIGURE 9. **Small Tim chaperones have only a minor role in the assembly of Oep37 and Oep24 into the mitochondrial OM.** *A,* rupturing of the outer membrane does not compromise the assembly of Oep37. Radiolabeled precursors of Oep37 and AAC (as a control) were incubated for the indicated time periods with isolated intact mitochondria or with mitochondria that had been subjected to osmotic swelling. After import, mitochondria were pelleted and subjected to alkaline extraction, and the pellet fractions were analyzed by SDS-PAGE followed by autoradiography. The amount of precursor proteins imported into intact mitochondria for 20 min was set to 100%. An autoradiographic representative of three independent repeats and quantification of three independent experiments are presented. *B,* insertion of Oep37 and Oep24 is hardly affected in mitochondria lacking the Tim8/Tim13 complex. Radiolabeled precursors of Oep37, Oep24, and Porin were imported into mitochondria isolated from either *tim8Δ/tim13Δ* or its corresponding parental strain. Imported proteins were analyzed and quantified as described in the legends to Fig. 7*A*. *C,* insertion of Oep37 is not affected in mitochondria mutated in *TIM10*. Radiolabeled precursors of Oep37 and AAC were imported into mitochondria isolated from a strain harboring a temperature-sensitive allele of *TIM10* (*TIM10-1* (58)) or from its corresponding parental strain. Imported proteins were analyzed and quantified as described in the legends to Fig. 7*A*. *D,* mitochondria isolated from nontransformed WT cells and those isolated from either *tim8/tim13Δ* or *TIM10-1* and their corresponding parental strains transformed with Oep37- or Oep24-encoding plasmid were analyzed by SDS-PAGE and immunodecoration with antibodies against Oep37 or Oep24, respectively. In addition, immunodecoration with antibodies against the indicated mitochondrial proteins was performed.

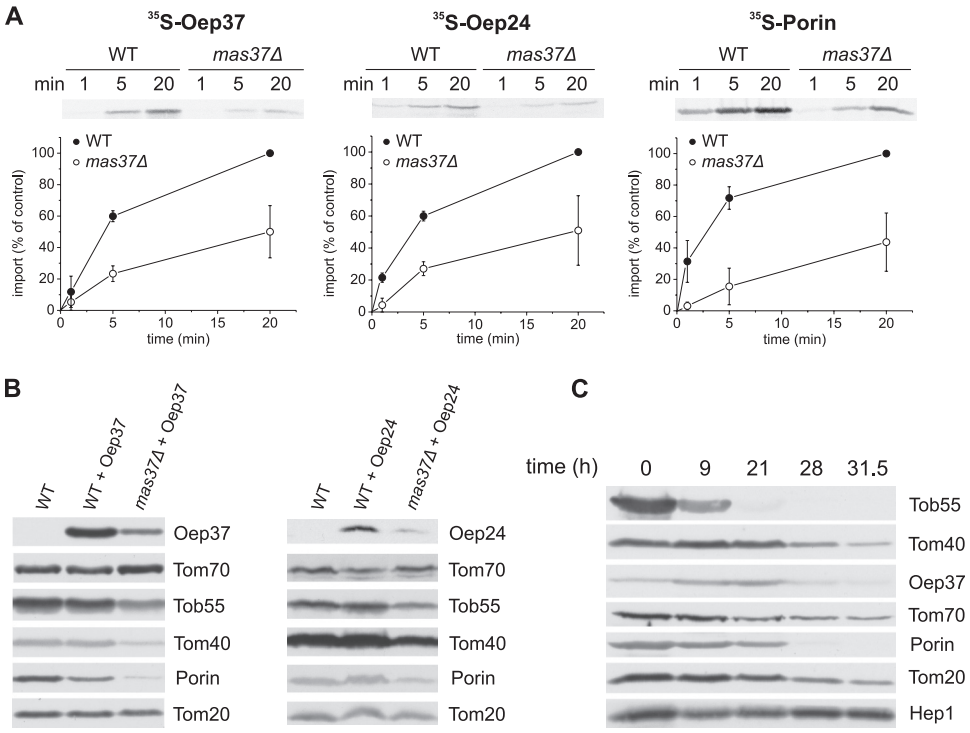


FIGURE 10. **TOB complex is crucial for the mitochondrial integration of Oep37 and Oep24**. *A,* radiolabeled precursors of Oep37, Oep24, and Porin were imported into mitochondria isolated from either WT or *mas37*Δ strains. Imported proteins were analyzed and quantified as described in the legends to Fig. 7*A*. *B,* mitochondria isolated from nontransformed WT cells and those isolated from *mas37*Δ and their corresponding wild-type cells transformed with either Oep37- or Oep24-encoding plasmid were analyzed by SDS-PAGE and immunodecoration with antibodies against Oep37 or Oep24, respectively. In addition, immunodecoration with antibodies against the indicated mitochondrial proteins was performed. *C,* Oep37 was transformed into cells expressing Tob55 under the control of the *GAL10* promoter. Cells were harvested at the indicated time points after a shift from galactose- to glucose-containing medium. Crude mitochondria were isolated, and proteins were analyzed by SDS-PAGE and immunodecoration with antibodies against Oep37 and the indicated mitochondrial proteins. Tob55, Tom40, and Porin are β-barrel proteins.

chondrial OM in a process that required the TOM complex and the TOB machinery. Thus, their assembly pathway was similar to the one taken by the *bona fide* mitochondrial β-barrel proteins. Of note, although Oep37 and Oep24 do not share sequence similarity with endogenous mitochondrial β-barrels, they appear to bear the signals required for recognition by the aforementioned fungal mitochondrial import components. Although the import receptors of plant mitochondria are somewhat altered in comparison with their counterparts in fungal cells, they recognize a similar set of substrate proteins (56). We believe that the plant TOM receptors can also recognize mitochondrial β-barrel proteins. However, because we did not address the recognition of Oep37 by plant TOM receptors, we cannot exclude the unlikely possibility that the *in vitro* import into plant mitochondria was mediated by other mitochondrial surface proteins.

Our findings underscore the importance of structural elements rather than mitochondrial specific sequences for the biogenesis of β-barrel proteins in mitochondria. They also might imply that the yet to be identified machinery that assembles β-barrel proteins into the chloroplast OM uses similar signals as the mitochondrial counterpart. Collectively, it seems that the evolutionary relations of mitochondrial and chloroplast β-barrel proteins to their bacterial ancestral proteins ensured a certain degree of similarity also among β-barrel proteins from both endosymbiotic organelles. Considering the evolutionary link, it is also tempting to speculate that the principles of β-barrel biogenesis have been conserved from a cyanobacterium to chloroplasts.

An interesting open question is how the precursors of eukaryotic β-barrel proteins are targeted from the cytosol to their target membrane. The signals that facilitate the specific targeting of such precursors to either mitochondria or chloroplasts are not yet characterized. So far, a linear well defined sequence that can function as an intracellular targeting signal was not identified. The only exception is the chloroplast Toc75-III where an N-terminal extension functions as a targeting sequence (10). Thus, it can be assumed that the mitochondrial and chloroplast protein import machineries recognize β-barrel-related structural elements (9, 57). However, this assumption raises the question whether such elements in chloroplast β-barrel proteins are distinct from those in β-barrel proteins destined to mitochondria. Our current results suggest that this is probably not the case as both Oep37 and Oep24 were imported *in vitro* into plant mitochondria and both *in vivo* and *in vitro* into yeast mitochondria.

Conversely, the mitochondrial VDAC protein was imported *in vivo* and *in vitro* only to plant mitochondria. One possibility to explain these observations is the difference in the availability of both organelles in plant cells and especially in mesophyll cells. Chloroplasts are predominant in these cells and expose a much larger surface as compared with mitochondria. Hence, the former organelles must ensure that only the correct proteins are inserted. Thus, a bouncing off mechanism in chloro-

plasts seems to exist. Because of the difference in their surfaces, the likelihood that chloroplast proteins are targeted to mitochondria by free diffusion is rather low, thus mitochondria did not evolve such a mechanism. An additional potential explanation to the absence of import of VDAC into chloroplasts might be its weak affinity to the import receptors of the chloroplasts. As currently it is not clear which proteins recognize β-barrel substrates on the surface of chloroplasts, a detailed study on such recognition depends on future studies that will shed light on this issue.

The aforementioned mechanisms are probably not sufficient to ensure specific targeting. Another potential quality control measure could be the degradation of mis-targeted chloroplast β-barrel proteins by mitochondrial proteases. In any case, it appears that plant cells should not allow those β-barrel proteins destined to chloroplasts any contact with mitochondria as this latter organelle can serve as a default target to all β-barrel proteins. This idea is in line with the common assumption that chloroplasts were integrated into cells that already contained mitochondria. Thus, whereas the mitochondrial β-barrels could follow a general default pathway, the recently arrived chloroplast ones had to develop a mechanism to avoid this destination. Part of such an evading pathway of the chloroplast-destined β-barrels could involve recognition by dedicated factors already in the cytosol. A challenge for future studies will be to identify such putative factors and to understand how they can recognize specifically chloroplast β-barrel proteins.

Acknowledgments—We thank K. Rehn, G. Englich, and E. Kracker for technical support and G. S. Waldo (Los Alamos National Laboratory, Los Alamos, NM) for providing templates for the self-assembling GFP.

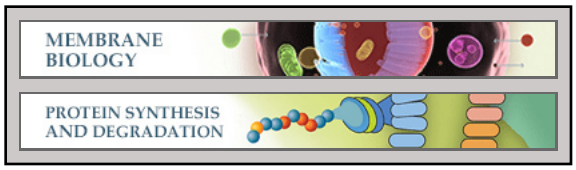
REFERENCES

1. Schleiff, E., Eichacker, L. A., Eckart, K., Becker, T., Mirus, O., Stahl, T., and Soll, J. (2003) Prediction of the plant β-barrel proteome. A case study of the chloroplast outer envelope. *Protein Sci.* **12,** 748–759
2. Goetze, T. A., Philippar, K., Ilkavets, I., Soll, J., and Wagner, R. (2006) OEP37 is a new member of the chloroplast outer membrane ion channels. *J. Biol. Chem.* **281,** 17989–17998
3. Hemmler, R., Becker, T., Schleiff, E., Bölter, B., Stahl, T., Soll, J., Götze, T. A., Braams, S., and Wagner, R. (2006) Molecular properties of Oep21, an ATP-regulated anion-selective solute channel from the outer chloroplast membrane. *J. Biol. Chem.* **281,** 12020–12029
4. Schleiff, E., Maier, U. G., and Becker, T. (2011) Omp85 in eukaryotic systems. One protein family with distinct functions. *Biol. Chem.* **392,** 21–27
5. Soll, J., and Schleiff, E. (2004) Protein import into chloroplasts. *Nat. Rev. Mol. Cell Biol.* **5,** 198–208
6. Schleiff, E., and Becker, T. (2011) Common ground for protein translocation. Access control for mitochondria and chloroplasts. *Nat. Rev. Mol. Cell Biol.* **12,** 48–59
7. Pfanner, N., Wiedemann, N., Meisinger, C., and Lithgow, T. (2004) Assembling the mitochondrial outer membrane. *Nat. Struct. Mol. Biol.* **11,** 1044–1048
8. Endo, T., and Yamano, K. (2009) Multiple pathways for mitochondrial protein traffic. *Biol. Chem.* **390,** 723–730
9. Walther, D. M., Rapaport, D., and Tommassen, J. (2009) Biogenesis of β-barrel membrane proteins in bacteria and eukaryotes. Evolutionary conservation and divergence. *Cell. Mol. Life Sci.* **66,** 2789–2804
10. Tranel, P. J., and Keegstra, K. (1996) A novel, bipartite transit peptide targets OEP75 to the outer membrane of the chloroplastic envelope. *Plant Cell* **8,** 2093–2104
11. Schleiff, E., and Klösgen, R. B. (2001) Without a little help from 'my' friends. Direct insertion of proteins into chloroplast membranes? *Biochim. Biophys. Acta* **1541,** 22–33
12. Inoue, K., Baldwin, A. J., Shipman, R. L., Matsui, K., Theg, S. M., and Ohme-Takagi, M. (2005) Complete maturation of the plastid protein translocation channel requires a type I signal peptidase. *J. Cell Biol.* **171,** 425–430
13. Eckart, K., Eichacker, L., Sohrt, K., Schleiff, E., Heins, L., and Soll, J. (2002) A Toc75-like protein import channel is abundant in chloroplasts. *EMBO Rep.* **3,** 557–562
14. Patel, R., Hsu, S. C., Bédard, J., Inoue, K., and Jarvis, P. (2008) The Omp85-related chloroplast outer envelope protein OEP80 is essential for viability in *Arabidopsis*. *Plant Physiol.* **148,** 235–245
15. Müller, A., Rassow, J., Grimm, J., Machuy, N., Meyer, T. F., and Rudel, T. (2002) VDAC and the bacterial Porin PorB of *Neisseria gonorrhoeae* share mitochondrial import pathways. *EMBO J.* **21,** 1916–1929
16. Walther, D. M., Papic, D., Bos, M. P., Tommassen, J., and Rapaport, D. (2009) Signals in bacterial β-barrel proteins are functional in eukaryotic cells for targeting to and assembly in mitochondria. *Proc. Natl. Acad. Sci. U.S.A.* **106,** 2531–2536
17. Müller, J. E., Papic, D., Ulrich, T., Grin, I., Schütz, M., Oberhettinger, P., Tommassen, J., Linke, D., Dimmer, K. S., Autenrieth, I. B., and Rapaport, D. (2011) Mitochondria can recognize and assemble fragments of a β-barrel structure. *Mol. Biol. Cell* **22,** 1638–1647
18. Kozjak-Pavlovic, V., Ott, C., Götz, M., and Rudel, T. (2011) Neisserial Omp85 protein is selectively recognized and assembled into functional complexes in the outer membrane of human mitochondria. *J. Biol. Chem.* **286,** 27019–27026
19. Röhl, T., Motzkus, M., and Soll, J. (1999) The outer envelope protein OEP24 from pea chloroplasts can functionally replace the mitochondrial VDAC in yeast. *FEBS Lett.* **460,** 491–494
20. Habib, S. J., Waizenegger, T., Lech, M., Neupert, W., and Rapaport, D. (2005) Assembly of the TOB complex of mitochondria. *J. Biol. Chem.* **280,** 6434–6440
21. Paschen, S. A., Rothbauer, U., Káldi, K., Bauer, M. F., Neupert, W., and Brunner, M. (2000) The role of the TIM8–13 complex in the import of Tim23 into mitochondria. *EMBO J.* **19,** 6392–6400
22. Kondo-Okamoto, N., Shaw, J. M., and Okamoto, K. (2008) Tetratricopeptide repeat proteins Tom70 and Tom71 mediate yeast mitochondrial morphogenesis. *EMBO Rep.* **9,** 63–69
23. Schleiff, E., Heard, T. S., and Weiner, H. (1999) Positively charged residues, the helical conformation and the structural flexibility of the leader sequence of pALDH are important for recognition by hTom20. *FEBS Lett.* **461,** 9–12
24. Kirwin, P. M., Meadows, J. W., Shackleton, J. B., Musgrove, J. E., Elderfield, P. D., Mould, R., Hay, N. A., and Robinson, C. (1989) ATP-dependent import of a lumenal protein by isolated thylakoid vesicles. *EMBO J.* **8,** 2251–2255
25. von Arnim, A. G., Deng, X. W., and Stacey, M. G. (1998) Cloning vectors for the expression of green fluorescent protein fusion proteins in transgenic plants. *Gene* **221,** 35–43
26. Daum, G., Böhni, P. C., and Schatz, G. (1982) Import of proteins into mitochondria. Cytochrome b_2 and cytochrome *c* peroxidase are located in the intermembrane space of yeast mitochondria. *J. Biol. Chem.* **257,** 13028–13033
27. Bionda, T., Tillmann, B., Simm, S., Beilstein, K., Ruprecht, M., and Schleiff, E. (2010) Chloroplast import signals. The length requirement for translocation *in vitro* and *in vivo*. *J. Mol. Biol.* **402,** 510–523
28. Day, D. A., Neuburger, M., and Douce, R. (1985) Biochemical characterization of chlorophyll-free mitochondria from pea leaves. *Aust. J. Plant Physiol.* **12,** 219–228
29. Hausmann, N., Werhahn, W., Huchzermeyer, B., Braun, H. P., and Papenbrock, J. (2003) How to document the purity of mitochondria prepared from green tissue of pea, tobacco, and *Arabidopsis thaliana*. *Phyton* **43,** 215–229
30. Rödiger, A., Baudisch, B., and Klösgen, R. B. (2010) Simultaneous isolation of intact mitochondria and chloroplasts from a single pulping of plant

tissue. *J. Plant Physiol.* **167,** 620–624
31. Rudhe, C., Chew, O., Whelan, J., and Glaser, E. (2002) A novel *in vitro* system for simultaneous import of precursor proteins into mitochondria and chloroplasts. *Plant J.* **30,** 213–220
32. Schleiff, E., Soll, J., Küchler, M., Kühlbrandt, W., and Harrer, R. (2003) Characterization of the translocon of the outer envelope of chloroplasts. *J. Cell Biol.* **160,** 541–551
33. Sommer, M. S., Daum, B., Gross, L. E., Weis, B. L., Mirus, O., Abram, L., Maier, U. G., Kühlbrandt, W., and Schleiff, E. (2011) Chloroplast Omp85 proteins change orientation during evolution. *Proc. Natl. Acad. Sci. U.S.A.* **108,** 13841–13846
34. Machettira, A. B., Gross, L. E., Sommer, M. S., Weis, B. L., Englich, G., Tripp, J., and Schleiff, E. (2011) The localization of Tic20 proteins in *Arabidopsis thaliana* is not restricted to the inner envelope membrane of chloroplasts. *Plant Mol. Biol.* **77,** 381–390
35. Schleiff, E., Tien, R., Salomon, M., and Soll, J. (2001) Lipid composition of outer leaflet of chloroplast outer envelope determines topology of OEP7. *Mol. Biol. Cell* **12,** 4090–4102
36. Hurt, E. C., Soltanifar, N., Goldschmidt-Clermont, M., Rochaix, J. D., and Schatz, G. (1986) The cleavable presequence of an imported chloroplast protein directs attached polypeptides into yeast mitochondria. *EMBO J.* **5,** 1343–1350
37. Brink, S., Flügge, U. I., Chaumont, F., Boutry, M., Emmermann, M., Schmitz, U., Becker, K., and Pfanner, N. (1994) Preproteins of chloroplast envelope inner membrane contain targeting information for receptor-dependent import into fungal mitochondria. *J. Biol. Chem.* **269,** 16478–16485
38. Rudolph, R., and Lilie, H. (1996) *In vitro* folding of inclusion body proteins. *FASEB J.* **10,** 49–56
39. Lee, Y. J., Kim, D. H., Kim, Y. W., and Hwang, I. (2001) Identification of a signal that distinguishes between the chloroplast outer envelope membrane and the endomembrane system *in vivo*. *Plant Cell* **13,** 2175–2190
40. Blachly-Dyson, E., Song, J., Wolfgang, W. J., Colombini, M., and Forte, M. (1997) Multicopy suppressors of phenotypes resulting from the absence of yeast VDAC encode a VDAC-like protein. *Mol. Cell. Biol.* **17,** 5727–5738
41. Rapaport, D., and Neupert, W. (1999) Biogenesis of Tom40, core component of the TOM complex of mitochondria. *J. Cell Biol.* **146,** 321–331
42. Krimmer, T., Rapaport, D., Ryan, M. T., Meisinger, C., Kassenbrock, C. K., Blachly-Dyson, E., Forte, M., Douglas, M. G., Neupert, W., Nargang, F. E., and Pfanner, N. (2001) Biogenesis of the major mitochondrial outer membrane protein Porin involves a complex import pathway via receptors and the general import pore. *J. Cell Biol.* **152,** 289–300
43. Yamano, K., Yatsukawa, Y., Esaki, M., Hobbs, A. E., Jensen, R. E., and Endo, T. (2008) Tom20 and Tom22 share the common signal recognition pathway in mitochondrial protein import. *J. Biol. Chem.* **283,** 3799–3807
44. Wiedemann, N., Pfanner, N., and Ryan, M. T. (2001) The three modules of ADP/ATP carrier cooperate in receptor recruitment and translocation into mitochondria. *EMBO J.* **20,** 951–960
45. Young, J. C., Hoogenraad, N. J., and Hartl, F. U. (2003) Molecular chaperones Hsp90 and Hsp70 Deliver preproteins to the mitochondrial import receptor Tom70. *Cell* **112,** 41–50
46. Becker, T., Wenz, L. S., Krüger, V., Lehmann, W., Müller, J. M., Goroncy, L., Zufall, N., Lithgow, T., Guiard, B., Chacinska, A., Wagner, R., Meisinger, C., and Pfanner, N. (2011) The mitochondrial import protein Mim1 promotes biogenesis of multispanning outer membrane proteins. *J. Cell Biol.* **194,** 387–395
47. Papic, D., Krumpe, K., Dukanovic, J., Dimmer, K. S., and Rapaport, D. (2011) Multispan mitochondrial outer membrane protein Ugo1 follows a unique Mim1-dependent import pathway. *J. Cell Biol.* **194,** 397–405
48. Hoppins, S. C., and Nargang, F. E. (2004) The Tim8-Tim13 complex of *Neurospora crassa* functions in the assembly of proteins into both mitochondrial membranes. *J. Biol. Chem.* **279,** 12396–12405
49. Wiedemann, N., Frazier, A. E., and Pfanner, N. (2004) The protein import machinery of mitochondria. *J. Biol. Chem.* **279,** 14473–14476
50. Smith, M., Hicks, S., Baker, K., and McCauley, R. (1994) Rupture of the mitochondrial outer membrane impairs Porin assembly. *J. Biol. Chem.* **269,** 28460–28464
51. Kübrich, M., Rassow, J., Voos, W., Pfanner, N., and Hönlinger, A. (1998) The import route of ADP/ATP carrier into mitochondria separates from the general import pathway of cleavable preproteins at the trans side of the outer membrane. *J. Biol. Chem.* **273,** 16374–16381
52. Paschen, S. A., Waizenegger, T., Stan, T., Preuss, M., Cyrklaff, M., Hell, K., Rapaport, D., and Neupert, W. (2003) Evolutionary conservation of biogenesis of β-barrel membrane proteins. *Nature* **426,** 862–866
53. Schleiff, E., and Soll, J. (2005) Membrane protein insertion. Mixing eukaryotic and prokaryotic concepts. *EMBO Rep.* **6,** 1023–1027
54. Alcock, F., Clements, A., Webb, C., and Lithgow, T. (2010) Evolution. Tinkering inside the organelle. *Science* **327,** 649–650
55. Gentle, I., Gabriel, K., Beech, P., Waller, R., and Lithgow, T. (2004) The Omp85 family of proteins is essential for outer membrane biogenesis in mitochondria and bacteria. *J. Cell Biol.* **164,** 19–24
56. Duncan, O., Murcha, M. W., and Whelan, J. (2012) Unique components of the plant mitochondrial protein import apparatus. *Biochim. Biophys. Acta*, in press
57. Rapaport, D. (2003) How to find the right organelle. Targeting signals in mitochondrial outer membrane proteins. *EMBO Rep.* **4,** 948–952
58. Koehler, C. M., Jarosch, E., Tokatlidis, K., Schmid, K., Schweyen, R. J., and Schatz, G. (1998) Import of mitochondrial carriers mediated by essential proteins of the intermembrane space. *Science* **279,** 369–373

Membrane Biology:

Evolutionary Conservation in Biogenesis of β-Barrel Proteins Allows Mitochondria to Assemble a Functional Bacterial Trimeric Autotransporter Protein



Thomas Ulrich, Philipp Oberhettinger, Monika Schütz, Katharina Holzer, Anne S. Ramms, Dirk Linke, Ingo B. Autenrieth and Doron Rapaport

J. Biol. Chem. 2014, 289:29457-29470.
doi: 10.1074/jbc.M114.565655 originally published online September 4, 2014

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 289, NO. 43, pp. 29457–29470, October 24, 2014

Evolutionary Conservation in Biogenesis of β-Barrel Proteins Allows Mitochondria to Assemble a Functional Bacterial Trimeric Autotransporter Protein*

Received for publication, March 24, 2014, and in revised form, August 4, 2014 Published, JBC Papers in Press, September 4, 2014, DOI 10.1074/jbc.M114.565655

Thomas Ulrich[‡], Philipp Oberhettinger[§], Monika Schütz[§], Katharina Holzer[‡], Anne S. Ramms[‡], Dirk Linke[¶1], Ingo B. Autenrieth[§], and Doron Rapaport[‡2]

From the [‡]Interfaculty Institute of Biochemistry, University of Tübingen, 72076 Tübingen, Germany, [§]Interfaculty Institute of Microbiology and Infection Medicine, University of Tübingen, 72076 Tübingen, Germany, and [¶]Department of Protein Evolution, Max-Planck Institute for Developmental Biology, 72076 Tübingen, Germany

Background: β-Barrel proteins are found in the outer membrane of Gram-negative bacteria, mitochondria, and chloroplasts.
Results: Mitochondria are able to assemble the bacterial trimeric autotransporter YadA in a functional form.
Conclusion: The lipoproteins of the BAM machinery are not absolutely required for the biogenesis of autotransporter protein.
Significance: The evolutionary link of mitochondria to bacteria allows the former to process even prokaryotic-specific proteins.

Yersinia adhesin A (YadA) belongs to a class of bacterial adhesins that form trimeric structures. Their mature form contains a passenger domain and a C-terminal β-domain that anchors the protein in the outer membrane (OM). Little is known about how precursors of such proteins cross the periplasm and assemble into the OM. In the present study we took advantage of the evolutionary conservation in the biogenesis of β-barrel proteins between bacteria and mitochondria. We previously observed that upon expression in yeast cells, bacterial β-barrel proteins including the transmembrane domain of YadA assemble into the mitochondrial OM. In the current study we found that when expressed in yeast cells both the monomeric and trimeric forms of full-length YadA were detected in mitochondria but only the trimeric species was fully integrated into the OM. The oligomeric form was exposed on the surface of the organelle in its native conformation and maintained its capacity to adhere to host cells. The co-expression of YadA with a mitochondria-targeted form of the bacterial periplasmic chaperone Skp, but not with SurA or SecB, resulted in enhanced levels of both forms of YadA. Taken together, these results indicate that the proper assembly of trimeric autotransporter can occur also in a system lacking the lipoproteins of the BAM machinery and is specifically enhanced by the chaperone Skp.

β-Barrel proteins are found in both prokaryotic and eukaryotic kingdoms. In prokaryotes, β-barrel proteins are found in the outer membrane (OM)[3] of Gram-negative bacteria whereas in eukaryotes, they reside exclusively in the OM of mitochondria and chloroplasts. Their presence in these organelles supports the endosymbiotic hypothesis, according to which mitochondria and chloroplasts evolved from prokaryotic ancestors (1). Indeed, the biogenesis of these proteins in the various systems bears considerable similarities (2).

Like other bacterial proteins, β-barrel proteins are synthesized in the cytoplasm and thus pass through both the inner membrane and the periplasm before reaching their final destination. To that goal they are synthesized with an N-terminal signal sequence that facilitates their transport across the inner membrane via the Sec system (3, 4). Upon entering the periplasm, the leader sequence is processed by a signal peptidase, and the nascent outer membrane proteins (OMP) associate with periplasmic chaperones, including SurA and Skp (4). Their subsequent integration into the OM is facilitated by the BAM complex. In *Escherichia coli* this complex is composed of five proteins: BamA to BamE. The central component of the complex is the essential protein BamA (also known as Omp85 or YaeT), a β-barrel protein itself (5, 6).

In eukaryotic cells, precursors of β-barrel proteins are synthesized on cytosolic ribosomes and then recognized by import receptors on the surface of mitochondria. Subsequently, they are translocated from the cytosol into the intermembrane space (IMS) via the translocase of the outer membrane (TOM) complex (7–9). Their transit through the IMS is facilitated by small chaperones (Tim9/Tim10 and Tim8/Tim13 complexes) and the assembly into the OM depends on a dedicated translocase, the TOB (also known as SAM) complex. The central member of this latter complex is the essential protein Tob55/Sam50 that bears sequence and functional homology to BamA (10–12). The other two subunits of the TOB complex, Mas37/Sam37 and Tob38/Sam35/Tom38, are peripheral membrane proteins exposed to the cytosol that share no obvious sequence similar-

* This work was supported by the Deutsche Forschungsgemeinschaft (SFB766/B1, to I. A. and M. S.; SFB766/B10, to D. L.; and SFB766/B11, to D. R.) and by the UKT fortüne program (F1433253, to P. O.).
[1] Present address: Dept. of Biosciences, University of Oslo, P.O. Box 1066 Blindern, 0316 Oslo, Norway.
[2] To whom correspondence should be addressed: Interfaculty Institute of Biochemistry, University of Tübingen, Hoppe-Seyler-Str. 4, 72076 Tübingen, Germany. Tel.: 49-7071-2974184; Fax: 49-7071-294016; E-mail: doron.rapaport@uni-tuebingen.de.
[3] The abbreviations used are: OM, outer membrane; AT, autotransporter; BAM, beta-barrel assembly machinery; HA, hemagglutinin; IMS, intermembrane space; OMP, outer membrane protein; TAA, trimeric autotransporter adhesins; TOM, translocase of the outer mitochondrial membrane; YadA, Yersinia adhesin A.

ity with the lipoproteins of the bacterial BAM complex (13–17). Thus, the biogenesis machineries in bacteria and mitochondria share certain characteristics: (i) insertion into the OM from the internal side of the membrane, (ii) involvement of soluble chaperones in delivering the precursor proteins to the target membrane, and (iii) sequence and functional homology between the central protein components of the inserting translocases. On the other hand, the assembly processes vary with respect to the accessory proteins and the fact that precursors of mitochondrial β-barrel proteins are synthesized in the cytosol without signal sequence and they initially have to cross the OM.

To better understand the assembly process of β-barrel proteins in both bacteria and mitochondria we expressed bacterial β-barrel proteins like OmpA, PhoE, and Omp85 in the yeast *Saccharomyces cerevisiae*. The proteins were imported into the mitochondrial OM and formed native-like oligomers. A detailed investigation of the import pathway revealed that the bacterial proteins required the TOM and TOB complexes for their assembly. Thus, they followed a route shared with mitochondrial β-barrel proteins (18). Similarly, the pathogenic bacterial PorB can target mitochondria in mammalian cells (19, 20). Moreover, we could demonstrate by reciprocal approach that expression of mitochondrial porin in *E. coli* cells resulted in a BamA-dependent assembly of the protein in the bacterial OM (21). Taken together, it appears that despite some differences the basic mechanism by which β-barrel proteins assemble in the OM of bacteria and mitochondria is evolutionary conserved. The aforementioned investigations revealed that canonical β-barrel proteins from one system can be dealt with and assembled by the other.

Despite these similarities in the biogenesis pathways and machineries, an open question is whether the evolutionary relations of mitochondria to bacteria will allow the former to process special forms of β-barrel proteins that are completely absent from eukaryotic cells. Such proteins are the autotransporter (AT) proteins and their sub-group of trimeric autotransporter adhesins (TAAs) that form a special subfamily of bacterial β-barrel proteins. These proteins have a characteristic arrangement of functional domains, including an N-terminal signal peptide, an internal passenger domain (also called the effector domain), and a relatively short C-terminal β-domain (also designated as a translocator domain). The passenger moiety mediates the various functions of the autotransporters, which are often associated with virulence, and the translocation domain forms a β-barrel that anchors the protein to the OM. This anchor is made by a single 12-stranded β-barrel structure to which in the case of TAAs each monomer is contributing four β-strands (22–25). The biogenesis of these proteins is thought to be a multi-step process, in which membrane insertion and β-barrel pore formation is followed by the export ("autotransport") of the passenger domain(s) through the newly formed pore of the C-terminal translocator domain (26).

Considering the special features of TAAs and the requirement to transfer a rather large passenger domain across the OM, we wondered whether mitochondria will be able to process such precursor proteins. In a first stage of our studies we initially expressed the β-domain of one of the prototypic members of this subfamily, Yersinia adhesin A (YadA) in yeast cells and analyzed its cellular localization and topology. We found that the β-domain of YadA was imported into mitochondria and got assembled into the OM of the organelle in its native trimeric structure (27). However, it is currently unclear how mitochondria can deal with the transfer of the passenger domain across the OM. Since very little is known about how newly synthesized TAAs cross the periplasm, integrate into the bacterial OM and assemble into oligomeric structures (24, 28), using mitochondria as a model system can shed light on these issues.

To that end we expressed full-length YadA molecules in yeast cells and analyzed their biogenesis and assembly. We could characterize two species in mitochondria of such transformed cells, a monomeric assembly intermediate and a native-like functional trimeric structure. We further observed that the co-expression of a mitochondrial-destined form of the bacterial chaperone Skp, but not of other bacterial chaperones, dramatically enhanced the assembly of the YadA molecules into functional trimeric structures. Taken together, these results indicate that the proper assembly of TAAs can occur even in the mitochondria of eukaryotic cells in a process that is facilitated by the periplasmic chaperone Skp.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Methods—Standard genetic techniques were used for growth and manipulation of yeast strains. In this study the wild-type strains YPH499 and W303α were utilized. The *tom20*Δ, *mas37*Δ and *GAL10-TOB55* strains were described before ((27, 29, 11), respectively). The *tom70*Δ/*tom71*Δ double-deletion strain, the *yme1*Δ strain, and the *tom40–25* strain are kind gifts of Dr. Okamoto (30), Dr. Langer (31), and Drs. Becker and Pfanner (32), respectively. Unless otherwise stated, cells were grown on rich or synthetic galactose-containing medium (YPGal or SGal, respectively). For some experiments, cycloheximide (100 $\mu g\ ml^{-1}$) was added to the medium.

Recombinant DNA Techniques—The sequence encoding full-length *Y. enterocolitica* YadA lacking the signal sequence was cloned by PCR amplification from the plasmid pASK-IBA2_yadA (33). The PCR products were inserted into the yeast expression vector pYX113 or pYX142. For construction of N-terminally HA-tagged YadA, the 3xHA-tag cassette was PCR amplified from pFA6a-3HA-KanMX4 and inserted into the target vectors using EcoRI and NcoI restriction sites. YadA-MA was cloned as previously described (27). The sequence encoding SecB from *E. coli* was subcloned from the plasmid p29SEN_SecB into the yeast expression vector pYX132 using EcoRI and HindIII restriction sites. The sequences encoding *E. coli* SurA and Skp lacking their signal sequences were cloned by PCR amplification from corresponding plasmids and inserted into the yeast expression vectors pYX122 and pYX113 carrying the N-terminal domain (aa 1–228) of the yeast mitochondrial protein Mgm1 lacking the first transmembrane segment (Mgm1-(1–228ΔTM1). The resulting fusion proteins Mgm1-(1–228 ΔTM1)-SurA and Mgm1-(1–228 ΔTM1)-Skp are referred to as mtSurA and mtSkp, respectively.

Biochemical Procedures—Mitochondria were isolated from yeast cells by differential centrifugation as described before (34). Subcellular fractionation of yeast cells was performed as

described previously (18). For swelling experiments mitochondria were subjected to hypoosmotic shock for 30 min on ice in swelling buffer (20 mM Hepes/KOH, pH 7.0).

For pull-down assays 600 μg purified mitochondria were solubilized at 4 °C for 1 h in lysis buffer A containing 50 mM NaH_2PO_4, 100 mM NaCl, 10% (v/v) glycerol, complete protease inhibitor mix (cOmplete, EDTA-free; Roche), and 1% (w/v) digitonin. Undissolved material was spun down (30,000 × *g*, 15 min, 4 °C), and 2% of the supernatant were kept as input. Slurry HA-beads (30 μl, Pierce) or protein G-beads pre-coupled with antibodies against Skp were equilibrated for 10 min on ice in 1000 μl lysis buffer A harboring 1% (*w*/*v*) bovine serum albumin (BSA) and washed with 1000 μl of lysis buffer A. Then, the beads were incubated at 4 °C overnight with the mitochondrial lysate, and 2% of the supernatant were kept as unbound materials. Next, the beads were washed five times with lysis buffer A containing 0.02% (w/v) digitonin, and finally proteins were eluted with Laemmli buffer at 95 °C.

Immunofluorescence Microscopy—For immunofluorescence staining mitochondria in SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS/KOH, pH 7.2) were centrifuged on polyethyleneimine-coated coverslips. Subsequently the coverslips were incubated with 1% (*w*/*v*) BSA in SEM buffer to block unspecific binding sites. HA tags were stained by overnight incubation of the samples with monoclonal mouse antibody (diluted 1:100) followed by an incubation at room temperature for 2 h in a dark chamber with Cy3-conjugated secondary anti-mouse antibody (diluted 1:100, Dianova). Finally, coverslips were mounted with Mowiol (Merck, Darmstadt, Germany). Fluorescence images were obtained with a DMRE fluorescence microscope (Leica).

Cell Culture and IL8-ELISA—HeLa cells (ATCC number: CCL-2) were cultivated in RPMI 1640 medium (Biochrom) supplemented with 2 mM L-glutamine (Sigma) and 10% fetal calf serum (FCS; Invitrogen). For infection assay, 1.5×10^5 HeLa cells per well were seeded in a 24-well microplate and grown overnight. The next day, cells were washed once with pre-warmed PBS and grown for another hour in RPMI 1640 medium supplemented with 10% FCS but without antibiotics. Afterward, HeLa cells were incubated with freshly isolated mitochondria samples with a multiplicity of infection (MOI) of 1200, and the cells were incubated for another 6 h. The cell culture supernatant was collected, and an IL-8 ELISA was carried out as described previously (35). IL-8 concentrations were calculated using recombinant human IL-8 (Becton Dickinson) as a standard.

RESULTS

Full-length YadA Is Targeted to Mitochondria—To investigate whether yeast mitochondria can deal with the biogenesis and assembly of the trimeric autotransporter adhesin YadA, we transformed a construct encoding full-length protein with N-terminal HA tag into yeast cells. Subcellular fractionation of the transformed cells revealed that, similarly to the mitochondrial proteins Tom70 and Tom40, YadA is located in mitochondria (Fig. 1*A*). This behavior resembles the exclusive mitochondrial targeting of a construct containing only the β-domain of the protein (27). Of note, the expression of YadA neither affected the growth of the transformed yeast cells nor altered the morphology of mitochondria in these cells (data not shown). It is well documented that YadA forms a trimeric species that is stable in SDS-PAGE (36–39). Hence, we isolated mitochondria from the YadA expressing strain and analyzed the migration behavior of YadA. As expected, we observed a band representing the trimeric form of the protein, but a significant portion of YadA molecules migrated as a monomeric form. The mature part of YadA contains 422 amino acid residues and together with the 3xHA-tag it has a molecular mass of approx. 48 kDa. Of note, both the monomer and the trimer species migrate at a higher apparent molecular mass than the expected one, probably due to the triple HA-tag (Fig. 1*B*, *lane 1*). As the monomeric form of native YadA is hardly detected in bacterial samples (37, 39), it seems that although YadA can be assembled in yeast mitochondria, this assembly is not as efficient as in bacteria.

Next, we were interested to study the differences between the monomeric and the trimeric forms. To that goal, the isolated mitochondria were subjected to carbonate extraction. Remarkably, the monomeric version of YadA was found partially in the supernatant of this treatment together with soluble proteins like the IMS isoform of Mcr1 and the matrix protein Hep1. In contrast, the trimeric species was exclusively detected in the pellet fraction together with other membrane-embedded mitochondrial proteins like Tom40 or Tom20 (Fig. 1*B*, *lanes 2* and *3*). Moreover, addition of increasing amounts of externally added protease to the isolated mitochondria resulted in complete disappearance of the signal corresponding to the trimeric form of YadA whereas a portion of the monomeric form was resistant to proteolytic degradation (Fig. 1*C*, *lanes 1–5*). The observed protease resistance of the monomeric form cannot be explained by aggregation of this species because it was completely degraded upon solubilization of the mitochondrial membranes by detergent (Fig. 1*C*, *lane 6*). As expected, the surface-exposed protein Tom20 was degraded already by low amounts of the protease. In contrast, the IMS protein Dld1 was protected, demonstrating the intactness of the isolated organelles. Collectively, these observations suggest that a portion of the monomeric form is associated with mitochondria in a topology where the N-terminally HA tag is exposed on the organelle's surface. This population represents probably early import intermediates. The other fraction resides already in the mitochondrial IMS where it is protected from the external proteases.

To directly test this hypothesis the proteolytic treatment was performed with organelles where their OM was either left intact or ruptured by hypotonic swelling. As observed before, the usage of the higher concentrations of proteinase K caused degradation of a sub-population of the monomeric form even in intact mitochondria (Fig. 1, *C*, *lane 5* and *D*, *lane 2*). This degradation became almost complete upon rupturing the OM (Fig. 1*D*, compare *lanes 2* and *4*). As expected, the short form of Mcr1 and Tim13, both residing in the IMS, were resistant to the protease treatment in intact organelles but got released from the ruptured organelles and thus are not detected in the corresponding samples (Fig. 1*D*, *lanes 3* and *4*).

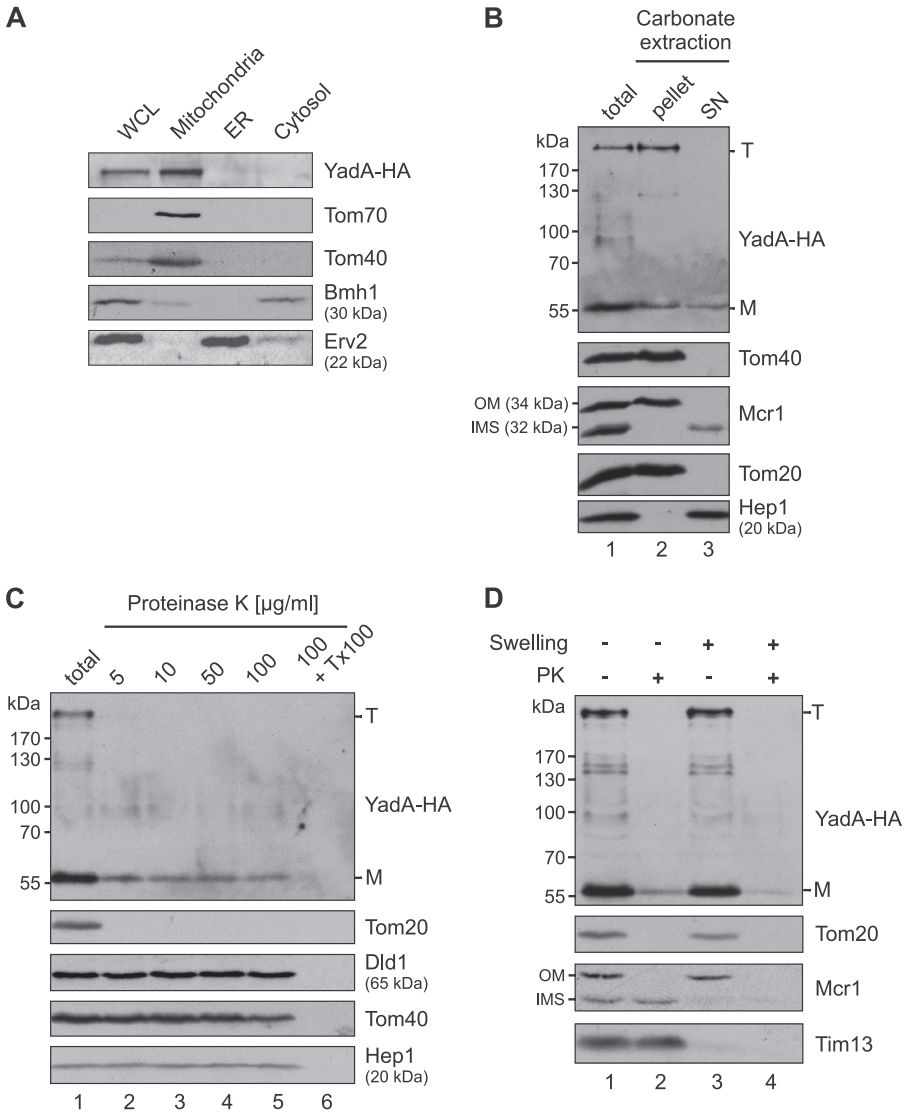


FIGURE 1. **The trimeric autotransporter YadA is targeted to the mitochondrial outer membrane.** *A*, whole cell lysate (*WCL*) of yeast cells expressing YadA-HA and fractions corresponding to mitochondria, endoplasmic reticulum (*ER*), and cytosol were analyzed by SDS-PAGE and immunodetection with antibodies against the HA tag, the mitochondrial OM proteins proteins Tom70 and Tom40, Bmh1 as a cytosolic marker protein as well as the ER protein Erv2. *B*, mitochondria isolated from cells expressing YadA-HA were directly analyzed (total) or subjected to carbonate extraction and centrifuged to discriminate between membrane proteins in the pellet fraction (*lane 2*) and soluble proteins in the supernatant (SN, *lane 3*). Proteins were analyzed by SDS-PAGE and immunodetection with antibodies against the HA-tag; Tom40; Tom20, an OM protein exposed on the surface of the organelle; Mcr1, a protein with two isoforms, a 34 kDa OM species and a soluble 32 kDa species in the IMS; Hep1, mitochondrial matrix protein. The positions of monomeric and trimeric YadA-HA are indicated with M and T, respectively. *C*, isolated mitochondria were either left intact (total, *lane 1*) or incubated with the indicated amounts of externally added proteinase K (PK, *lanes 2–5*). In one sample, the protease was added in the presence of Triton X-100 (*lane 6*). Further analysis was as described for *part B*. Dld1, an IMS protein. *D*, isolated mitochondria from yeast cells expressing YadA-HA were either left intact (*lanes 1–2*) or subjected to osmotic swelling to rupture the OM (*lanes 3–4*). Half of each of the two samples was incubated with PK. Proteins were analyzed by SDS-PAGE, and immunodetection with antibodies against the HA tag and the indicated mitochondrial proteins. Tim13, an IMS protein.

Taken together, these observations indicate that the trimeric form of YadA is embedded in the mitochondrial OM where the passenger domain is exposed to the cytosol. In contrast, the monomeric species contains most likely two populations: (i) a portion, which probably represents early import intermediates, associated with the OM and still (at least partially) facing the cytosol, and (ii) a soluble intermediate in the IMS that is protected from external protease. Of note, such a soluble monomeric intermediate was not characterized so far in bacteria.

YadA Import into Mitochondria Is Independent of Import Receptors but Requires the TOM Pore and the TOB Complex—It was previously observed that efficient import of mitochondrial and bacterial β-barrel proteins into yeast mitochondria require the import receptors Tom20 and Tom70 where the former is the more important one (18, 40–42). Interestingly, this requirement for import receptors was shared by other bacterial β-barrel proteins expressed in yeast cells but not by the β-domain of YadA (18, 27). This receptor-independency could result from either the small size of this domain in comparison to other β-barrel proteins or from the absence of trimeric autotransporter proteins in eukaryotes, which means that the mitochondrial import receptors were never exposed to such substrates.

To discriminate between these two alternatives we asked whether the import receptors of the TOM complex play a role

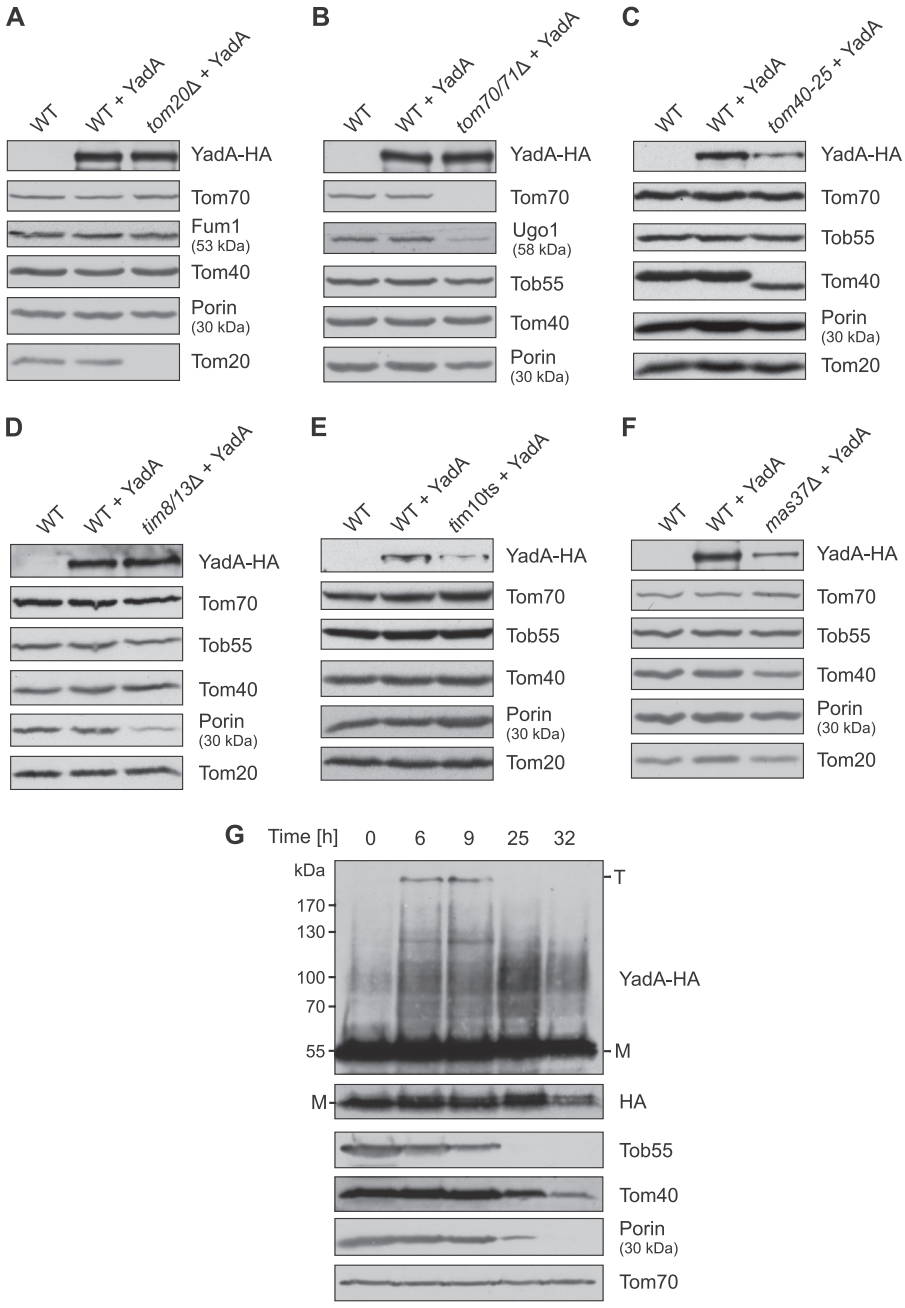


FIGURE 2. **The assembly of YadA into the mitochondrial OM depends on the TOM and TOB complexes.** Mitochondria were isolated from *A*, *tom20*Δ, *B*, *tom70*Δ*/tom71*Δ, *C*, *tom40–25*, *D*, *tim8*Δ*/tim13*Δ, *E*, *tim10ts,* and *F*, *mas37*Δ cells and their corresponding WT strain transformed with YadA-HA encoding plasmid. Mitochondrial proteins were analyzed by SDS-PAGE, and immunodetection with antibodies against the HA tag and the indicated mitochondrial proteins. Only the trimeric YadA species is shown. *G*, YadA-HA was transformed into a strain expressing Tob55 under the control of an inducible *GAL10* promoter. Cells were harvested at the indicated time points after a shift from galactose- to glucose-containing medium. Crude mitochondria were isolated and analyzed by SDS-PAGE and immunodetection with antibodies against the HA-tag and the indicated mitochondrial proteins. A shorter exposure depicting only the monomeric form is shown to demonstrate the reduced levels of this form after the longest incubation period. Tob55, Tom40, and porin are β-barrel proteins and substrates of the TOB complex; Tom70, an OM signal-anchored protein. The positions of monomeric and trimeric YadA-HA are indicated with *M* and *T*, respectively.

in the import of full-length YadA. We expressed YadA in cells deleted for either Tom20 or Tom70/Tom71 and monitored its level in these cells. Tom71 is a low-expressed paralog of Tom70 that can partially complement the absence of Tom70 (43, 44). Hence, to avoid any remaining Tom70/Tom71 activity we used the double deletion strain (30). As expected, the absence of Tom70 and Tom71 resulted in reduced levels of their substrate Ugo1 (45). However, similarly to the construct containing only the β-domain of YadA (27), mitochondria isolated from strains lacking either Tom20 or Tom70/71 import receptors had comparable levels of YadA to those in wild type organelles (Fig. 2, *A* and *B*). Thus, it appears that in contrast to their importance for the biogenesis of precursors of mitochondrial and other bacterial β-barrel proteins, the TOM import receptors are not crucial for the import of a trimeric autotransporter like YadA. Unfortunately, bacterial β-barrel proteins could not be

imported *in vitro* into yeast mitochondria (Refs. 18, 27).[4] Thus the dependence on import receptors could not be further studied with the well-established cell-free import assays.

To investigate whether Tom40 is involved in the assembly pathway of YadA we utilized a conditional Tom40 yeast mutant strain which was reported to be hampered in import of TOM-dependent precursor proteins (32). The detected levels of YadA were strongly reduced in this strain suggesting that proper function of the TOM pore is required for an optimal biogenesis of YadA (Fig. 2*C*).

In mitochondria there are four small TIM chaperones that assist the relay of β-barrel precursor proteins to the TOB complex: Tim9, Tim10, Tim8, and Tim13 that form two heterohexameric complexes of Tim9/Tim10 and Tim8/Tim13 (46, 47). Next, we investigated whether YadA requires the small chaperones in the IMS for its assembly in mitochondria. To that end, YadA was transformed into a double-deletion strain lacking both Tim8 and Tim13 or into a strain harboring a temperature-sensitive (ts) allele of *TIM10*. Crude mitochondria were isolated from these cells and subjected to SDS-PAGE and immunodetection. It can be observed that the steady-state levels of porin are indeed reduced in cells lacking the Tim8/Tim13 complex but those of YadA are unaltered in comparison to the wild type cells (Fig. 2*D*). In contrast, the levels of the mitochondrial β-barrel proteins are unaffected in the strain containing the conditional *tim10* allele but those of YadA were moderately reduced (Fig. 2*E*). Hence, it seems that the relevance of these small chaperones is substrate-specific and Tim10 is involved in the assembly of YadA in mitochondria.

The mitochondrial TOB complex is essential for the membrane integration of all β-barrel proteins analyzed so far. Mas37 is the only non-essential subunit of this complex and thus a strain deleted for this component allows investigation on the involvement of the TOB complex. To that end, we transformed *mas37*Δ cells with a plasmid encoding YadA. Mitochondria were isolated from these cells and subjected to SDS-PAGE and immunodetection. Obviously, this deletion caused a dramatic reduction in the detected levels of YadA (Fig. 2*F*). As reported previously, the absence of Mas37 caused also a reduction, although moderate, in the steady-state levels of mitochondrial β-barrel proteins like Tom40 and porin (Fig. 2*F*).

Next, we asked whether the down-regulation of the essential central subunit of the TOB complex, Tob55 will affect the mitochondrial levels of YadA. To that end, we employed a strain where the expression of Tob55 is under the control of the inducible promoter *GAL10* (11, 18, 48). Growing this strain on galactose-containing medium results in overexpression of Tob55, a β-barrel protein itself. Such high amounts of Tob55 compete out the assembly of YadA and other bacterial β-barrel proteins expressed in yeast cells (Fig. 2*G*, $t = 0$ and Refs. 18, 27). Shifting these cells to growth on glucose for extended time periods resulted in gradual depletion of Tob55 and subsequently of other β-barrel proteins like porin and Tom40 (Fig. 2*G*). Apparently, depletion of Tob55 moderately affected the levels of monomeric YadA only after 32 h but resulted in a complete disappearance of the trimeric species already after 25 h of growth on glucose (Fig. 2*G*). Thus, we conclude that the TOB complex is absolutely essential for the integration of YadA into the OM and its trimerization on the surface of the organelle but is less important for the initial mitochondrial association of the monomeric form and its subsequent import. As Tob55 is the mitochondrial homologue of the bacterial BamA, these findings are in agreement with a previous report on the important role of BamA in the biogenesis of YadA (33).

Bacterial Chaperones Have Differential Effects on the Biogenesis of YadA—Soluble chaperones are involved in both bacteria and mitochondria in the delivery of β-barrel precursor proteins to the corresponding insertion machineries, BAM and TOB complexes, respectively. The major periplasmic chaperones in the translocation pathway of bacterial β-barrel proteins are SurA and Skp while others like DegP might also play a role. In addition, the cytoplasmic chaperone SecB is thought to be involved in the stabilization of such proteins upon their synthesis in the cytoplasm (2, 24, 49–51).

Interestingly, both SurA and the TIM10 complex of mitochondria shared binding selectivity to peptides rich in aromatic residues and with net positive charge. However, SurA failed to completely replace TIM10 in yeast cells *in vivo* (52). The determination of the precise role of the different chaperones in bacteria is hampered since upon the deletion of any single chaperone the remaining ones might take over its task. Furthermore, mutation of periplasmic chaperones can have pleiotropic effects so one has to verify that the observed impact is a direct one (24). In contrast, the expression of any single bacterial chaperone in yeast cells can give a clear effect. Hence, the evolutionary link of mitochondria to bacteria can facilitate the usage of the former to shed light on this topic.

We tested whether the expression of bacterial SecB, SurA or Skp in cells expressing YadA can improve the assembly of full-length YadA molecules. SecB was expressed in its native form in the cytosol of yeast cells, a location that resembles its normal function in the bacterial cytoplasm. In contrast, we aimed to target SurA and Skp to the mitochondrial IMS that corresponds in many aspects to the bacterial periplasm. To that goal, both proteins were expressed in yeast cells as a fusion protein downstream of the N-terminal 228 amino acid residues of the mitochondrial protein Mgm1 lacking the first transmembrane segment (1–228ΔTM1). This latter protein contains in its N-terminal domain a bipartite signal sequence composed of a canonical matrix targeting signal upstream of a stop-transfer segment. Processing of the protein by the Pcp1 peptidase assures the release of the passenger domain into the mitochondrial IMS (53). As expected, upon subcellular fractionation of a strain expressing these proteins, Skp was detected solely in the mitochondrial fraction whereas SecB was found in the cytosol (Fig. 3*A*). The precursor form of SurA was detected exclusively in mitochondria but a significant portion of the processed form was found also in the cytosol (Fig. 3*A*). We suggest that this cytosolic population of the mature form may result from processing event before the import process was completed and then retrograde transport into the cytosol. Nevertheless, comparison of the ratio of detected levels of SurA and Skp in the mitochondrial fraction to those found in *E. coli* cells revealed

[4] T. Ulrich and D. Rapaport, unpublished results.

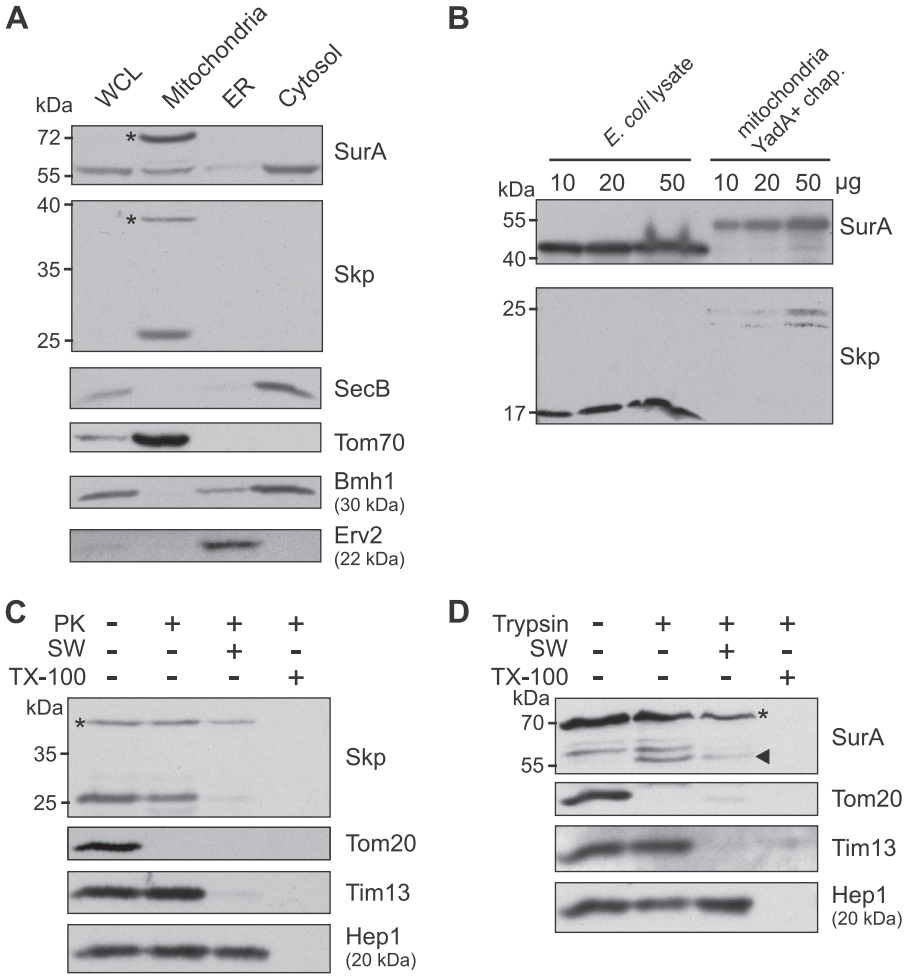


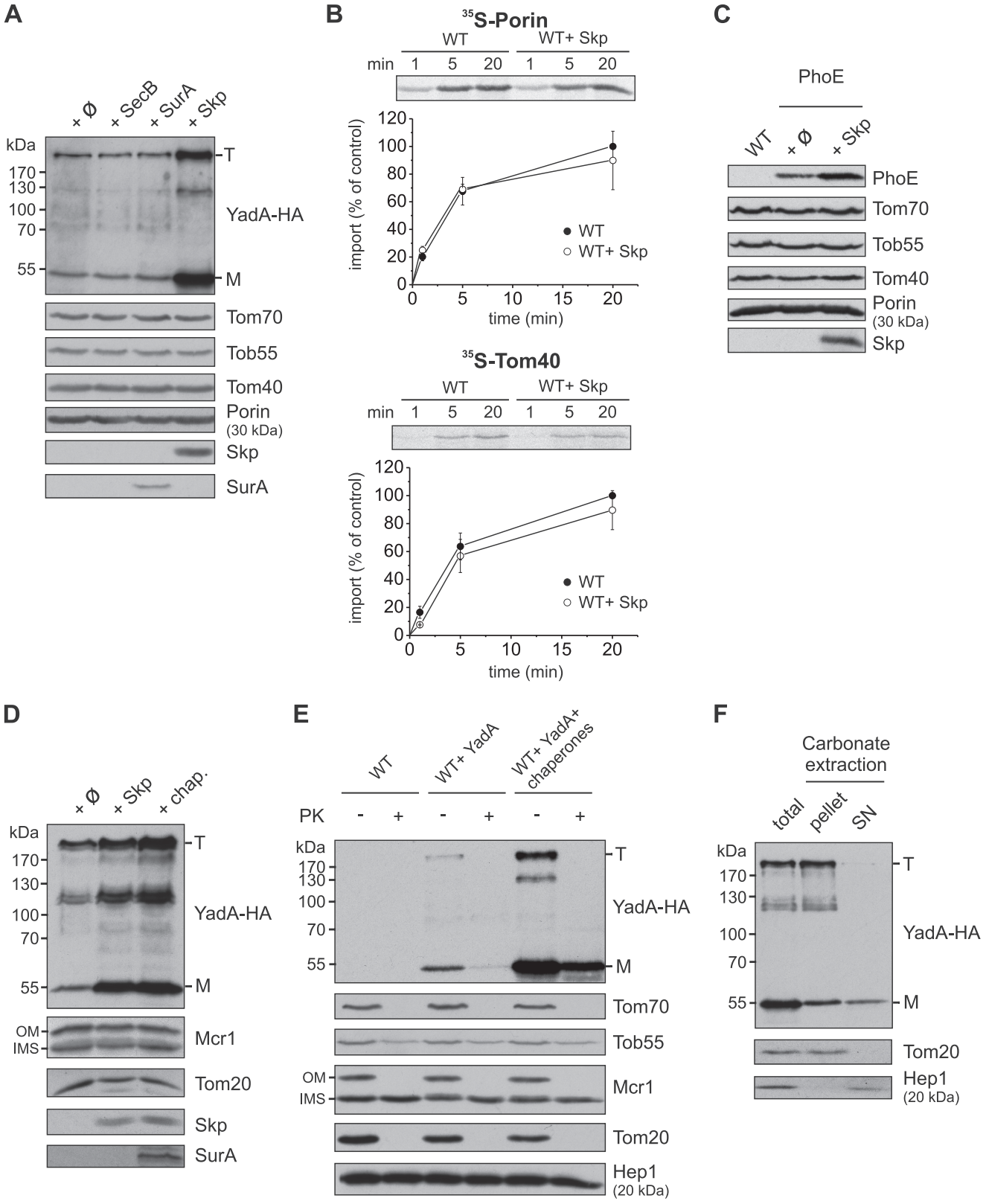
FIGURE 3. **Bacterial chaperones can be expressed in yeast cells.** *A*, Whole cell lysate (*WCL*) of yeast cells co-expressing YadA-HA together with the three bacterial chaperones and fractions corresponding to mitochondria, ER, and cytosol were analyzed by SDS-PAGE and immunodecoration with the indicated antibodies. *Asterisks* indicate unprocessed forms. *B*, comparison of the steady-state levels of SurA and Skp in *E. coli* cells to those in yeast mitochondria. The indicated amounts of *E. coli* lysate and mitochondria derived from yeast cells described in part A were analyzed by SDS-PAGE and immunodetection. *C*, isolated mitochondria from yeast cells expressing Skp were either left intact (*lanes 1–2*) or subjected to osmotic swelling to rupture the OM (*lane 3*). The indicated samples were incubated with PK. In one sample the protease was added in the presence of Triton X-100. Proteins were analyzed by SDS-PAGE and immunodetection with antibodies against the indicated proteins. *Asterisk* indicates the unprocessed forms. *D*, isolated mitochondria from yeast cells expressing SurA were treated as in part *C* with the only difference that trypsin was used instead of PK. *Asterisk* and *arrowhead* indicate the unprocessed form and a proteolytic fragment, respectively.

that the relative mitochondrial levels of SurA are still higher than those of Skp (Fig. 3*B*). Since both antibodies (against SurA and Skp) have different affinities toward their antigens, the intensities of the immunodetection bands do not allow determination of absolute amounts but rather only correlation of the mitochondrial amounts to those in bacteria.

We then asked whether the bacterial chaperones were indeed targeted to the mitochondrial IMS. To that goal we added proteases to either intact organelles or mitochondria where the OM was ruptured by osmotic swelling. As expected, the OM receptor Tom20 that is exposed toward the cytosol was degraded even in intact organelles whereas the IMS protein Tim13 became protease-sensitive only after rupturing the OM. Of note, the processed forms of both chaperones behaved like Tim13 suggesting that they are located in the IMS (Fig. 3, *C* and *D*). In contrast, a portion of the unprocessed forms of both Skp and SurA demonstrated protease sensitivity similar to the matrix protein Hep1 that was degraded only after lysis of the organelles by detergent (Fig. 3, *C* and *D*). Hence it seems that some molecules of the chaperone fusion-proteins were not halted in the inner membrane but rather were mis-targeted all the way to the matrix and thus could not be cleaved by the IMS peptidase Pcp1.

Next, we analyzed the amounts of YadA upon co-expression with each one of the chaperones. Whereas the presence of SecB or SurA did not affect the detected amounts of YadA, co-expression with Skp caused a dramatic increase in the mitochondrial levels of both monomeric and trimeric forms of YadA (Fig. 4*A*). The relative amounts of Skp and SurA in mitochondria in comparison to their levels in bacteria (Fig. 3*B*), argue against the possibility that the superior stabilization effect of Skp is due to its relative higher amounts. Interestingly, the presence of Skp or the other bacterial chaperones did not alter the levels of mitochondrial β-barrel proteins like porin, Tom40 or Tob55 (Fig. 4*A*). Similarly, the capacity of isolated organelles harboring bacterial chaperones to import *in vitro* mitochondrial β-barrel proteins like porin and Tom40 was equal to that of control organelles (Fig. 4*B*). These observations demonstrate that Skp

specifically contributes to the biogenesis of the bacterial YadA but not to that of mitochondrial β-barrel proteins. They are also in line with a previous study reporting that Skp can interact *in vitro* with recombinant bacterial β-barrel proteins but not with recombinant mammalian Porin, VDAC1 (54). We previously observed that other bacterial β-barrel proteins like PhoE can be expressed in yeast cells and targeted to mitochondria (18). Thus, we next wondered if the co-expression of Skp can stabilize also canonical β-barrel proteins. Indeed, co-expression of Skp together with PhoE enhanced the detected levels of the



latter by at least 2-fold (Fig. 4*C*), suggesting a more general effect of Skp.

To test whether the three chaperones might have synergistic effect, we created a strain where SecB, SurA, and Skp were simultaneously co-expressed. As expected, mitochondria isolated from this strain contained both SurA and Skp (Fig. 4*D*). Co-expression of all three chaperones only slightly improved the amounts of YadA beyond the contribution of Skp alone (Fig. 4*D*). However, the levels of Skp were also somewhat enhanced in this strain. Hence we cannot exclude the possibility that the slightly higher levels of YadA in this strain results from elevated amounts of Skp. Taken together, these findings indicate that in yeast cells Skp can enhance the biogenesis of YadA and PhoE whereas SurA and SecB have only minor effect, if at all.

Next, we tested whether the co-expression of the chaperones with YadA changed the oligomerization behavior of the protein. As was observed when YadA was expressed alone, the N-terminal HA tag in the trimeric form was exposed on the surface of the organelle and readily accessible to externally added protease while the monomeric form was partially protected under these conditions (Fig. 4*E*). Similarly, also in the presence of the chaperones the trimeric species behaved upon carbonate extraction as a membrane protein whereas the monomeric form was partially extracted by the alkaline solution (Fig. 4*F*). Thus, it seems that also under these conditions the monomeric form is composed of two populations: one that is associated with the membrane (maybe as an early import intermediate) and the other fraction of soluble molecules in the IMS.

We then asked how Skp can cause such an increase in the observed YadA levels. Two non-mutual exclusive alternatives are that either Skp improved the biogenesis and/or it reduced degradation of newly imported YadA molecules. To test the latter option, we added to the yeast culture cycloheximide that blocks protein synthesis and monitored the levels of YadA and control proteins after various incubation periods. Importantly, we observed that when YadA was expressed alone its monomeric form had a relatively short half-life and most of it was degraded already after 30 min (Fig. 5*A*). The co-expression of SurA did not change this behavior. In sharp contrast, the presence of Skp stabilizes the monomeric form and no difference in its levels was observed even after two hours of incubation. Of note, the assembled trimeric form remained stable under all the tested conditions (Fig. 5*A*).

Observing that the monomeric form of YadA undergoes degradation, we rationalized that a good candidate for this proteolytic activity is Yme1 which is known to degrade proteins in the mitochondrial IMS (55). Thus, we next investigated the life-span of the monomeric form of YadA in a strain deleted for *YME1*. The absence of Yme1 reduced the degradation rate of monomeric YadA by two to 3-fold (Fig. 5, compare *panel B to panel A*), suggesting that Skp indeed protects YadA from degradation. Of note, the steady-state levels and stability of both bacterial chaperones are not affected by the deletion of *YME1* (data not shown).

To better understand the effect of Skp we asked whether the chaperone interacts directly with YadA. To that goal, we solubilized mitochondria isolated from cells co-expressing YadA-HA and Skp and from control cells expressing only Skp with the mild detergent digitonin and performed pull-down assay with anti-HA beads. Although there is some residual binding of Skp to the beads, we observed about 3-fold stronger binding when YadA-HA was present (Fig. 5*C*). The weak direct binding of Skp to the anti-HA beads is probably due to some cross-reactivity of the anti-HA antibody with Skp (data not shown). To confirm this physical interaction we subjected organelles expressing both Skp and YadA-HA to immunoprecipitation with antibodies against Skp. Together with Skp itself also substantial amounts of YadA were pulled-down. The specificity of this interaction is reflected by the observation that only neglectable amounts of the mitochondrial OM proteins Tom20 and porin were found in the bound material (Fig. 5*D*). Collectively, it appears that Skp supports the biogenesis of YadA by a direct interaction that stabilizes the latter protein and reduces its turnover.

Skp Improves the Biogenesis of the Membrane-Anchor Domain of YadA—We then tested whether the stabilization effect of Skp depends on the interaction of the chaperone with the passenger domain. To that end, we co-expressed each of the three bacterial chaperones with the membrane-anchor (MA) domain of YadA (YadA-MA). Similarly to the results with the full-length protein, Skp caused a major increase in the detected levels of YadA-MA whereas the presence of the other two chaperones did not result in any observable enhancement (Fig. 6*A*). When we next analyzed the life-span of YadA-MA in the presence of the various chaperones, we observed that Skp can stabilize both the monomeric and the trimeric forms (Fig. 6*B*). Of note, the membrane-embedded trimeric form remained stable for the duration of the experiment. However, in contrast to the full-length protein, some monomeric form of YadA-MA was degraded even in the presence of Skp (Fig. 6*B*). The co-expression of Yad-MA with all three chaperones did not result in any

FIGURE 4. **Coexpression of the bacterial chaperone Skp increases the detected levels of YadA.** *A*, mitochondria were isolated from cells co-transformed with a plasmid expressing YadA-HA together with an empty plasmid, or together with a plasmid encoding SecB, mtSurA or mtSkp. Mitochondrial proteins were analyzed by SDS-PAGE and immunodetection with the indicated antibodies. *B*, mitochondria isolated from either a wild type or Skp-expressing strain were incubated with radiolabeled precursors of porin and Tom40 for the indicated time periods. At the end of the import reactions samples were treated with PK to remove non-imported molecules and analyzed by SDS-PAGE and autoradiography. Bands corresponding to imported material from three independent experiments were quantified and the intensity of the bands representing imported material into wild-type mitochondria for the longest time period was set as 100%. *C*, mitochondria were isolated from WT cells or from cells co-transformed with a plasmid expressing PhoE together with an empty plasmid, or together with a plasmid encoding mtSkp. Mitochondrial proteins were analyzed by SDS-PAGE and immunodetection with the indicated antibodies. *D*, mitochondria were isolated from cells transformed with a plasmid expressing YadA-HA or from cells co-expressing YadA with either mtSkp alone or with three plasmids encoding SecB, mtSurA and mtSkp (+*chap*.). Mitochondrial proteins were analyzed by SDS-PAGE and immunodetection with the indicated antibodies. *E*, mitochondria from control cells (*WT*), cells expressing YadA-HA, or cells expressing YadA-HA together with the bacterial chaperones were either left intact (−) or treated with PK (+). Mitochondrial proteins were analyzed by SDS-PAGE and immunodetection. *F*, mitochondria from cells expressing YadA-HA and the three bacterial chaperones were either left intact (total) or were subjected to carbonate extraction. Proteins were analyzed by SDS-PAGE and immunodetection.

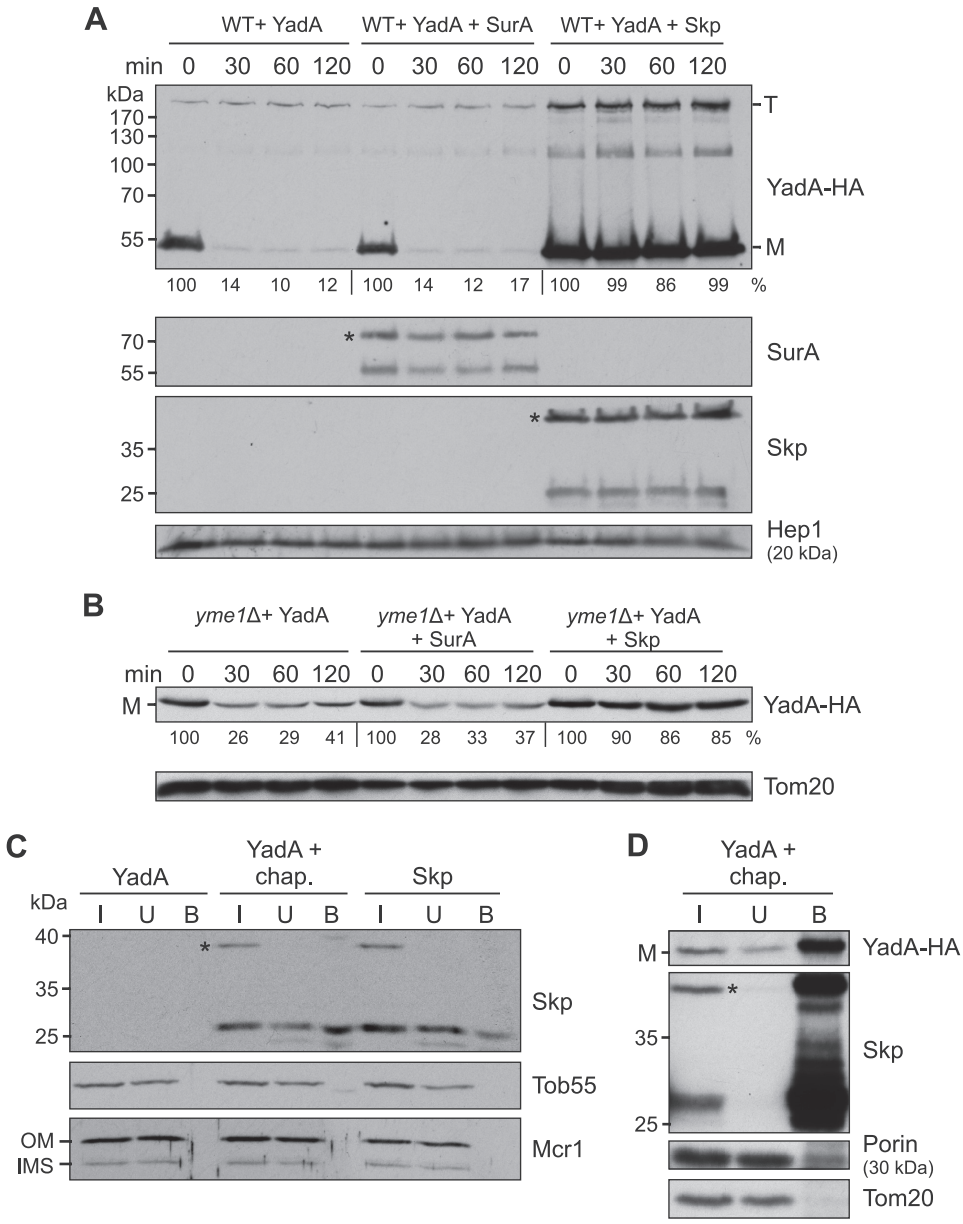


FIGURE 5. **Skp supports the biogenesis of YadA by protection from degradation.** *A*, yeast cells expressing YadA-HA, YadA-HA and mtSurA, or YadA-HA together with mtSkp were grown in liquid medium. Cycloheximide was then added to the cultures ($t = 0$), and cells were further incubated for the specified time periods. Cells were then harvested and whole cell lysates were analyzed by SDS-PAGE, and immunodetection with the indicated antibodies. *Asterisks* indicate unprocessed forms. The intensities of the bands corresponding to the monomeric form of YadA were quantified, and the band representing $t = 0$ was set as 100%. *B*, yeast cells lacking Yme1 and expressing YadA-HA alone or together with either mtSurA or mtSkp were grown in liquid medium. Cycloheximide was then added to the cultures and further treatment and analysis was as described for part *A*. *C*, mitochondria were isolated from cells expressing YadA-HA alone, YadA-HA together with the bacterial chaperones, or mtSkp alone. Organelles were solubilized with buffer containing 1% digitonin and cleared-supernatant were incubated with anti-HA beads. Supernatants before (input (*I*), 2% of total) and after (unbound (*U*), 2% of total) binding to the beads as well as bound material (*B*, 100% of total) were analyzed by SDS-PAGE and immunodetection with the indicated antibodies. *D*, mitochondria as in part *C* were solubilized with buffer containing 1% digitonin and cleared supernatant was incubated with protein G-Sepharose beads that were pre-incubated with antibodies against Skp. Further treatment and analysis is as in part *C*.

synergistic effect and the levels of YadA-MA did not increase beyond those observed upon co-expression with Skp alone (Fig. 6*C*). Finally, we checked whether the absence of the IMS protease Yme1 will result in increase in the detected levels of YadA-MA. As for the full-length YadA, such a deletion indeed slowed-down the turn-over rate of the monomeric form of YadA-MA (Fig. 6, compare *panel D to B*). These findings suggest that Yme1 is involved in the degradation of YadA-MA. Collectively, it seems that although the membrane-anchor domain of YadA can interact with and become stabilized by Skp, the presence of the passenger domain of the autotransporter enhances such interactions.

Trimeric YadA on the Surface of Mitochondria Is Functional—The aforementioned experiments strongly suggest that YadA is exposed on the surface of mitochondria in its native structure. To provide further support for this assumption we performed immunofluorescence microscopy with anti-HA antibody and mitochondria isolated from cells expressing N-terminally HA-tagged YadA. Of note, no fixation was used in this experiment to assure the intactness of the isolated organ-

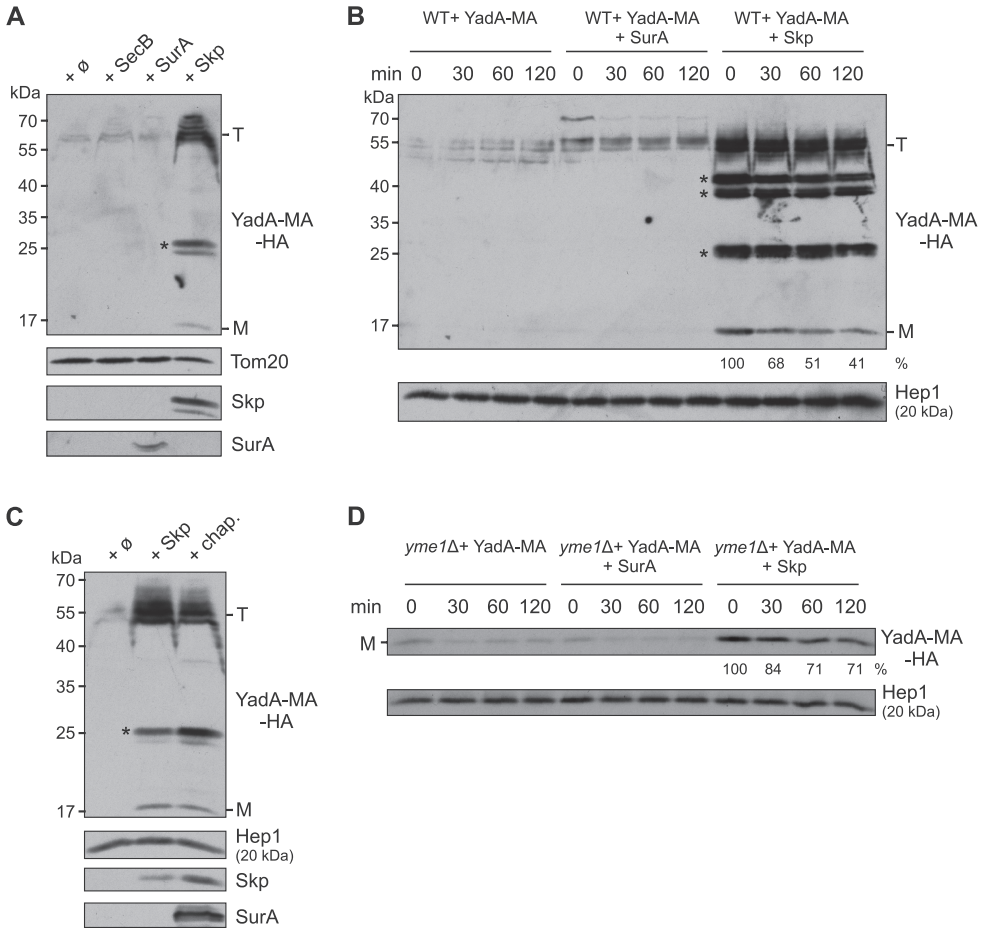


FIGURE 6. **Skp enhances the steady-state levels of YadA-MA.** *A*, mitochondria were isolated from cells co-transformed with a plasmid expressing YadA-MA together with an empty plasmid, or together with a plasmid encoding SecB, mtSurA, or mtSkp. Mitochondrial proteins were analyzed by SDS-PAGE and immunodetection with the indicated antibodies. The positions of monomeric and trimeric YadA-MA are indicated with M and T, respectively. *Asterisks* in *panels A-C* indicate bands corresponding to Skp that are unspecifically recognized by the HA antibody. *B*, yeast cells expressing YadA-MA alone or together with either mtSurA or mtSkp were grown in liquid medium. Cycloheximide was then added to the cultures, and further treatment was as described in the legend to Fig. 5*A*. *C*, mitochondria were isolated from cells expressing YadA-MA alone or together with either mtSkp or the three chaperones (+*chap*.). Mitochondrial proteins were analyzed by SDS-PAGE and immunodetection with the indicated antibodies. *D*, yeast cells lacking Yme1 and expressing YadA-HA alone or together with either mtSurA or mtSkp were grown in liquid medium. Cycloheximide was then added to the cultures, and further treatment was as described in the legend to Fig. 5*A*.

elles. In agreement with the immunodetection results in Fig. 4, we observed a rather weak staining when YadA was expressed alone and much stronger signal upon co-expression of YadA with the bacterial chaperones (Fig. 7*A*). As expected, we observed a strong signal with the control OM exposed protein Fis1-HA. The specificity of the signal and the intactness of the isolated organelles are demonstrated by the absence of signal in organelles from cells that either do not express YadA or contain HA-tagged protein in the inner membrane of mitochondria, Mdm38 (Fig. 7*A*). Thus, as anticipated for the native trimeric structure, these results indicate that the N-terminal HA tag is indeed exposed on the surface of the organelle.

Finally, we asked whether the mitochondrial-targeted YadA molecules preserve also their physiological function namely, adhering to host cells. To address this question we employed an assay that was originally used to monitor the activation of the proinflammatory host cell response upon exposure to bacteria expressing YadA as such adherence results in the secretion of IL-8 (35, 39). HeLa cells were exposed to isolated control mitochondria or to organelles harboring either YadA alone or YadA expressed in the presence of the bacterial chaperones. Then, IL-8 levels in the cell culture supernatant were determined after 6 h. Our results clearly indicate that the production of IL-8 is significantly increased if YadA is present on the surface of the organelles and this production is further stimulated by the co-expression of the bacterial chaperones (Fig. 7*B*). Of note, the co-expression of the chaperones resulted in a lower increase in the secretion of IL-8 as compared with the increase in the fluorescence signal (Fig. 7*A*) or the immunodetection signal (Fig. 4, *C* and *D*). We suggest that this difference resulted from the fact that only a small portion of the added isolated mitochondria and not the whole mitochondrial surface are actually in contact with the HeLa cells and induce secretion, whereas in the latter two assays all the YadA molecules are contributing to the signal. Collectively, our results demonstrate that mitochondria can recognize and assemble newly synthesized molecules of the TAA protein YadA and expose the protein in its native functional form.

DISCUSSION

In this study we demonstrate that the evolutionary link between mitochondria and Gram-negative bacteria allows the

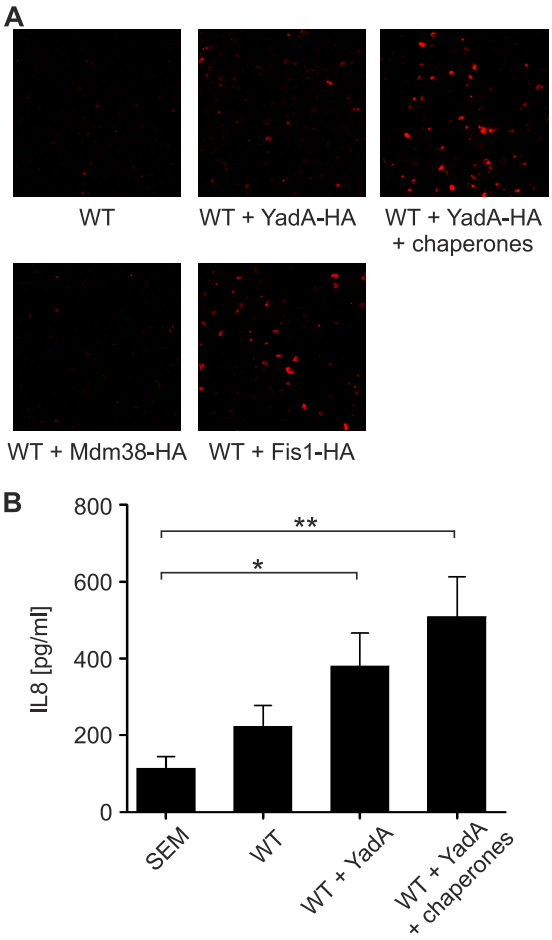


FIGURE 7. **YadA is assembled at the mitochondrial OM in its native functional conformation.** *A*, mitochondria were isolated from control cells (*WT*), cells expressing YadA-HA alone, or cells expressing YadA-HA together with the three bacterial chaperones. Mitochondria isolated from cells expressing HA-tagged version of either the OM protein Fis1 or the IM protein Mdm38 were used as control. Organelles were analyzed by immunofluorescence microscopy using the anti-HA antibody, and images are shown. *B*, mitochondria were isolated from the first three strains described in part *A*. HeLa cells were incubated with the isolated organelles, and IL-8 concentrations were measured after 6 h of incubation. Values are mean ± S.E. The statistical significance of the changes was evaluated using a two-sided *t* test. *, $p < 0.05$; **, $p < 0.01$.

former to assemble trimeric prokaryotic β-barrel proteins in a functional form although such proteins are completely absent from eukaryotic cells. This surprising capacity allows the usage of the yeast mitochondrial system to shed some light on various aspects of the biogenesis of TAAs.

These proteins cross the inner membrane via the SEC machinery, traverse the periplasm and then integrate into the outer membrane. In the present study we investigated the potential contribution of chaperones to their passage and stability within the periplasm and the mechanism by which trimeric autotransporters oligomerize to their trimeric form. To address these issues we utilized the yeast model system that provides two advantages: a detailed dissection of the biogenesis pathway and the ability to investigate the contribution of single components to the overall process. Our results indicate that upon its expression in yeast cells, the TAA protein YadA could assemble on the surface of mitochondria to its native and functional trimeric form. This trimeric form is embedded into the mitochondrial outer membrane and completely exposed on the surface of the organelle. Our observations further indicate that the TOB complex plays an important role in the assembly of YadA in yeast cells. We could further characterize a monomeric form that was partially soluble in the mitochondrial IMS. This monomeric form was rather unstable and eventually degraded by mitochondrial proteases. Co-expression of the periplasmic chaperone Skp together with YadA or with the membrane anchor domain of YadA resulted in overall increase in the detected levels of both monomeric and trimeric forms of the protein and dramatic stabilization of the monomeric species. These effects were not observed upon co-expression with the other chaperones SurA or SecB.

Our results might reflect the relative importance of chaperones for the biogenesis of TAAs. Whereas all three chaperones (SecB, SurA, and Skp) were suggested to contribute to various stages of the biogenesis of monomeric OMPs (24, 28), very little is known about the chaperone requirements of TAAs. In addition to Skp and SurA, also DegP was proposed to play a role in OMP biogenesis. However since the chaperone function of DegP in this process is less defined (28, 56), we did not include DegP in our assays. Our findings indicate a special importance of Skp for YadA biogenesis in the yeast model system and they might provide the first indication for the involvement of periplasmic chaperones in the biogenesis of TAAs. However, the applicability of these findings to the bacterial system has still to be confirmed. Skp was suggested to promote the overall biogenesis of OMPs by interacting with unfolded precursor forms and thus preventing their unproductive aggregation or degradation (57). The capacity of Skp to stabilize the monomeric form of YadA suggests a similar effect on YadA. Interestingly, our results suggest that Skp can interact with both the membrane anchor and the passenger domains of YadA. Such a special role of Skp is in agreement with a previous study where Skp was suggested to play an important role in the initial stages of the periplasm transit of the autotransporter EspP (58). It is also in line with other studies on the variable relative contribution of periplasmic chaperones to the biogenesis of different OMPs (28). For example, a study in *N. meningitidis* has revealed an important role for Skp but not for SurA or DegP in OMP biogenesis (59).

After crossing the periplasm the BAM machinery is required for the targeting of autotransporters to the OM and for the integration of the membrane-anchor domain into this membrane (24, 28). Recently, a new transport system named translocation and assembly module (TAM) was suggested to play a role in the membrane integration of some autotransporters (60). However, it is currently unclear whether the TAM system is also involved in the biogenesis of TAAs and the biogenesis of YadA was shown to require the BAM complex (32). The contribution of the BAM complex to the subsequent translocation of the passenger domain of autotransporters across the membrane and the role of the other Bam subunits in this process are not clear yet (24, 26, 61, 62). The mitochondrial Tob55 is homologous to the bacterial BamA but homolog yeast proteins to the other Bam subunits, BamB-E were not identified (1, 2, 11, 63). Moreover, the set of accessory lipoproteins (BamB-E) differs from species to species, suggesting that not all of them have

a crucial role (28). Considering the limited similarity of the TOB complex to the BAM machinery, our aforementioned findings suggest that a BamA-like structure is required for the targeting and translocation processes of YadA. In contrast, the other BAM components seem not to be absolutely necessary for these biogenesis stages. It is interesting to note that the evolutionary conservation between the systems that process β-barrel proteins in prokaryotes (BAM complex) and eukaryotes (TOB complex) is sufficient to provide the eukaryotic import system in yeast cells the capacity to fold into the correct native structure TAAs although such proteins are completely absent from eukaryotes.

Each of the subunits of TAAs is separately synthesized in the cytoplasm and most likely crosses the Sec translocon in an unfolded monomeric form. This situation raises the question at which stage the oligomerization of TAAs occurs. The possibilities range from a full trimerization already in the periplasm to oligomerization only after membrane insertion of each of the three subunits. Our inability to detect a YadA trimeric form that is both soluble and protected from externally-added protease strongly suggests that fully assembled trimeric structure forms only at the outer membrane, probably upon interaction with the TOB complex. These findings are in line with the recent report that precursors of mitochondrial β-barrel proteins start to acquire their β-barrel structure only upon their interaction with the TOB complex (64).

Taken together, our findings suggest that despite the evolutionary drift of mitochondria while becoming an organelle in eukaryotic cells and afterward, they still kept the capacity to process prokaryotic-specific proteins. Such a capacity can be utilized to investigate the potential importance of periplasmic chaperones to the biogenesis process of trimeric autotransporter proteins.

Acknowledgments—We thank E. Kracker and C. Schönfeld for excellent technical support, K.S. Dimmer for helpful discussions, Drs. K. Okamoto, T. Langer, N. Pfanner, T. Becker, and B. Guiard for yeast strains, and Drs. S. Behrens-Kneip, A. Driessen, P. Genevaux, M. Müller, L. Randall, A. Reichert, and J. Tommassen for constructs and antibodies.

REFERENCES

1. Dolezal, P., Likic, V., Tachezy, J., and Lithgow, T. (2006) Evolution of the molecular machines for protein import into mitochondria. *Science* **313,** 314–318
2. Walther, D. M., Rapaport, D., and Tommassen, J. (2009) Biogenesis of β-barrel membrane proteins in bacteria and eukaryotes: evolutionary conservation and divergence. *Cell. Mol. Life Sci.* **66,** 2789–2804
3. Bos, M. P., Robert, V., and Tommassen, J. (2007) Biogenesis of the gram-negative bacterial outer membrane. *Annu. Rev. Microbiol.* **61,** 191–214
4. Knowles, T. J., Scott-Tucker, A., Overduin, M., and Henderson, I. R. (2009) Membrane protein architects: the role of the BAM complex in outer membrane protein assembly. *Nature Rev. Microbiol.* **7,** 206–214
5. Voulhoux, R., Bos, M. P., Geurtsen, J., Mols, M., and Tommassen, J. (2003) Role of a highly conserved bacterial protein in outer membrane protein assembly. *Science* **299,** 262–265
6. Wu, T., Malinverni, J., Ruiz, N., Kim, S., Silhavy, T. J., and Kahne, D. (2005) Identification of a multicomponent complex required for outer membrane biogenesis in Escherichia coli. *Cell* **121,** 235–245
7. Pfanner, N., Wiedemann, N., Meisinger, C., and Lithgow, T. (2004) Assembling the mitochondrial outer membrane. *Nat. Struct. Mol. Biol.* **11,** 1044–1048
8. Paschen, S. A., Neupert, W., and Rapaport, D. (2005) Biogenesis of β-barrel membrane proteins of mitochondria. *Trends Biochem. Sci.* **30,** 575–582
9. Endo, T., and Yamano, K. (2009) Multiple pathways for mitochondrial protein traffic. *Biol. Chem.* **390,** 723–730
10. Kozjak, V., Wiedemann, N., Milenkovic, D., Lohaus, C., Meyer, H. E., Guiard, B., Meisinger, C., and Pfanner, N. (2003) An Essential Role of Sam50 in the Protein Sorting and Assembly Machinery of the Mitochondrial Outer Membrane. *J. Biol. Chem.* **278,** 48520–48523
11. Paschen, S. A., Waizenegger, T., Stan, T., Preuss, M., Cyrklaff, M., Hell, K., Rapaport, D., and Neupert, W. (2003) Evolutionary conservation of biogenesis of β-barrel membrane proteins. *Nature* **426,** 862–866
12. Gentle, I., Gabriel, K., Beech, P., Waller, R., and Lithgow, T. (2004) The Omp85 family of proteins is essential for outer membrane biogenesis in mitochondria and bacteria. *J. Cell Biol.* **164,** 19–24
13. Wiedemann, N., Kozjak, V., Chacinska, A., Schönfisch, B., Rospert, S., Ryan, M. T., Pfanner, N., and Meisinger, C. (2003) Machinery for protein sorting and assembly in the mitochondrial outer membrane. *Nature* **424,** 565–571
14. Ishikawa, D., Yamamoto, H., Tamura, Y., Moritoh, K., and Endo, T. (2004) Two novel proteins in the mitochondrial outer membrane mediate β-barrel protein assembly. *J. Cell Biol.* **166,** 621–627
15. Milenkovic, D., Kozjak, V., Wiedemann, N., Lohaus, C., Meyer, H. E., Guiard, B., Pfanner, N., and Meisinger, C. (2004) Sam35 of the mitochondrial protein sorting and assembly machinery is a peripheral outer membrane protein essential for cell viability. *J. Biol. Chem.* **279,** 22781–22785
16. Waizenegger, T., Habib, S. J., Lech, M., Mokranjac, D., Paschen, S. A., Hell, K., Neupert, W., and Rapaport, D. (2004) Tob38, a novel essential component in the biogenesis of β-barrel proteins of mitochondria. *EMBO Rep.* **5,** 704–709
17. Chan, N. C., and Lithgow, T. (2008) The Peripheral Membrane Subunits of the SAM Complex Function Codependently in Mitochondrial Outer Membrane Biogenesis. *Mol. Biol. Cell* **19,** 126–136
18. Walther, D. M., Papic, D., Bos, M. P., Tommassen, J., and Rapaport, D. (2009) Signals in bacterial β-barrel proteins are functional in eukaryotic cells for targeting to and assembly in mitochondria. *Proc. Natl. Acad. Sci. U.S.A.* **106,** 2531–2536
19. Müller, A., Rassow, J., Grimm, J., Machuy, N., Meyer, T. F., and Rudel, T. (2002) VDAC and the bacterial porin PorB of Neisseria gonorrhoeae share mitochondrial import pathways. *EMBO J.* **21,** 1916–1929
20. Kozjak-Pavlovic, V., Ott, C., Götz, M., and Rudel, T. (2011) Neisserial omp85 protein is selectively recognized and assembled into functional complexes in the outer membrane of human mitochondria. *J. Biol. Chem.* **286,** 27019–27026
21. Walther, D. M., Bos, M. P., Rapaport, D., and Tommassen, J. (2010) The mitochondrial porin, VDAC, has retained the ability to be assembled in the bacterial outer membrane. *Mol. Biol. Evol.* **27,** 887–895
22. Hoiczyk, E., Roggenkamp, A., Reichenbecher, M., Lupas, A., and Heesemann, J. (2000) Structure and sequence analysis of Yersinia YadA and Moraxella UspAs reveal a novel class of adhesins. *EMBO J.* **19,** 5989–5999
23. Linke, D., Riess, T., Autenrieth, I. B., Lupas, A., and Kempf, V. A. (2006) Trimeric autotransporter adhesins: variable structure, common function. *Trends Microbiol.* **14,** 264–270
24. Leyton, D. L., Rossiter, A. E., and Henderson, I. R. (2012) From self sufficiency to dependence: mechanisms and factors important for autotransporter biogenesis. *Nature Rev. Microbiol.* **10,** 213–225
25. Shahid, S. A., Bardiaux, B., Franks, W. T., Krabben, L., Habeck, M., van Rossum, B. J., and Linke, D. (2012) Membrane-protein structure determination by solid-state NMR spectroscopy of microcrystals. *Nature Methods* **9,** 1212–1217
26. Leo, J. C., Grin, I., and Linke, D. (2012) Type V secretion: mechanism(s) of autotransport through the bacterial outer membrane. *Phil. Trans. R. Soc. B* **367,** 1088–1101
27. Müller, J. E., Papic, D., Ulrich, T., Grin, I., Schütz, M., Oberhettinger, P., Tommassen, J., Linke, D., Dimmer, K. S., Autenrieth, I. B., and Rapaport, D. (2011) Mitochondria can recognize and assemble fragments of a β-bar-

rel structure. *Mol. Biol. Cell* **22,** 1638–1647
28. Grijpstra, J., Arenas, J., Rutten, L., and Tommassen, J. (2013) Autotransporter secretion: varying on a theme. *Res. Microbiol.* **164,** 562–582
29. Habib, S. J., Waizenegger, T., Lech, M., Neupert, W., and Rapaport, D. (2005) Assembly of the TOB complex of mitochondria. *J. Biol. Chem.* **280,** 6434–6440
30. Kondo-Okamoto, N., Shaw, J. M., and Okamoto, K. (2008) Tetratricopeptide repeat proteins Tom70 and Tom71 mediate yeast mitochondrial morphogenesis. *EMBO Rep.* **9,** 63–69
31. Leonhard, K., Herrmann, J. M., Stuart, R. A., Mannhaupt, G., Neupert, W., and Langer, T. (1996) AAA proteases with catalytic sites on opposite membrane surface comprise a proteolytic system for the ATP-dependent degradation of inner membrane proteins in mitochondria. *EMBO J.* **15,** 4218–4229
32. Wenz, L. S., Opaliński, L., Schuler, M. H., Ellenrieder, L., Ieva, R., Böttinger, L., Qiu, J., van der Laan, M., Wiedemann, N., Guiard, B., Pfanner, N., and Becker, T. (2014) The presequence pathway is involved in protein sorting to the mitochondrial outer membrane. *EMBO Rep.* **15,** 678–685
33. Lehr, U., Schütz, M., Oberhettinger, P., Ruiz-Perez, F., Donald, J. W., Palmer, T., Linke, D., Henderson, I. R., and Autenrieth, I. B. (2010) C-terminal amino acid residues of the trimeric autotransporter adhesin YadA of *Yersinia enterocolitica* are decisive for its recognition and assembly by BamA. *Mol. Microbiol.* **78,** 932–946
34. Daum, G., Böhni, P. C., and Schatz, G. (1982) Import of proteins into mitochondria: cytochrome b2 and cytochrome *c* peroxidase are located in the intermembrane space of yeast mitochondria. *J. Biol. Chem.* **257,** 13028–13033
35. Schmid, Y., Grassl, G. A., Bühler, O. T., Skurnik, M., Autenrieth, I. B., and Bohn, E. (2004) Yersinia enterocolitica adhesin A induces production of interleukin-8 in epithelial cells. *Infect. Immun.* **72,** 6780–6789
36. Wollmann, P., Zeth, K., Lupas, A. N., and Linke, D. (2006) Purification of the YadA membrane anchor for secondary structure analysis and crystallization. *Int. J. Biol. Macromol.* **39,** 3–9
37. Grosskinsky, U., Schütz, M., Fritz, M., Schmid, Y., Lamparter, M. C., Szczesny, P., Lupas, A. N., Autenrieth, I. B., and Linke, D. (2007) A conserved glycine residue of trimeric autotransporter domains plays a key role in Yersinia adhesin A autotransport. *J. Bacteriol.* **189,** 9011–9019
38. Ackermann, N., Tiller, M., Anding, G., Roggenkamp, A., and Heesemann, J. (2008) Contribution of trimeric autotransporter C-terminal domains of oligomeric coiled-coil adhesin (Oca) family members YadA, UspA1, EibA, and Hia to translocation of the YadA passenger domain and virulence of *Yersinia enterocolitica*. *J. Bacteriol.* **190,** 5031–5043
39. Schütz, M., Weiss, E. M., Schindler, M., Hallström, T., Zipfel, P. F., Linke, D., and Autenrieth, I. B. (2010) Trimer stability of YadA is critical for virulence of *Yersinia enterocolitica*. *Infect. Immun.* **78,** 2677–2690
40. Rapaport, D., and Neupert, W. (1999) Biogenesis of Tom40, core component of the TOM complex of mitochondria. *J. Cell Biol.* **146,** 321–331
41. Krimmer, T., Rapaport, D., Ryan, M. T., Meisinger, C., Kassenbrock, C. K., Blachly-Dyson, E., Forte, M., Douglas, M. G., Neupert, W., Nargang, F. E., and Pfanner, N. (2001) Biogenesis of the major mitochondrial outer membrane protein porin involves a complex import pathway via receptors and the general import pore. *J. Cell Biol.* **152,** 289–300
42. Model, K., Meisinger, C., Prinz, T., Wiedemann, N., Truscott, K. N., Pfanner, N., and Ryan, M. T. (2001) Multistep assembly of the protein import channel of the mitochondrial outer membrane. *Nat. Struct. Biol.* **8,** 361–370
43. Schlossmann, J., Lill, R., Neupert, W., and Court, D. A. (1996) Tom71, a novel homologue of the mitochondrial preprotein receptor Tom70. *J. Biol. Chem.* **271,** 17890–17895
44. Koh, J. Y., Hájek, P., and Bedwell, D. M. (2001) Overproduction of PDR3 suppresses mitochondrial import defects associated with a TOM70 null mutation by increasing the expression of TOM72 in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* **21,** 7576–7586
45. Papic, D., Krumpe, K., Dukanovic, J., Dimmer, K. S., and Rapaport, D. (2011) Multispan mitochondrial outer membrane protein Ugo1 follows a unique Mim1-dependent import pathway. *J. Cell Biol.* **194,** 397–405
46. Tokatlidis, K., and Schatz, G. (1999) Biogenesis of Mitochondrial Inner Membrane Proteins. *J. Biol. Chem.* **274,** 35285–35288
47. Koehler, C. M. (2004) The small Tim proteins and the twin Cx3C motif. *Trends Biochem. Sci* **29,** 1–4
48. Ulrich, T., Gross, L. E., Sommer, M. S., Schleiff, E., and Rapaport, D. (2012) Chloroplast β-barrel proteins are assembled into the mitochondrial outer membrane in a process that depends on the TOM and TOB complexes. *J. Biol. Chem.* **287,** 27467–27479
49. Randall, L. L., and Hardy, S. J. (2002) SecB, one small chaperone in the complex milieu of the cell. *Cell Mol. Life Sci.* **59,** 1617–1623
50. Ullers, R. S., Luirink, J., Harms, N., Schwager, F., Georgopoulos, C., and Genevaux, P. (2004) SecB is a bona fide generalized chaperone in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **101,** 7583–7588
51. Sklar, J. G., Wu, T., Kahne, D., and Silhavy, T. J. (2007) Defining the roles of the periplasmic chaperones SurA, Skp, and DegP in *Escherichia coli*. *Genes Dev.* **21,** 2473–2484
52. Alcock, F. H., Grossmann, J. G., Gentle, I. E., Likić, V. A., Lithgow, T., and Tokatlidis, K. (2008) Conserved substrate binding by chaperones in the bacterial periplasm and the mitochondrial intermembrane space. *Biochem. J.* **409,** 377–387
53. Herlan, M., Bornhövd, C., Hell, K., Neupert, W., and Reichert, A. S. (2004) Alternative topogenesis of Mgm1 and mitochondrial morphology depend on ATP and a functional import motor. *J. Cell Biol.* **165,** 167–173
54. Qu, J., Mayer, C., Behrens, S., Holst, O., and Kleinschmidt, J. H. (2007) The trimeric periplasmic chaperone Skp of *Escherichia coli* forms 1:1 complexes with outer membrane proteins via hydrophobic and electrostatic interactions. *J. Mol. Biol.* **374,** 91–105
55. Leonhard, K., Stiegler, A., Neupert, W., and Langer, T. (1999) Chaperone-like activity of the AAA domain of the yeast Yme1 AAA protease. *Nature* **398,** 348–351
56. Krojer, T., Sawa, J., Schäfer, E., Saibil, H. R., Ehrmann, M., and Clausen, T. (2008) Structural basis for the regulated protease and chaperone function of DegP. *Nature* **453,** 885–890
57. Walton, T. A., Sandoval, C. M., Fowler, C. A., Pardi, A., and Sousa, M. C. (2009) The cavity-chaperone Skp protects its substrate from aggregation but allows independent folding of substrate domains. *Proc. Natl. Acad. Sci. U.S.A.* **106,** 1772–1777
58. Ieva, R., Tian, P., Peterson, J. H., and Bernstein, H. D. (2011) Sequential and spatially restricted interactions of assembly factors with an autotransporter beta domain. *Proc. Natl. Acad. Sci. U.S.A.* **108,** 383–391
59. Volokhina, E. B., Grijpstra, J., Stork, M., Schilders, I., Tommassen, J., and Bos, M. P. (2011) Role of the periplasmic chaperones Skp, SurA, and DegQ in outer membrane protein biogenesis in Neisseria meningitidis. *J. Bacteriol.* **193,** 1612–1621
60. Selkrig, J., Mosbahi, K., Webb, C. T., Belousoff, M. J., Perry, A. J., Wells, T. J., Morris, F., Leyton, D. L., Totsika, M., Phan, M. D., Celik, N., Kelly, M., Oates, C., Hartland, E. L., Robins-Browne, R. M., Ramarathinam, S. H., Purcell, A. W., Schembri, M. A., Strugnell, R. A., Henderson, I. R., Walker, D., and Lithgow, T. (2012) Discovery of an archetypal protein transport system in bacterial outer membranes. *Nat. Struct. Mol. Biol.* **19,** 506–510
61. Saurí, A., Oreshkova, N., Soprova, Z., Jong, W. S., Sani, M., Peters, P. J., Luirink, J., and van Ulsen, P. (2011) Autotransporter beta-domains have a specific function in protein secretion beyond outer-membrane targeting. *J. Mol. Biol.* **412,** 553–567
62. Pavlova, O., Peterson, J. H., Ieva, R., and Bernstein, H. D. (2013) Mechanistic link between beta barrel assembly and the initiation of autotransporter secretion. *Proc. Natl. Acad. Sci. U.S.A.* **110,** 938–947
63. Noinaj, N., Kuszak, A. J., Gumbart, J. C., Lukacik, P., Chang, H., Easley, N. C., Lithgow, T., and Buchanan, S. K. (2013) Structural insight into the biogenesis of β-barrel membrane proteins. *Nature* **501,** 385–390
64. Qiu, J., Wenz, L. S., Zerbes, R. M., Oeljeklaus, S., Bohnert, M., Stroud, D. A., Wirth, C., Ellenrieder, L., Thornton, N., Kutik, S., Wiese, S., Schulze-Specking, A., Zufall, N., Chacinska, A., Guiard, B., Hunte, C., Warscheid, B., van der Laan, M., Pfanner, N., Wiedemann, N., and Becker, T. (2013) Coupling of mitochondrial import and export translocases by receptor-mediated supercomplex formation. *Cell* **154,** 596–608

Accepted for publication in "Methods in Molecular Biology – The BAM complex: Methods and protocols". Buchanan S. and Noinaj N., editors. Humana Press.

Yeast mitochondria as a model system to study the biogenesis of bacterial β-barrel proteins

Thomas Ulrich[1], Philipp Oberhettinger[2], Ingo B. Autenrieth[2] and Doron Rapaport[1,*]

[1] Interfaculty Institute of Biochemistry, University of Tübingen, 72076 Tübingen, Germany
[2] Interfaculty Institute of Microbiology and Infection Medicine, University of Tübingen, 72076 Tübingen, Germany

* To whom correspondence should be addressed: Doron Rapaport, Interfaculty Institute of Biochemistry, University of Tübingen, Hoppe-Seyler-Str. 4, 72076 Tübingen, Germany. Tel.: +49-7071-2974184; Fax: +49-7071-294016; E-mail: doron.rapaport@uni-tuebingen.de

Abstract

Beta-barrel proteins are found in the outer membrane of Gram-negative bacteria, mitochondria and chloroplasts. The evolutionary conservation in the biogenesis of these proteins allows mitochondria to assemble bacterial β-barrel proteins in their functional form. In this chapter we describe exemplarily how the capacity of yeast mitochondria to process the trimeric autotransporter YadA can be used to study the role of bacterial periplasmic chaperones in this process.

Key words

BAM complex, β-barrel proteins, chaperones, evolutionary conservation, mitochondria, Skp, TOB complex

1 Introduction

Integral β-barrel proteins are exclusively found in the outer membrane of Gram-negative bacteria and in the outer membranes of eukaryotic organelles derived from prokaryotic ancestors namely, mitochondria and chloroplasts. Although most of the proteins in the bacterial outer membrane are members of this protein class, only five mitochondrial β-barrel proteins were identified in yeast so far ***(1)***. Like the vast majority of mitochondrial genes they have undergone gene transfer to the host genome ***(2,3)***. Hence, precursors of β-barrel proteins are synthesized on cytosolic ribosomes and therefore have to contain all the information required to ensure an efficient and specific targeting to the mitochondrial outer membrane (MOM). Furthermore, translocases in the MOM had to evolve or adapt in order to facilitate the post-translational import and assembly of precursor β-barrel proteins.

1.1 Biogenesis of β-barrel proteins in bacteria

Bacterial β-barrel proteins are synthesized on cytoplasmic ribosomes with N-terminal signal sequences. Upon they appearance at the exit channel of the ribosome they can be stabilized by the highly conserved trigger factor ***(4,5)***. Subsequently, the chaperone SecB is proposed to bind the nascent polypeptide chain and directs it to the Sec translocase (see Fig. 1) ***(6)***. Proteins destined for the outer membrane are translocated across the inner membrane through the Sec translocase in a process dependent on the hydrolysis of ATP by SecA ***(7)***. Reaching the periplasmic side of the inner membrane, the signal peptide is cleaved off and the precursor proteins are escorted by periplasmic chaperones to the BAM complex ***(8,9)***. The precise roles of the chaperones SurA, Skp and DegP are still under debate and seem to differ depending on the substrate and the organism ***(10-13)***. The subsequent insertion into the outer bacterial membrane is facilitated by the BAM machinery. In *Escherichia coli*, this complex is composed of the central β-barrel protein BamA (Omp85/YaeT) associated with four lipoproteins (BamB, BamC, BamD and BamE) ***(14-16)***. Despite remarkable progress in characterizing the various components in the biogenesis pathway of β-barrel proteins in bacteria, the exact mechanism by which the proteins are assembled into the lipid bilayer still remains unresolved.

1.2 Biogenesis of β-barrel proteins in mitochondria

The requirement for N-terminal signal sequences in the sorting of β-barrel proteins got lost in the evolutionary transformation from bacteria to mitochondria. Upon their synthesis on cytosolic ribosomes mitochondrial β-barrel proteins are recognized at the organelle's surface by import receptors of the translocase of the outer membrane (TOM) complex and transferred

across the MOM through Tom40, the general entry gate of the TOM complex, a β-barrel protein itself (see Fig. 1) ***(1,17,18)***. Within the intermembrane space (IMS) the precursor proteins are then protected from misfolding and aggregation by the small heterohexameric Tim chaperone complexes Tim8/13 and Tim9/10. Finally, assembly of the precursor proteins into the MOM occurs with the help of a dedicated protein complex termed topogenesis of outer-membrane β-barrel proteins (TOB complex) or sorting and assembly machinery (SAM complex) ***(19-21)***. This complex is composed of the central highly conserved β-barrel protein Tob55/Sam50 and the peripheral subunits Tob38/Sam35/Tom38 and Mas37/Sam37. The latter two are located on the cytosolic side of the membrane and do not show any sequence similarity with their bacterial counterparts of the BAM complex ***(20,22-24)***. The essential subunit Tob38 was previously shown to be involved in intramitochondrial substrate recognition through the so called β-signal, whereas Mas37 acts at a late stage of β-barrel protein assembly with a putative role in precursor release from the TOB complex ***(25-27)***.

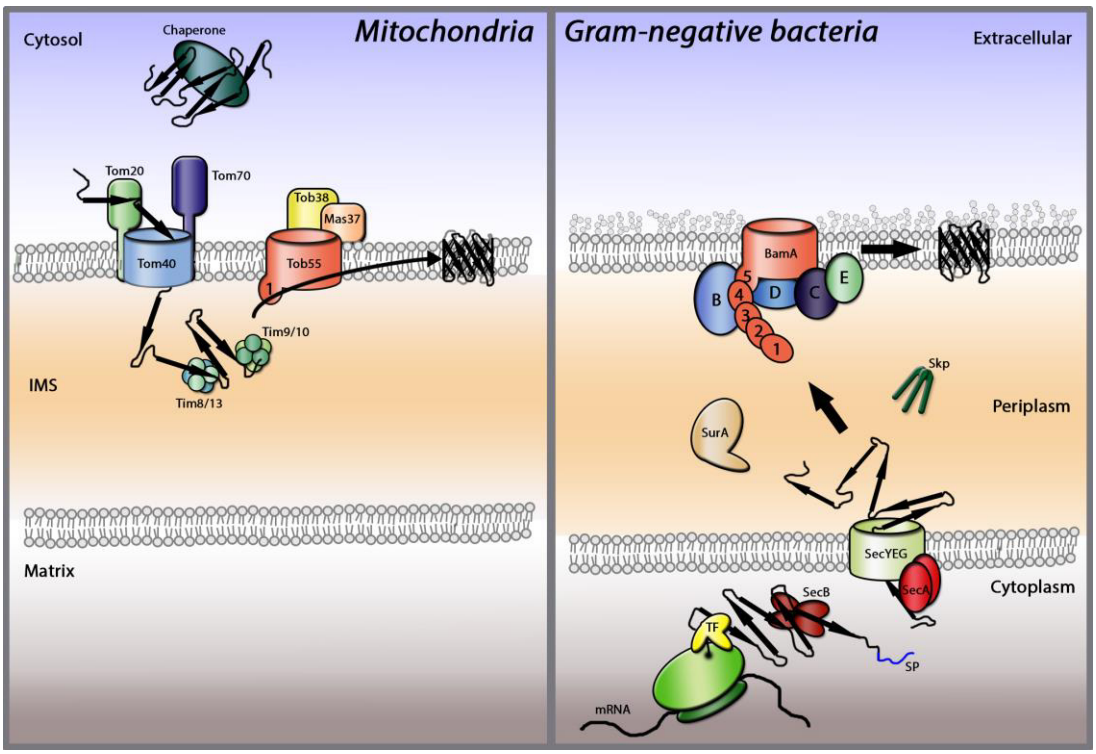


Fig. 1: Evolutionary conservation in the biogenesis pathways of β-barrel proteins between mitochondria and Gram-negative bacteria. For details see text.

1.3 Evolutionary conservation in the biogenesis of β-barrel proteins

The incorporation of an ancestral α-proteobacterium into the eukaryotic cell led to a major transfer of DNA to the host genome ***(2,28)***. Thereby the developing organelle had to adapt in order to ensure post-translational import of proteins. However, functional expression of bacterial β-barrel proteins in eukaryotic cells suggests that during this adaptation process the ability of mitochondria to facilitate the assembly of prokaryotic β-barrel proteins was conserved ***(29-31)***. In a reciprocal approach, the mitochondrial VDAC could also be assembled into the bacterial outer membrane upon its expression in *Escherichia coli* ***(32)***. A closer look at the biogenesis pathways of β-barrel proteins reveals that many characteristics are shared among Gram-negative bacteria and mitochondria. In both cases the precursor proteins are initially translocated across a membrane and prevented from misfolding and aggregation in the intermembranal space by specialized soluble chaperones. Insertion into the outer membrane occurs in each instance from the inner side of the membrane. The most striking similarity, however, is the sequential and functional homology in the central components of the assembly machineries, Tob55/Sam50 and BamA, both being members of the Omp85 superfamily. Homologs of this family are present in all Gram-negative bacteria and in the outer membranes of the eukaryotic organelles mitochondria and chloroplasts ***(14,21,33-35)***. A common feature of all proteins belonging to this family is the presence of N-terminal polypeptide-transport-associated (POTRA) domains followed by a 16-stranded C-terminal β-barrel domain. However, the number of POTRA domains can range from one in the mitochondrial Tob55/Sam50 and three in the chloroplast homolog Toc75-V up to seven in Omp85 from *Myxococcus xanthus* ***(36,37)***. It seems that the POTRA domains facilitate the transfer of the β-barrel precursors from the soluble chaperones to the membrane embedded part of the translocase although, at least for mitochondria, a role in the release of the precursor from the TOB/SAM complex was also suggested ***(38-41)***. Apart from the aforementioned similarities, the assembly processes differs in terms of accessory proteins and the requirement of N-terminal signal sequences. Whereas in mitochondria, the two accessory subunits are located at the cytosolic side of the MOM, all accessory lipoprotein subunits of the BAM complex reside on the internal side of the membrane similar to the N-terminal POTRA domains.

Due to this evolutionary conservation, yeast mitochondria provide a powerful system to study the biogenesis of prokaryotic β-barrel proteins. By using this system we investigated, for example, the involvement of periplasmic chaperones in the biogenesis pathway of the trimeric autotransporter protein Yersinia adhesin A (YadA) (see Fig. 2). Mature YadA is composed of an internal passenger domain (also called the effector domain), and a relatively short C-terminal

β-domain that anchors the protein to the OM. This anchor is made by a single 12-stranded β-barrel structure to which each monomer is contributing four β-strands. In this chapter we describe the expression of YadA in yeast cells, how to target periplasmic chaperones to the mitochondrial IMS and illustrate a method to examine the functionality of correctly assembled YadA by monitoring its ability to induce the secretion of IL8 from HeLa cells ***(42)***.

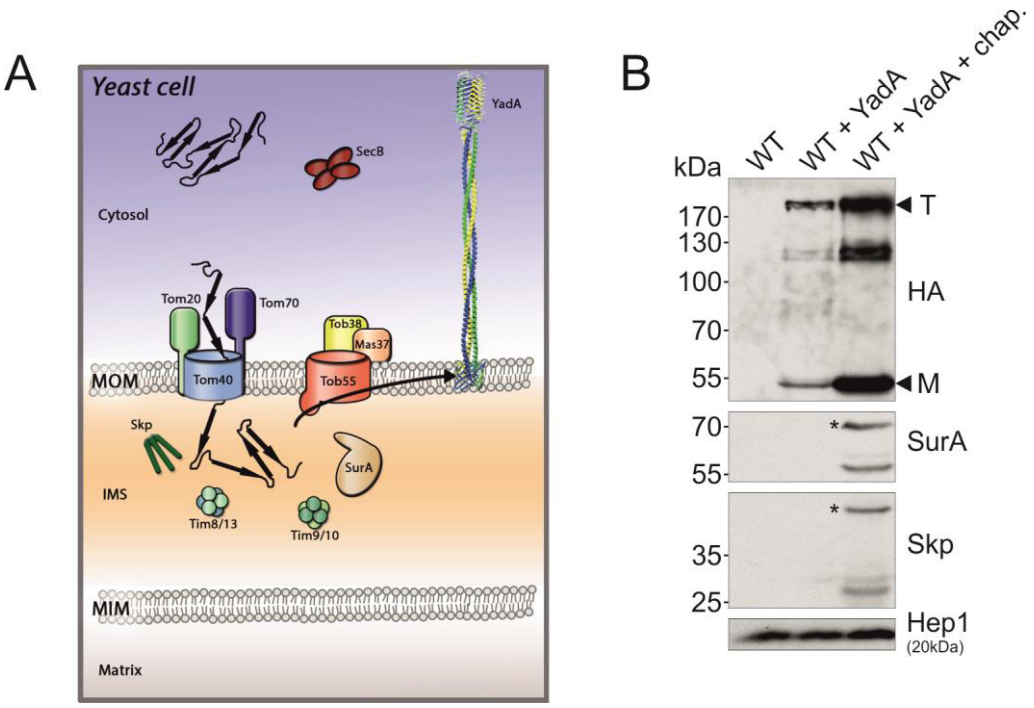


Fig. 2: (A) Schematic representation of the coexpression of YadA and the bacterial chaperones SecB, SurA and Skp in yeast cells. **(B)** Comparison of the mitochondrial steady-state levels of YadA-HA in yeast cells expressing either empty plasmid, YadA-HA alone or coexpressing YadA-HA and the bacterial chaperones SurA, Skp and SecB. Crude mitochondria were analysed by SDS-PAGE and immunodecoration with antibodies against the HA-tag, Skp, SurA and Hep1 (a mitochondrial matrix protein that serves as a loading control). Asterisks indicate unprocessed forms of SurA and Skp. The positions of monomeric and trimeric YadA-HA are indicated with M and T, respectively.

2 Materials

2.1 Yeast transformation

1. 100 mM LiOAc (sterile). Store at room temperature (RT).
2. 1 M LiOAc (sterile). Store at RT.
3. 50% polyethylene glycol (PEG), sterile: 50 g PEG-3350 in 100 ml water. Store at RT.
4. Salmon sperm DNA: 10 mg/ml, store in small aliquots at -20°C.
5. Vector to be transformed.

2.2 Targeting of proteins to the mitochondrial IMS

1. Yeast expression vector containing the protein of interest fused to the first 228 amino acids of *S. cerevisiae* Mgm1 lacking the first transmembrane domain (see Note 1).

2.3 Analysis of steady-state levels of bacterial proteins expressed in yeast cells

2.3.1 Isolation of crude mitochondria by lysis with glass beads

1. SEM buffer: 250 mM sucrose, 10 mM MOPS, 1 mM EDTA in water, pH 7.2 (adjusted with KOH) (see Note 2)
2. 200 mM Phenylmethylsulfonylfluoride (PMSF) in isopropanol
3. Reaction tubes (2 ml).
4. Glass beads, 0.25-0.5 mm
5. Bradford solution
6. 2x Laemmli buffer: 16 mL 1 M Tris-HCl, pH 6.8, 4 g SDS, 20 mL glycerol, 20 mg bromophenol blue, 5% 2-mercaptoethanol. Add water to 100 mL. Store at RT.
7. S-medium: 0.17% (w/v) yeast nitrogen base, 0.5% (w/v) ammonium sulfate, 0.3 µM adenine and 0.5 µM uracil in water, pH 5.5 (adjusted with KOH). Add amino acids separately as 100x stock solution (200 mg arginine, 400 mg tryptophane, 1 g leucine, 400 mg lysine, 200 mg histidine, 600 mg phenylalanine and 200 mg methionine in 100 ml water). Autoclave carbon source separately and add to 2% (w/v) final D-glucose (SD), 2% (w/v) final D-galactose (SGal), or 2% (w/v) glycerol (SG). For selection media leave out amino acids corresponding to the auxotrophic marker of the employed plasmid.

2.3.2 SDS-PAGE

1. 15% bottom gel: 2.39 mL water, 3.75 mL 1 M Tris/HCl (pH 8.8), 3.75 mL acrylamide/*bis*-acrylamide (40%/0.8%), 0.1 mL 10% ammonium persulfate (APS), 8 µL tetramethyl-ethylenediamine (TEMED). Mix thoroughly before use.
2. 10% separating gel: 4.55 mL distilled water, 4.69 mL 1 M Tris/HCl (pH 8.8), 3.13 mL acrylamide/*bis*-acrylamide (40%/0.8%), 0.125 ml 10% APS, 10 µL TEMED. Mix thoroughly before use.
3. 4% stacking gel: 3.76 mL distilled water, 0.625 mL 1 M Tris/HCl (pH 6.8), 0.563 mL acrylamide/*bis*-acrylamide (40%/0,8%), 0.05 mL 10% APS, 4 µL TEMED. Mix thoroughly before use.

4. 5x running buffer: 30 g Tris-HCl, 145 g glycine, 5 g sodium dodecyl sulfate (SDS), add distilled water to 1 L. Store at RT. Working concentration is 1x.

2.3.3 Western blotting

1. Filter papers (thickness 0.35 mm)
2. Nitrocellulose membrane (pore size 0.2 µm)
3. 10x blotting buffer: 60.57 g Tris-HCl, 281.51 g glycine, 5 g SDS, add distilled water to 2.5 L, store at 4°C. Working concentration is 1x: 100 mL 10x blotting buffer, 200 mL methanol (20% final), add distilled water to 1 L.
4. Ponceau S solution: 0.4 g Ponceau S, 8.5 mL 72% trichloroacetic acid (TCA), add distilled water to 200 mL. Store at RT.
5. 5% Blocking buffer: 5 g skim milk powder in 100 mL TBS-buffer.
6. 20x TBS: 121.16 g Tris-HCl, 900 g NaCl, add distilled water to 5 L, pH 7.5 (adjust with HCl). Working concentration is 1x. Store at room temperature.
7. 1x TBS-T: add 500 µl Tween-20 to 1 L 1x TBS solution. Store at RT.
8. Enhanced chemiluminescence (ECL) solution: 25 mL cold 1 M Tris-HCl (pH 8.5), 1.25 mL 4.4% luminol, 0.55 mL 1.5% p-coumaric acid, add cold distilled water to 250 mL. Store light-protected at 4°C.
9. Secondary antibody: horseradish peroxidase conjugated immunoglobulin G specific for the corresponding primary antibody.

2.3.4 Cell culture

1. HeLa cells, ATCC number CCL-2 (see Note 3).
2. RPMI-1640 medium (e.g. Biochrom).
3. Penicillin/Streptomycin (Pen/Strep, e.g. Gibco).
4. Fetal calf serum (FCS; e.g. Gibco) (see Note 4).
5. Phosphate buffered saline (PBS; e.g. Gibco) (see Note 5).
6. Trypsin EDTA (0.05%; e.g. Gibco) (see Note 6).
7. Cell culture flask (e.g. Nunc).
8. 24 well plate (e.g. Greiner).
9. Tabletop centrifuge (e.g. Heraeus Multifuge 3S-R).
10. Microcentrifuge tubes (1.5 mL).
11. Neubauer counting chamber.

12. Microscope (e.g. Zeiss Axiovert 25).
13. Cell culture incubator.

2.3.5 ELISA to determine amounts of IL8

1. RPMI-1640 medium (Biochrom), supplemented with 10% FCS and 1% Pen/Strep.
2. 96 well plate (e.g. Nunc) (see Note 7).
3. Binding solution: 0.1 M Na_2HPO_4 (pH 9.0): Weigh 14.17 g of Na_2HPO_4 and dissolve in 900 mL ultra-pure H_2O. Adjust pH to 9.0 with NaOH and add ultra-pure H_2O to 1000 mL. For long term storage, autoclave the solution and store at RT.
4. Blocking buffer: 1xPBS/10% FCS. For one 96 well plate, freshly prepare 18 mL PBS supplemented with 2 mL FCS.
5. Blocking/Tween buffer: 1x PBS/10% FCS/0.05% Tween20. For one 96 well plate, mix 9 mL 1xPBS with 1 mL 10% FCS and add 5 µL Tween20.
6. Substrate buffer: 0.05 M Na_2CO_3, 0.05 M $NaHCO_3$, 1 mM $MgCl_2$. Weight 5.3 g of Na_2CO_3, 4.2 g of $NaHCO_3$ and 0.094 g of $MgCl_2$ in 900 mL ultra-pure H_2O. Adjust pH to 9.8 with NaOH and fill up to 1000 mL with ultra-pure H_2O. Store the substrate solution at 4°C.
7. Washing buffer: 1x PBS/0.05% Tween20. Store the washing buffer at 4°C.
8. Capture antibody: purified anti-human IL-8 (Becton Dickinson).
9. Detection antibody: biotin mouse anti-human IL-8 (Becton Dickinson).
10. Conjugate: Streptavidin alkaline phosphatase (Roche).
11. Substrate: 5 mg p-Nitrophenyl-phosphate (PNPP) tablets.
12. Recombinant human IL8 (Becton Dickinson) as standard.
13. Multichannel pipet.
14. Microplate washer (e.g. TECAN HydroFlex™).
15. Microplate reader (e.g. TECAN sunrise™).

3 Methods

3.1 Yeast transformation

1. Scrape yeast cells from agar plate and wash in 1 mL sterile water (1.5 mL reaction tube).
2. Pellet the cells by centrifugation (5 sec, top speed, tabletop centrifuge).

3. Discard the water and resuspend the cells in 1 mL 100 mM LiOAc. Incubate the cells at 30°C for 5 min. In the meantime incubate salmon sperm carrier DNA for 5 min at 95°C and place immediately on ice.
4. Pellet the cells by centrifugation (5 sec, top speed, tabletop centrifuge)
5. Discard the supernatant and add in the following order: 240 µl PEG 3350 (50%), 36 µl 1 M LiOAc, 10 µl salmon sperm carrier DNA (denatured), 60 µl sterile water and 5 µl plasmid DNA (see Note 8).
6. Mix thoroughly and incubate for 20 min at 42°C.
7. Collect the cells by centrifugation (10 sec, top speed, tabletop centrifuge)
8. Discard supernatant and resuspend pellet in 100 µl sterile water.
9. Plate cells on selective SD-agar plates and incubate at 30°C. Depending on the yeast strain, colonies appear after approximately two days.

3.2 Analysis of steady-state levels of bacterial proteins expressed in yeast cells

3.2.1 Isolation of crude mitochondria by lysis with glass beads

1. Inoculate one yeast colony into 30 mL of liquid SGal-medium (see Note 9) and grow overnight at 30°C while shaking (120 rpm).
2. Dilute the overnight culture in fresh medium to 200 mL (OD_{600} = 0.2) and grow to OD_{600} = 0.8-1.5 (see Note 10).
3. Harvest cells by centrifugation (3000g, 5 min, RT).
4. Discard the supernatant and wash cells in 50 ml water.
5. Recollect cells by centrifugation (3000g, 5 min, RT) (see Note 11).
6. Resuspend the cells pellet in 2 mL SEM buffer + 2 mM PMSF (final conc.) and distribute into four 2 ml reaction tubes containing each 600 mg glass beads.
7. Vortex five times for 30 sec each at max speed and cool cells for 30 sec on ice in between.
8. To pellet down nuclei, unbroken cells and cell debris, spin the samples (1000g, 3 min, 4°C).
9. Pool the supernatants of the four reaction tubes for each strain and measure protein concentration by Bradford method. In case of yeast cells grown on glucose, a rough estimation is that 7% of total cellular proteins can be considered as mitochondria. If the cells are grown on galactose, ca. 15% can be estimated to be mitochondrial proteins.
10. Collect crude mitochondria by centrifugation (13200g, 10 min, 4°C).

11. The pellet is the crude mitochondrial fraction. Supernatant contains proteins from the cytosolic fraction. Resuspend the mitochondrial fraction in 2x Laemmli buffer to a concentration of 2 µg/µL and boil samples for 5 min at 95°C.

3.2.2 SDS-PAGE

1. Carefully remove the comb and wash the wells with 1x running buffer.
2. Prior to loading, centrifuge the samples shortly at 1000g in a tabletop centrifuge.
3. Load 15-30 µl (30-60 µg) of the samples per each well.
4. Run at 20 mA for each gel until the dye front reaches the bottom gel.
5. Remove the gel from the electrophoresis chamber and discard the stacking and bottom gel.

3.2.3 Western blotting

For electrophoretic protein transfer from the SDS-gel to the nitrocellulose membrane, we employ the semi-dry western blotting.

1. Prior to the assembly of the blotting sandwich, incubate six filter papers (depending on the thickness of the filter papers), the nitrocellulose membrane and the SDS-gel in 1x blotting buffer.
2. Assemble the blotting sandwich in the following order:
    a. Three wet filter papers
    b. Nitrocellulose membrane
    c. SDS-gel
    d. Three wet filter papers
3. After assembly, carefully role a glass pipett over the sandwich to get rid of residual air bubbles.
4. Connect the blotting apparatus to the power supply and run for 1 h at 1 mA/cm^2 at RT.
5. Disassemble the blotting sandwich and transfer the nitrocellulose membrane to an incubation chamber (avoid touching the membrane without gloves). To control for successful blotting, incubate the membrane with Ponceau S solution for 2 min.
6. Wash the membrane with distilled water until protein bands appear. If decoration with different antibodies is required, cut the membrane into respective slices with a scalpel.
7. Block the membrane in 5% blocking buffer for 1 h at RT under agitation.
8. Discard the blocking buffer and wash the membrane once with 1x TBS.

9. Incubate the membrane with the primary antibody for 1 h at RT while shaking (see Note 12).
10. Remove the primary antibody and wash three times 5 min with 1x TBS, once with TBS-T and again with 1x TBS.
11. Incubate the membrane for 1 h at RT with a secondary antibody that was raised against your first antibody and is conjugated to horseradish peroxidase.
12. Remove the secondary antibody and wash at least three times for 5 min with 1x TBS.
13. After brief incubation with ECL solution (add 1:1000 fresh H_2O_2 before use), chemioluminescence can be detected.

3.3 Cell culture

1. Quickly thaw at 37°C in a water bath human HeLa cervical epithelial cells out of liquid nitrogen. Seed cells subsequently in 25 mL RPMI-1640 medium containing 10% FCS and 1% Pen/Strep provided in a sterile cell culture flask with 175 cm^2 growth area.
2. Incubate cells in a cell culture incubator at 37°C with 5% CO_2 und 95% air humidity. Check growth behavior of the cells under the microscope every day. If cell growth on the bottom of the flask is nearly confluent, cells can be splitted, counted and seeded for further assays.
3. Extract old medium from the flask and add 8 mL of prewarmed trypsin to remove HeLa cells from the bottom of the flask. Incubate for 5 min at 37°C.
4. Afterwards add 20 mL of RPMI-1640 medium to dilute trypsin and transfer cells into a 50 mL Falcon tube.
5. Centrifuge cells for 5 min at 400g and discard supernatant. Resuspend cell pellet carefully in 5 mL prewarmed RPMI-1640 medium and avoid air bubbles.
6. Prepare a 1:10 dilution (10 µL of HeLa cells in 90 µL trypane blue) and count cells under the microscope with the help of a Neubauer counting chamber.
7. Provide 1 mL of prewarmed RPMI-1640 medium supplemented with 10% FCS and 1% Pen/Strep in each slot of a 24-well plate and add 1.5 x 10^5 HeLa cells per well (see Note 13).
8. Grow HeLa cells overnight at 37°C in a cell culture incubator.
9. Next day, one hour before adding either bacteria or isolated mitochondria (see 3.2.1), wash cells twice with 1 mL prewarmed PBS and append 1 mL RPMI-1640 medium with 10% FCS, but without antibiotics.

10. Afterwards add bacteria at a multiplicity of infection (MOI) of 100 (1.5 x 10^7) or mitochondria (100 µg) to desired wells and centrifuged at 400g for 5 min (see Note 14).
11. After one hour of incubation in the cell culture incubator, add 100 µg gentamicin (10 µL from a 10 mg/mL stock solution in cell culture PBS) to avoid further growth of bacteria in each well.
12. Incubate cells for further 5 hours at 37°C in the cell culture incubator and subsequently collect supernatant in 1.5 mL microcentrifuge tubes. Store tubes at -20°C until further analysis.

3.4 IL8-ELISA Assay

1. Coat a 96-well plate with capture antibody diluted in binding solution. Then, mix 30 µL of capture antibody (ab) with 5 mL binding solution and pipet 50 µL per well with a multichannel pipette (see Note 15).
2. Store the 96-well plate at 4°C overnight.
3. Wash the plate four times with a microplate washer. Afterwards add 200 µL blocking buffer to each well. Incubate at room temperature (RT) for 2 hours.
4. Thaw on ice cell culture supernatants to be analyzed (from 3.3.12). Additionally prepare IL8 standard by a serial two-fold dilution of known protein concentrations from 800 to 12.5 pg/mL. Prepare one microcentrifuge tube containing medium without protein.
5. Wash the ELISA plate again four times. Pipet 100 µL of prepared standard in duplicates in the first two columns starting with 0 pg/mL (blank), followed by increasing IL8 concentrations from 12.5 to 800 pg/mL.
6. Distribute 100 µL of each cell culture supernatant sample in the 96-well plate and incubate for 2 hours at RT or overnight at 4°C. Perform technical duplicates.
7. Dilute 20 µL detection antibody in 10 mL blocking/tween buffer.
8. Wash ELISA plate four times and add 100 µL of the prepared detection antibody solution in each well. Incubate for 1 hour at RT.
9. Prepare conjugate solution: add 10 µL conjugate in 10 mL blocking/tween buffer.
10. After washing the plate four times, add 100 µL conjugate solution per well and incubate for 1 hour at RT.
11. Add a PNPP pill to 5 mL chilled substrate buffer and be aware that the pill is dissolved completely. After another washing step of the 96 well plate, add 50 µL of the substrate

solution to each well and incubate again for 10-60 min at 37°C in the dark. Check reaction intensity of the alkaline phosphatase every ten minutes (see Note 15).

12. If a yellowish staining is visible, put the plate in a microplate reader and measure reaction intensity of the alkaline phosphatase at 405 nm.

4 Notes

1. Such construct contains a bipartite targeting and sorting signal that targets the protein first to the mitochondrial inner membrane where the protein is then being processed by a specific peptidase and a soluble moiety is released to the IMS (43). We used the yeast expression plasmid pYX113 but any other plasmid can be employed.
2. We always refer to purified water when "water" is mentioned.
3. HeLa cells can be stored as single use aliquots at -80°C. Thaw an aliquot quickly at 37°C and transfer the cells subsequently to a cell culture flask filled with prewarmed 1640 medium supplemented with 10% FCS and appropriate antibiotics.
4. If applicable, FCS can be heat-inactivated for 30 minutes at 56°C in a water bath.
5. PBS contains $CaCl_2$ and $MgCl_2$.
6. All cell culture media and chemicals have to be stored at 4°C after opening. Before usage and contact with cells, prewarm all reagents to 37°C. Sterile working is essential when dealing with cell culture.
7. Use a maxisorb ELISA plate with a flat top.
8. It is important to keep this exact order as direct contact of yeast cells with 1 M LiOAc can severely harm them.
9. The yield of mitochondria increases when yeast cells are grown in media with non-fermentable carbon sources such as glycerol, ethanol or lactate. Growth on galactose combines a decent yield with a moderate doubling time. The use of glucose as a carbon source should be avoided since in fungi glucose represses the expression of numerous mitochondrial genes. For selection, media should be prepared lacking the corresponding auxothropic marker(s).
10. Try to harvest the cells in the mid-logarithmic growth phase. Cells from the stationary phase are harder to lyse with glass beads and differ from cells in the logarithmic phase in the composition and amount of mitochondrial proteins.
11. Yeast cell pellets can be kept at -20°C for several days.
12. In some cases over-night incubation with the primary antibody at 4°C is beneficial.

13. Carefully agitate the 24-well plate hourglass-shaped to spread the cells over the whole well.
14. As a positive control for the assay, you can add 10 µg purified TNFα in one well which results in a strong IL8 secretion into the supernatant. Biological duplicates are recommended.
15. Check if every well is covered completely and remove all air bubbles.

Acknowledgments

Our work is supported by the Deutsche Forschungsgemeinschaft (SFB766/TP B11 and RA 1028/7-1 to D.R. and SFB766/TP B1 to I.A.) and by the UKT fortüne program (F1433253 to P.O.).

References

1. Paschen SA, Neupert W, Rapaport D (2005) Biogenesis of beta-barrel membrane proteins of mitochondria. *Trends Biochem Sci* 30:575-582
2. Gray MW, Burger G, Lang BF (1999) Mitochondrial evolution. *Science* 283:1476-1481
3. Esser C, Ahmadinejad N, Wiegand C, et al. (2004) A genome phylogeny for mitochondria among alpha-proteobacteria and a predominantly eubacterial ancestry of yeast nuclear genes. *Mol Biol Evol* 21:1643-1660
4. Ferbitz L, Maier T, Patzelt H, et al., (2004) Trigger factor in complex with the ribosome forms a molecular cradle for nascent proteins. *Nature* 431:590-596
5. Bos MP, Robert V, Tommassen J (2007) Biogenesis of the gram-negative bacterial outer membrane. *Annu Rev Microbiol* 61:191-214
6. Bechtluft P, Nouwen N, Tans SJ, et al. (2010) SecB-a chaperone dedicated to protein translocation. *Mol BioSys* 6:620-627
7. Zimmer J, Nam Y, Rapoport T A (2008) Structure of a complex of the ATPase SecA and the protein-translocation channel. *Nature* 455:936-943
8. De Keyzer J, Van Der Does C, Driessen AJ (2003) The bacterial translocase: a dynamic protein channel complex. Cellular and molecular life sciences *CMLS* 60:2034-2052
9. Paetzel M (2013) Structure and mechanism of Escherichia coli type I signal peptidase. Biochim Biophys Acta, in press. doi: 10.1016/j.bbamcr.2013.12.003.
10. Sklar JG, Wu T, Kahne D, et al. (2007) Defining the roles of the periplasmic chaperones SurA, Skp, and DegP in Escherichia coli. *Genes Dev* 21:2473-2484
11. Knowles TJ, Scott-Tucker A, Overduin M, et al. (2009) Membrane protein architects: the role of the BAM complex in outer membrane protein assembly. *Nature Rev Microbiol* 7:206-214

12. Patel GJ, Behrens-Kneip S, Holst O, et al. (2009) The periplasmic chaperone Skp facilitates targeting, insertion, and folding of OmpA into lipid membranes with a negative membrane surface potential. *Biochemistry* 48:10235-10245
13. Volokhina EB, Grijpstra J, Stork M, et al. (2011) Role of the periplasmic chaperones Skp, SurA, and DegQ in outer membrane protein biogenesis in Neisseria meningitidis. *J Bact* 193:1612-1621
14. Voulhoux R, Bos MP, Geurtsen J, et al. (2003) Role of a highly conserved bacterial protein in outer membrane protein assembly. *Science* 299:262-265
15. Wu T, Malinverni J, Ruiz N, et al. (2005) Identification of a multicomponent complex required for outer membrane biogenesis in Escherichia coli. *Cell* 121:235-245
16. Hagan CL, Silhavy TJ, Kahne D (2011) β-Barrel membrane protein assembly by the Bam complex. *Annu Rev Biochem* 80:189-210
17. Pfanner N, Wiedemann N, Meisinger C, et al. (2004) Assembling the mitochondrial outer membrane. *Nat. Struct. Mol. Biol*. 11:1044-1048
18. Endo T, Yamano K (2009) Multiple pathways for mitochondrial protein traffic. *Biol Chem* 390:723-730
19. Paschen SA, Waizenegger T, Stan T, et al. (2003) Evolutionary conservation of biogenesis of β-barrel membrane proteins. *Nature* 426:862-866
20. Wiedemann N, Kozjak V, Chacinska A, et al. (2003) Machinery for protein sorting and assembly in the mitochondrial outer membrane. *Nature* 424:565-571
21. Gentle I, Gabriel K, Beech P, et al. (2004) The Omp85 family of proteins is essential for outer membrane biogenesis in mitochondria and bacteria. *J Cell Biol* 164:19-24
22. Ishikawa D, Yamamoto H, Tamura Y, et al. (2004) Two novel proteins in the mitochondrial outer membrane mediate β-barrel protein assembly. *J Cell Biol* 166:621-627
23. Milenkovic D, Kozjak V, Wiedemann N, et al. (2004) Sam35 of the mitochondrial protein sorting and assembly machinery is a peripheral outer membrane protein essential for cell viability. *J Biol Chem* 279:22781-22785
24. Waizenegger T, Habib SJ, Lech M, et al. (2004) Tob38, a novel essential component in the biogenesis of β-barrel proteins of mitochondria. *EMBO Rep* 5:704-709
25. Chan NC, Lithgow T (2008) The Peripheral Membrane Subunits of the SAM Complex Function Codependently in Mitochondrial Outer Membrane Biogenesis. *Mol Biol Cell* 19:126-136
26. Kutik S, Stojanovski D, Becker L, et al. (2008) Dissecting membrane insertion of mitochondrial beta-barrel proteins. *Cell* 132:1011-1024
27. Dukanovic J, Dimmer KS, Bonnefoy N, et al. (2009) Genetic and functional interactions between the mitochondrial outer membrane proteins Tom6 and Sam37. *Mol Cell Biol* 29:5975-5988
28. Gray MW (2011) The incredible shrinking organelle. *EMBO Rep* 12:873
29. Walther DM, Papic D, Bos MP, et al. (2009) Signals in bacterial β-barrel proteins are functional in eukaryotic cells for targeting to and assembly in mitochondria. *Proc Natl Acad Sci USA* 106:2531-2536
30. Kozjak-Pavlovic V, Ott C, Gotz M, et al. (2011) Neisserial omp85 protein is selectively recognized and assembled into functional complexes in the outer membrane of human mitochondria. *J Biol Chem* 286:27019-27026

31. Müller JE, Papic D, Ulrich T, et al. (2011) Mitochondria can recognize and assemble fragments of a beta-barrel structure. *Mol Biol Cell* 22:1638-1647
32. Walther DM, Bos MP, Rapaport D, et al. (2010) The mitochondrial porin, VDAC, has retained the ability to be assembled in the bacterial outer membrane. Mol Biol Evol 27:887-895
33. Reumann S, Davila-Aponte J, Keegstra K (1999) The evolutionary origin of the protein-translocating channel of chloroplastic envelope membranes: identification of a cyanobacterial homolog. *Proc Natl Acad Sci USA* 96:784-789
34. Gentle IE, Burri L, Lithgow T (2005) Molecular architecture and function of the Omp85 family of proteins. *Mol Microbiol* 58:1216-1225
35. Moslavac S, Mirus O, Bredemeier R, et al. (2005) Conserved pore-forming regions in polypeptide-transporting proteins. *FEBS J* 272:1367-1378
36. Sanchez-Pulido L, Devos D, Genevrois S, et al. (2003) POTRA: a conserved domain in the FtsQ family and a class of beta-barrel outer membrane proteins. *Trends Biochem Sci* 28:523-526
37. Arnold T, Zeth K, Linke D (2010) Omp85 from the thermophilic cyanobacterium Thermosynechococcus elongatus differs from proteobacterial Omp85 in structure and domain composition. *J Biol Chem* 285:18003-18015
38. Habib SJ, Waizenegger T, Niewienda A, et al. (2007) The N-terminal domain of Tob55 has a receptor-like function in the biogenesis of mitochondrial beta-barrel proteins. *J Cell Biol* 176:77-88
39. Kim S, Malinverni JC, Sliz P, et al. (2007) Structure and function of an essential component of the outer membrane protein assembly machine. *Science* 317:961-964
40. Koenig P, Mirus O, Haarmann R, et al. (2010) Conserved properties of polypeptide transport-associated (POTRA) domains derived from cyanobacterial Omp85. *J Biol Chem* 285:18016-18024
41. Stroud DA, Becker T, Qiu J, et al. (2011) Biogenesis of mitochondrial beta-barrel proteins: the POTRA domain is involved in precursor release from the SAM complex. *Mol Biol Cell* 22:2823-2833
42. Schmid Y, Grassl GA, Buhler OT, et al. (2004) Yersinia enterocolitica adhesin A induces production of interleukin-8 in epithelial cells. *Infect Immun* 72:6780-6789
43. Herlan M, Bornhovd C, Hell K, et al. (2004) Alternative topogenesis of Mgm1 and mitochondrial morphology depend on ATP and a functional import motor. *J Cell Biol* 165:167-173

Accepted for publication in Intl. J. Med. Microbiol.

Biogenesis of beta-barrel proteins in evolutionary context

Thomas Ulrich and Doron Rapaport*

Interfaculty Institute of Biochemistry, University of Tübingen, 72076 Tübingen, Germany

* To whom correspondence should be addressed: Doron Rapaport, Interfaculty Institute of Biochemistry, University of Tübingen, Hoppe-Seyler-Str. 4, 72076 Tübingen, Germany.
Tel.: +49-7071-2974184; Fax: +49-7071-294016; E-mail: doron.rapaport@uni-tuebingen.de

Keywords: BamA, BAM complex, β-barrel proteins, periplasmic chaperones, TOB complex, Tob55

Abstract

The vast majority of outer membrane (OM) proteins in Gram-negative bacteria belongs to the class of membrane-embedded β-barrel proteins. Besides Gram-negative bacteria, the presence of β-barrel proteins is restricted to the OM of the eukaryotic organelles mitochondria and chloroplasts that were derived from prokaryotic ancestors. The assembly of these proteins into the corresponding OM is in each case facilitated by a dedicated protein complex that contains a highly conserved central β-barrel protein termed BamA/YaeT/Omp85 in Gram-negative bacteria and Tob55/Sam50 in mitochondria. However, little is known about the exact mechanism by which these complexes mediate the integration of β-barrel precursors into the lipid bilayer. Interestingly, previous studies showed that during evolution, these complexes retained the ability to functionally assemble β-barrel proteins from different origins. In this review we summarize the current knowledge on the biogenesis pathway of β-barrel proteins in Gram-negative bacteria, mitochondria and chloroplasts and focus on the commonalities and divergences that evolved between the different β-barrel assembly machineries.

Introduction

Integral β-barrel proteins play important roles in the functions of outer membranes (OM) of Gram-negative bacteria, mitochondria and chloroplasts. They span the membrane with 8-26 amphiphatic anti-parallel β-strands that are arranged in a cylindrical shape. Despite this similar basic structure, their functional diversity ranges from passive and active metabolite transporters, through enzymes and receptors to structural proteins and translocation machineries (Wimley, 2003). Supporting the endosymbiotic theory, β-barrel proteins can be found exclusively in the OM of Gram-negative bacteria, mitochondria and chloroplasts.

In all systems, the assembly of these proteins is facilitated by dedicated protein complexes that contain a highly conserved central β-barrel protein termed BamA/YaeT/Omp85 in Gram-negative bacteria and Tob55/Sam50 in mitochondria (Schleiff and Becker, 2011; Voulhoux et al., 2003; Walther et al., 2009b). Much less is known about the assembly of these proteins in chloroplasts. Understanding the basic principles underlying the biogenesis of β-barrel proteins is in the focus of many recent studies. It is remarkable, that in the process of evolution from bacterial ancestors to semi-autonomous cell organelles, mitochondria retained a member of the Omp85 superfamily to facilitate the assembly of a diverse subset of β-barrel proteins into their OM. Although in the long process of organellogenesis, mitochondria and chloroplast had to adapt to the requirements of a post-translational import of precursor proteins, they conserved the ability to assemble β-barrel proteins of different origin (Kozjak-Pavlovic et al., 2011; Ulrich et al., 2012; Walther et al., 2009a). Interestingly, even proteins of a specific class of β-barrel proteins that cannot be found in eukaryotes, namely trimeric autotransporters, could be recognized and assembled into the mitochondrial OM (Müller et al., 2011). This is even more astonishing, considering the obvious differences in the accessory proteins between the bacterial and the mitochondrial biogenesis systems.

In this review we summarize the current knowledge on the biogenesis pathways of β-barrel proteins in Gram-negative bacteria, mitochondria and chloroplast. A special focus is set on the similarities and divergences that evolved between the distinct β-barrel assembly machineries.

Biogenesis of β-barrel proteins in Gram-negative bacteria

Most integral proteins of the OM of Gram-negative bacteria belong to the class of β-barrel proteins and are synthesized on cytoplasmic ribosomes with an N-terminal signal peptide, which ensures translocation across the inner membrane (Driessen and Nouwen, 2008; Papanikou et al., 2007). In contrast to inner membrane α-helical proteins, precursors of β-barrel proteins are guided to the Sec-machinery in a post-translational manner. The cytoplasmic trigger factor recognizes the nascent polypeptide chain as it emerges at the exit channel of the ribosome (Ferbitz et al., 2004; Hagan et al., 2011; Valent et al., 1995). Subsequently, the cytoplasmic chaperone SecB binds the precursor protein and escorts it to the Sec translocon of the inner membrane (Bechtluft et al., 2010; Randall and Hardy, 2002; Ullers et al., 2004). Whereas hydrophobic transmembrane segments in α-helical proteins destined to the inner membrane trigger the lateral opening of SecYEG translocon, β-barrel precursors are translocated across the inner membrane in a process depending on the hydrolosis of ATP (Van den Berg et al., 2004; Xie et al., 2007; Zimmer et al., 2008).

On the periplasmic side of the inner membrane, the signal sequence is cleaved off by the signal peptidase (Paetzel, 2013). To keep the precursor protein in an unfolded-state and to avoid misfolding and aggregation, periplasmic chaperones bind to the precursor proteins. It is assumed that two parallel pathways of periplasmic chaperones can act in the biogenesis of β-barrel proteins. One of them includes SurA that contains two peptidyl-prolyl domains and was previously shown using *Escherichia coli* cells to directly interact with the dedicated insertion machinery of the OM (Bennion et al., 2010; Lazar and Kolter, 1996; Rouviere and Gross, 1996). The second branch is build up by the periplasmic chaperone Skp that can either act alone or together with the chaperone/protease DegP. In *E. coli*, SurA seems to chaperone the vast majority of OM proteins with a minor role of DegP and Skp. It appears that the significance of the latter chaperone is increased in the absence of SurA (Sklar et al., 2007b; Vertommen et al., 2009). In contrast, deletion studies in *Neisseria meningitidis* proposed a major role for Skp in the biogenesis of the OM proteins PorA and PorB, whereas deletion of SurA did not show a comparable phenotype (Volokhina et al., 2011). These apparent contradictory results suggest that the precise roles of the periplasmic chaperones seem to depend on the substrate and organism.

Once having reached the inner surface of the OM, the β-barrel proteins are assembled into the lipid bilayer with the help of a dedicated protein assembly machinery termed β-barrel

assembly machinery (BAM)-complex (see figure 1). In the last years, remarkable progress has been made in identifying and characterizing the roles of the components of the BAM-complex in the biogenesis pathway of β-barrel proteins. In *E. coli*, the complex is composed of the central β-barrel protein BamA, homologs of which can be found in all organisms from Gram-negative bacteria to humans, and the four lipoproteins BamB, BamC, BamD and BamE (named in *E. coli* as YfgL, NlpB, YfiO and SmpA, respectively) (Hagan et al., 2011; Voulhoux et al., 2003; Wu et al., 2005). Of those proteins solely BamA and BamD were found to be essential for viability (Malinverni et al., 2006; Wu et al., 2005). BamD was previously shown to bind to POTRA domain 5 of BamA and to scaffold the interaction of BamC, BamE and BamA. Furthermore its N-terminal domain was suggested to interact with incoming precursor proteins (Gatsos et al., 2008; Hagan et al., 2011; Sklar et al., 2007a; Vuong et al., 2008). Although the lipoproteins BamB, BamC and BamE are not essential for cell viability and are less conserved among different bacterial species, deletion of each one of them results in a decrease of the steady-state levels of β-barrel proteins in the OM (Hagan et al., 2011).

Despite this recent progress, the exact mechanism by which precursor proteins are finally assembled into the lipid bilayer is still ill defined. Recently, the structure of the central component of the BAM-complex, BamA from *Neisseria gonorrhoeae* and *Haemophilus ducreyi*, has been solved. (Noinaj et al., 2013). Analysis of the structural features revealed a reduction in the hydrophobicity on one side of the barrel, which leads to a local destabilization of the OM. Furthermore it was shown that the β-barrel might undergo lateral opening that can allow the release of the precursor proteins from the cavity of BamA into the lipid bilayer. Accordingly, prevention of this proposed lateral opening by formation of disulfide bridges between β-strands 1 and 16 resulted in a loss of BamA function (Noinaj et al., 2014).

Membrane assembly of β-barrel proteins in mitochondria

In comparison to Gram-negative bacteria, mitochondria harbor a rather low number of β-barrel proteins in their OM. So far, only five members of this class have been identified in yeast. These are the two essential proteins Tom40 and Tob55, two isoforms of Porin/VDAC and Mdm10 (Paschen et al., 2005; Walther and Rapaport, 2009). According to the endosymbiotic theory, mitochondria were derived by the engulfment of an α-proteobacterium into an ancestral

eukaryotic cell (Gray et al., 1999). In the process of organelle evolution many of the genes encoding mitochondrial proteins have undergone a DNA transfer to the host genome. More than 99% of the proteins of present-day mitochondria are encoded in the nucleus. As a consequence, the vast majority of mitochondrial proteins is synthesized on cytosolic ribosomes and thus precursor proteins have to contain all the information required to ensure a specific and efficient sorting to their final destination within mitochondria. Due to the fact that precursors of mitochondrial β-barrel proteins do no longer have to cross the bacterial inner membrane, the requirement for N-terminal signal sequences got lost in the transformation from bacteria to a semi-autonomous eukaryotic organelle. Newly synthesized mitochondrial β-barrel proteins do not contain any cleavable targeting signal.

Upon their synthesis on cytosolic ribosomes precursors of mitochondrial β-barrel proteins are initially recognized on the mitochondrial surface by import receptors of the translocase of the outer membrane (TOM) complex (see figure 1) (Chacinska et al., 2009; Endo and Yamano, 2009; Paschen et al., 2005; Pfanner et al., 2004). Next, they are relayed to Tom40, the central unit of the TOM complex, which is a β-barrel protein itself and builds the general entry gate for most of the mitochondrial precursor proteins. It is thought that the stepwise increasing affinity of precursor proteins to components of the TOM complex is the driving force for the protein translocation across the mitochondrial OM (Komiya et al., 1998; Rapaport et al., 1998; Schatz, 1997). Upon the appearance of the β-barrel precursor proteins at the intermembrane space (IMS), they are protected from misfolding and aggregation by the hexameric small chaperone complexes Tim8/13 and Tim9/10 (Habib et al., 2005; Hoppins and Nargang, 2004; Wiedemann et al., 2004). The final step of protein assembly into the lipid bilayer of the OM is facilitated by a protein complex termed topogenesis of outer-membrane β-barrel proteins (TOB-complex) also called sorting and assembly machinery (SAM-complex) (Gentle et al., 2004; Paschen et al., 2003; Wiedemann et al., 2003). This protein complex consists of the β-barrel protein Tob55/Sam50, which is homologous to BamA, and the two subunits Tob38/Sam35/Tom38 and Mas37/Sam37 which are peripherally associated to the cytosolic side of the OM. Of these three components that make up the core structure of the TOB complex, only Tob55 and Tob38 were shown to be essential for cell viability.

Whereas the essential subunit Tob38 was previously shown to be involved in the intra-mitochondrial recognition of β-barrel precursor proteins through a C-terminal recognition

sequence termed β-signal, the second subunit Mas37 was suggested to act at a later stage of β-barrel assembly with a putative role in the release of the precursor into the lipid bilayer (Ishikawa et al., 2004; Kutik et al., 2008; Milenkovic et al., 2004; Waizenegger et al., 2004). Since in mitochondria the assembly of precursor proteins into the lipid bilayer occurs from the IMS side, it remains elusive how Tob38 can act in the recognition of precursor proteins. One possible explanation is that a cavity in the proteinaceous components of the complex allows Tob38 a direct contact with substrate proteins (Kutik et al., 2008).

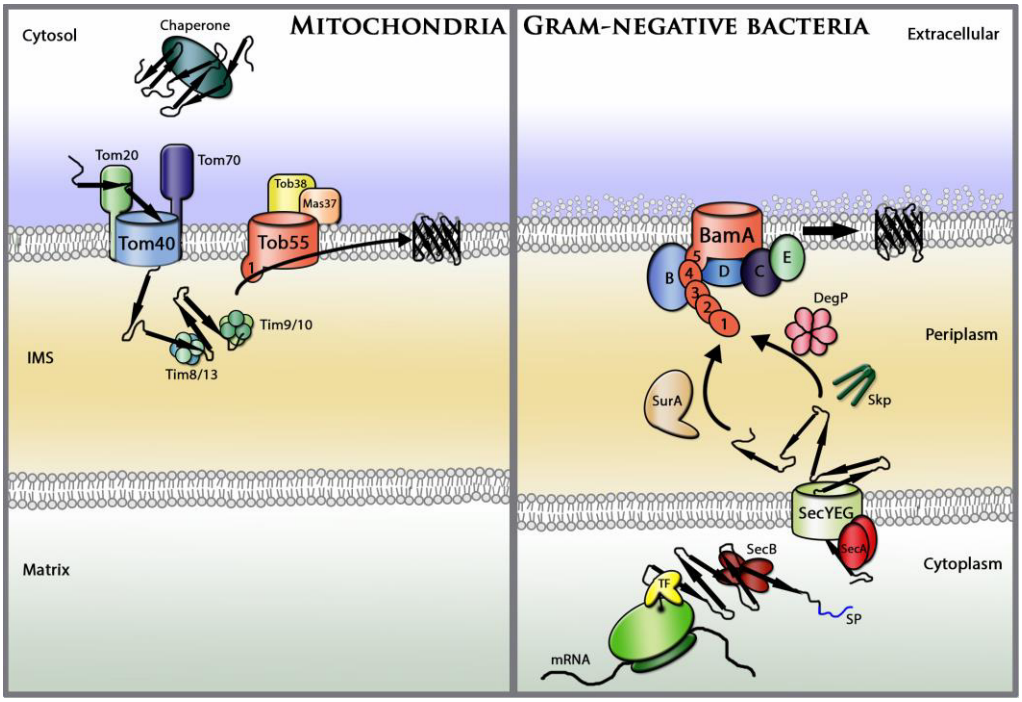


Figure 1 Comparison of the β-barrel biogenesis pathways in Gram-negative bacteria and mitochondria. Precursors of bacterial β-barrel proteins (right) are synthesized in the cytoplasm with an N-terminal signal sequences (SP) which guides them to the Sec translocon where they are translocated across the inner membrane. In the periplasm the precursor proteins are protected from misfolding and aggregation by the chaperones SurA, Skp and the chaperone/protease DegP. Finally the precursor proteins are inserted into the lipid bilayer by the BAM complex, which is build up by the central component BamA and the four lipoproteins BamB-E. Mitochondrial β-barrel proteins (left) are initially recognized at the mitochondrial surface by receptors of the TOM complex. Upon translocation across the mitochondrial OM through the Tom40 pore, they are escorted by the hexameric chaperone complexes Tim8/13 and Tim9/10. The assembly of precursor proteins into the mitochondrial OM is facilitated by the TOB complex, which is composed of the β-barrel Tob55 and the peripherally associated proteins Mas37 and Tob38.

Membrane integration of β-barrel proteins in chloroplasts

Chloroplasts or plastids in general, are the second organelles harboring membrane-embedded β-barrel proteins in the OM. According to the endosymbiotic theory, the plastids originated by the incorporation of an endosymbiotic prokaryote into an eukaryotic ancestor cell. Whereas mitochondria are thought to descend from α-proteobacteria, it is believed that the engulfment of a cyanobacterium was the initial step for the organellogenesis of plastids (McFadden, 2001). Of the approximately 2500-3500 proteins in chloroplasts only about 80-100 genes are still encoded on the plastome (chloroplast own genome), mainly encoding ribosomal RNAs and proteins, tRNAs, the large subunit of the Rubisco, and proteins required for photosynthesis (Joyard et al., 2009). The residual 95% of the chloroplasts proteins are encoded by the nuclear genome and thus have to be imported into the chloroplasts in a post-translational manner.

Although progress has been made in understanding the biogenesis of chloroplast proteins containing a cleavable targeting sequence, currently very little is known about how chloroplast β-barrel proteins are assembled into their corresponding membrane. Like in mitochondria, most chloroplast precursors of β-barrel proteins are devoid of N-terminal targeting signals. The only known exception is the central component of the translocase of the chloroplast outer membrane (TOC-complex), Toc75-III. It is a highly abundant protein in the OM that is synthesized in the cytoplasm with an N-terminal bipartite transit peptide (Cline et al., 1981; Tranel and Keegstra, 1996). Interestingly, the first half guides the precursor protein to the chloroplast stroma, whereas the second half of the signal contains a stop-transfer segment which is thought to prevent the full translocation across the inner membrane. After cleavage of the N-terminal part of the signal by the stromal processing peptidase (SPP), the precursor protein was shown to undergo further processing by a type I signal peptidase before it is finally assembled into the chloroplast OM (Inoue et al., 2005; Patel et al., 2008). The precursor translocation across the OM and the insertion into the lipid bilayer from the inner face of the membrane resemble the biogenesis pathway of β-barrel proteins in mitochondria. Another isoform of Toc75, Toc75-V or AtOep80, is closely related to the mitochondrial Tob55 and is also a member of the Omp85 superfamily. Toc75-V is essential for viability in *Arabidopsis thaliana* and was previously suggested to facilitate the insertion of β-barrel proteins into the outer envelope of chloroplasts (Patel et al., 2008; Soll and Schleiff, 2004). However, so far there is no experimental support for this claim. According to phylogenic analysis of Toc75 from numerous organisms, Toc75-III and Toc75-V

belong to two distinct branches of the Omp85 superfamily (Bredemeier et al., 2007; Moslavac et al., 2005). Whilst Toc75-V might have retained its ancestral function in the assembly of β-barrel proteins, Toc75-III evolved to form the general entry gate for chloroplasts-destined proteins that are synthesized in the cytosol and have to be imported into chloroplasts.

Common grounds and divergences in the biogenesis of β-barrel proteins

Three common characteristics are shared in the β-barrel biogenesis pathways of bacteria and mitochondria: (i) insertion via the internal side of the membrane, (ii) participation of soluble chaperones in the periplasm or IMS, and (iii) homology among the central subunit of the assembly complex. Homologs of BamA, the central component of the BAM-complex can be found in all Gram-negative bacteria and in the OM of the eukaryotic organelles mitochondria and chloroplasts. The most striking similarity among all members of the Omp85 superfamily is their unique structural organization. It was predicted that the structure contains N-terminal polypeptide-transport-associated (POTRA) domains that are followed by a 16-stranded C-terminal β-barrel pore. Indeed, a recent structure determination of BamA from *Neisseria gonorrhoeae* and *Haemophilus ducreyi* confirmed this structure (Noinaj et al., 2013).

Despite such apparent similarity, comparison of several members of the Omp85 superfamily reveals that the number of POTRA domains varies from one in the mitochondrial Tob55 to three in the chloroplast homolog Toc75-V, whereas up to seven POTRA domains were predicted for the Omp85 of *Myxococcus xanthus* (Arnold et al., 2010; Sanchez-Pulido et al., 2003). Regardless of the number of POTRA domains, a comparative study of 567 POTRA domains identified the most C-terminal POTRA domain as the best conserved one, followed by the most N-terminal one (Arnold et al., 2010). Accordingly, it can be speculated that in the process of organelle evolution the mitochondrial Omp85 homolog, Tob55 evolved in a way that it retained the most C-terminal POTRA domain as a minimal motif for β-barrel assembly in mitochondria. This assumption goes in line with the discovery that POTRA domains 1-4 are dispensable for viability in *Neisseria meningitidis* and their deletion results in only mild assembly defects of OM proteins (Bos et al., 2007). However, in sharp contrast to the situation in *Neisseria meningitidis*, POTRA domains 3-5 were shown to be essential in *E. coli* (Kim et al., 2007).

Interestingly, the accessory lipoproteins BamC, BamD and BamE were reported to bind to POTRA domain 5, whereas the binding of BamB, which is not present in *Neisseria meningitides*, to the BAM complex was affected in a strain deleted for POTRA domains 2-5 (Kim et al., 2007). According to these differences in the necessity of POTRA domains in the different organisms, it is also conceivable that Omp85 homologs with multiple POTRA domains evolved from a simple ancestral Omp85 harboring only one POTRA domain. It is still controversially discussed how the different numbers of POTRA might be explained. It is conspicuous that the number of POTRA domains seems to correlates with the predicted number of β-barrel proteins in Gram-negative bacteria, mitochondria and chloroplasts (Habib et al., 2007; Schleiff et al., 2003). On the other hand, the deletion of POTRA domains 1-4 in *Neisseria meningitides* had less effect on the assembly of the 8-stranded β-barrel protein NspA than on that of a 22-stranded β-barrel protein. This difference might reflect the requirement for multiple POTRA domains in order to facilitate an efficient assembly of larger and more complex substrates (Bos et al., 2007).

Besides the varying number of POTRA domains that can be found among the distinct Omp85 homologs, a crucial difference persists regarding the orientation of the POTRA domains. Here, an exceptional position among the members of the Omp85 superfamily is held by the cyanobacterial and plant homologs Toc75/Oep80. Whilst the varying number of POTRA domains in all other Omp85/BamA homologs protrude into the periplasm in bacteria or IMS in mitochondria, a recent study demonstrated that during evolution Toc75/Oep80 changed its orientation with the N-terminal POTRA domains exposed towards the cytosol (Sommer et al., 2011). This would however, exclude the putative involvement of the POTRA domains as a receptor or scaffold in the biogenesis of β-barrel proteins from the inner side of the membrane, as it was previously shown for bacteria and mitochondria. Therefore it remains elusive, whether the cytoplasmic orientation of the POTRA domains in Toc75/Oep80 allows for the recognition and assembly of incoming β-barrel precursor proteins without even entering chloroplasts through the TOC complex.

Even though structural data of the POTRA domains in different Omp85 homologs are available, very little is known about the exact mechanism by which the individual POTRA domains assist in the assembly of β-barrel precursor proteins. One possible mechanism that is often discussed is β-augmentation, which describes the interaction of β-strands of two different proteins by extending the β-sheet motif (Bennion et al., 2010; Harrison, 1996; Kim et al., 2007;

Knowles et al., 2008; Koenig et al., 2010). It is suggested, that exposed β-sheets in POTRA domains pair with β-sheets in precursors of β-barrel proteins, thus facilitating a sequential substrate sliding to the core of the corresponding Omp85 homolog. This idea would allow for the recognition and handling of a broad variety of OM proteins. Such a mechanism could be rather selective for alternating hydrophobic and polar patches in β-barrel proteins without a demand for specific linear sequence information (Gatzeva-Topalova et al., 2008; Knowles et al., 2008).

Heterologous assembly of β-barrel proteins

In accordance with the aforementioned similarities, functional expression of bacterial β-barrel proteins in eukaryotic cells suggests that mitochondria are able to recognize and assemble prokaryotic β-barrel proteins. Interestingly, in *Saccharomyces cerevisiae* several bacterial β-barrel proteins could be assembled into the mitochondrial OM. Detailed analysis showed that PhoE from *Escherichia coli* is assembled into the mitochondrial OM in its trimeric form by a process similar to the one taken by *bona fide* mitochondrial β-barrel proteins (Walther et al., 2009a). Another interesting example is provided by the trimeric autotransporter protein YadA from *Yersinia enterocolitica*. The β-barrel domain of this protein is composed from three monomers, whereas each one contributes four β-stands to the 12-stranded β-barrel. Expression of the transmembrane domain of YadA showed that four β-strands of monomeric YadA contain sufficient information to be recognized and assembled into the mitochondrial OM (Müller et al., 2011).

In a very recent study we extended our investigations to the full-length version of YadA. We found that when expressed in yeast cells both the monomeric and trimeric forms of the protein were detected in mitochondria but only the trimeric species was fully integrated into the mitochondrial OM (Ulrich et al., 2014). The oligomeric form was exposed on the surface of the organelle in its native conformation and preserved its ability to adhere to host cells. The assembly of YadA in mitochondria was dependent on the import machinery of the mitochondrial OM (TOM and TOB complexes). Interestingly, the co-expression of YadA with a mitochondria-targeted form of the bacterial periplasmic chaperone Skp, but not with SurA or SecB, resulted in elevated levels of both forms of YadA (Ulrich et al., 2014). Collectively, these results indicate that the proper assembly of trimeric autotransporter can occur also in a system lacking the

lipoproteins of the BAM machinery and is specifically enhanced by the chaperone Skp. Furthermore, these findings demonstrate that the evolutionary conservation of β-barrel assembly allows mitochondria to deal even with a class of β-barrel proteins that is not present in any eukaryotic cell.

In a reciprocal approach, the mitochondrial voltage-dependent anion-selective channel VDAC1 from *Neurospora crassa* was shown to be assembled into the bacterial OM upon its expression in *E. coli* cells (Walther et al., 2010). Interestingly, the ability to exchange substrates among the different β-barrel assembly machineries is not restricted to bacteria and mitochondria. A recent study demonstrated that the chloroplast β-barrel proteins Oep37 and Oep24 are assembled into the mitochondrial OM, when expressed in yeast cells (Ulrich et al., 2012). In contrast to the broad variety of bacterial and chloroplast β-barrel proteins that could be assembled into the mitochondrial OM in yeast cells, mammalian mitochondria did not show such a general assembly capacity for bacterial β-barrel proteins. It seems that bacterial porins like PorB from pathogenic strains were assembled into mammalian mitochondria whereas β-barrel proteins from non-pathogetic strain failed in doing so. Surprisingly, co-expression of neisserial Omp85 with non-pathogenic PorB allowed assembly of such PorB molecules into the OM of mitochondria. Nevertheless, Omp85 was not able to substitute for the loss of its mitochondrial homolog Tob55 (Kozjak-Pavlovic et al., 2011).

Apart from the aforementioned evolutionary conservation in the function and structure among the members of the Omp85 superfamily, the β-barrel assembly machineries in bacteria and mitochondria severely differ regarding their distinct accessory proteins. Chloroplasts proteins that assist Toc75/Oep80 in the assembly process have not yet been identified. A closer look at the protein complexes that facilitate the insertion of β-barrel proteins into the OM of bacteria and mitochondria reveals that the accessory proteins in the mitochondrial TOB complex do not share any homology with their bacterial counterparts. The most striking difference regarding the accessory proteins of the BAM and the TOB complexes is their contrarious localization. The bacterial lipoproteins BamB-E are localized on the inner side of the OM and hence are able to directly interact with incoming precursor proteins before their engagement by the cavity of BamA. In contrast, Tob38 and Mas37, the two mitochondrial subunits of the TOB complex, are peripherally associated at the cytosolic side of the OM.

Concluding remarks

Despite remarkable progress in characterizing the factors involved in the assembly of β-barrel proteins into the OM of Gram-negative bacteria and mitochondria, still very little is known about the machinery that assembles such proteins into the OM of chloroplasts. It is however, clear that a common ground of all membranes harboring β-barrel proteins is the presence of dedicated assembly machineries containing a central component from the Omp85 superfamily. The exact mechanism that allows the Omp85 homologs to assemble precursor proteins into the lipid bilayer still remains elusive. However, the recently reported structure of BamA and the proposed mechanism of a lateral release might serve as an initial point for further studies and is possibly also applicable to the Omp85 homologs in mitochondria and chloroplasts. Apart from the evolutionary conservation in their central unit, the machineries for assembly of β-barrel proteins display considerable differences regarding the number and the orientation of the accessory proteins. In future studies it will be interesting to analyze the exact function of such accessory proteins in order to understand how completely different subunits evolved to facilitate a basically similar process. In this regard, characterizing the factors involved in the biogenesis of β-barrel proteins in chloroplasts might also shed light on the question of how plant cells that harbor both mitochondria and chloroplasts avoid mistargeting of β-barrel proteins to the wrong organelle.

Acknowledgements

Our work is supported by the Deutsche Forschungsgemeinschaft (SFB766/B11 and RA1028/8-1 to D.R.).

References

Arnold, T., Zeth, K., Linke, D., 2010. Omp85 from the thermophilic cyanobacterium Thermosynechococcus elongatus differs from proteobacterial Omp85 in structure and domain composition. J. Biol. Chem. 285, 18003-18015.

Bechtluft, P., Nouwen, N., Tans, S.J., Driessen, A.J., 2010. SecB--a chaperone dedicated to protein translocation. Mol. Biosyst. 6, 620-627.

Bennion, D., Charlson, E.S., Coon, E., Misra, R., 2010. Dissection of beta-barrel outer membrane protein assembly pathways through characterizing BamA POTRA 1 mutants of Escherichia coli. Mol. Microbiol. 77, 1153-1171.

Bos, M.P., Robert, V., Tommassen, J., 2007. Functioning of outer membrane protein assembly factor Omp85 requires a single POTRA domain. EMBO Rep. 8, 1149-1154.

Bredemeier, R., Schlegel, T., Ertel, F., Vojta, A., Borissenko, L., Bohnsack, M.T., Groll, M., von Haeseler, A., Schleiff, E., 2007. Functional and phylogenetic properties of the pore-forming beta-barrel transporters of the Omp85 family. J. Biol. Chem. 282, 1882-1890.

Chacinska, A., Koehler, C.M., Milenkovic, D., Lithgow, T., Pfanner, N., 2009. Importing mitochondrial proteins: machineries and mechanisms. Cell 138, 628-644.

Cline, K., Andrews, J., Mersey, B., Newcomb, E.H., Keegstra, K., 1981. Separation and characterization of inner and outer envelope membranes of pea chloroplasts. Proc. Natl. Acad. Sci. U. S. A. 78, 3595-3599.

Driessen, A.J., Nouwen, N., 2008. Protein translocation across the bacterial cytoplasmic membrane. Annu. Rev. Biochem. 77, 643-667.

Endo, T., Yamano, K., 2009. Multiple pathways for mitochondrial protein traffic. Biol. Chem. 390, 723-730.

Ferbitz, L., Maier, T., Patzelt, H., Bukau, B., Deuerling, E., Ban, N., 2004. Trigger factor in complex with the ribosome forms a molecular cradle for nascent proteins. Nature 431, 590-596.

Gatsos, X., Perry, A.J., Anwari, K., Dolezal, P., Wolynec, P.P., Likic, V.A., Purcell, A.W., Buchanan, S.K., Lithgow, T., 2008. Protein secretion and outer membrane assembly in Alphaproteobacteria. FEMS Microbiol. Rev. 32, 995-1009.

Gatzeva-Topalova, P.Z., Walton, T.A., Sousa, M.C., 2008. Crystal structure of YaeT: conformational flexibility and substrate recognition. Structure 16, 1873-1881.

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

Gray, M.W., Burger, G., Lang, B.F., 1999. Mitochondrial evolution. Science 283, 1476-1481.

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

Habib, S.J., Waizenegger, T., Niewienda, A., Paschen, S.A., Neupert, W., Rapaport, D., 2007. The N-terminal domain of Tob55 has a receptor-like function in the biogenesis of mitochondrial β-barrel proteins. J. Cell Biol. 176, 77-88.

Hagan, C.L., Silhavy, T.J., Kahne, D., 2011. beta-Barrel membrane protein assembly by the Bam complex. Annu. Rev. Biochem. 80, 189-210.

Harrison, S.C., 1996. Peptide-surface association: the case of PDZ and PTB domains. Cell 86, 341-343.

Hoppins, S.C., Nargang, F.E., 2004. The Tim8-Tim13 complex of Neurospora crassa functions in the assembly of proteins into both mitochondrial membranes. J. Biol. Chem. 279, 12396-12405.

Inoue, K., Baldwin, A.J., Shipman, R.L., Matsui, K., Theg, S.M., Ohme-Takagi, M., 2005. Complete maturation of the plastid protein translocation channel requires a type I signal peptidase. J. Cell Biol. 171, 425-430.

Ishikawa, D., Yamamoto, H., Tamura, Y., Moritoh, K., Endo, T., 2004. Two novel proteins in the mitochondrial outer membrane mediate β-barrel protein assembly. J. Cell Biol. 166, 621-627.

Joyard, J., Ferro, M., Masselon, C., Seigneurin-Berny, D., Salvi, D., Garin, J., Rolland, N., 2009. Chloroplast proteomics and the compartmentation of plastidial isoprenoid biosynthetic pathways. Mol. Plant 2, 1154-1180.

Kim, S., Malinverni, J.C., Sliz, P., Silhavy, T.J., Harrison, S.C., Kahne, D., 2007. Structure and function of an essential component of the outer membrane protein assembly machine. Science 317, 961-964.

Knowles, T.J., Jeeves, M., Bobat, S., Dancea, F., McClelland, D., Palmer, T., Overduin, M., Henderson, I.R., 2008. Fold and function of polypeptide transport-associated domains responsible for delivering unfolded proteins to membranes. Mol. Microbiol. 68, 1216-1227.

Koenig, P., Mirus, O., Haarmann, R., Sommer, M.S., Sinning, I., Schleiff, E., Tews, I., 2010. Conserved properties of polypeptide transport-associated (POTRA) domains derived from cyanobacterial Omp85. J. Biol. Chem. 285, 18016-18024.

Komiya, T., Rospert, S., Koehler, C., Looser, R., Schatz, G., Mihara, K., 1998. Interaction of Mitochondrial Targeting Signals with Acidic Receptor Domains along the Protein Import Pathway: Evidence for the "Acidic Chain" hypothesis. EMBO J. 17, 3886-3898.

Kozjak-Pavlovic, V., Ott, C., Gotz, M., Rudel, T., 2011. Neisserial omp85 protein is selectively recognized and assembled into functional complexes in the outer membrane of human mitochondria. J. Biol. Chem. 286, 27019-27026.

Kutik, S., Stojanovski, D., Becker, L., Becker, T., Meinecke, M., Kruger, V., Prinz, C., Meisinger, C., Guiard, B., Wagner, R., Pfanner, N., Wiedemann, N., 2008. Dissecting membrane insertion of mitochondrial β-barrel proteins. Cell 132, 1011-1024.

Lazar, S.W., Kolter, R., 1996. SurA assists the folding of Escherichia coli outer membrane proteins. J. Bacteriol. 178, 1770-1773.

Malinverni, J.C., Werner, J., Kim, S., Sklar, J.G., Kahne, D., Misra, R., Silhavy, T.J., 2006. YfiO stabilizes the YaeT complex and is essential for outer membrane protein assembly in Escherichia coli. Mol. Microbiol. 61, 151-164.

McFadden, G.I., 2001. Chloroplast origin and integration. Plant Physiol. 125, 50-53.

Milenkovic, D., Kozjak, V., Wiedemann, N., Lohaus, C., Meyer, H.E., Guiard, B., Pfanner, N., Meisinger, C., 2004. Sam35 of the mitochondrial protein sorting and assembly machinery is a peripheral outer membrane protein essential for cell viability. J. Biol. Chem. 279, 22781-22785.

Moslavac, S., Mirus, O., Bredemeier, R., Soll, J., von Haeseler, A., Schleiff, E., 2005. Conserved pore-forming regions in polypeptide-transporting proteins. FEBS J. 272, 1367-1378.

Müller, J.E., Papic, D., Ulrich, T., Grin, I., Schutz, M., Oberhettinger, P., Tommassen, J., Linke, D., Dimmer, K.S., Autenrieth, I.B., Rapaport, D., 2011. Mitochondria can recognize and assemble fragments of a beta-barrel structure. Mol. Biol. Cell 22, 1638-1647.

Noinaj, N., Kuszak, A.J., Balusek, C., Gumbart, J.C., Buchanan, S.K., 2014. Lateral Opening and Exit Pore Formation Are Required for BamA Function. Structure 22, 1055-1062.

Noinaj, N., Kuszak, A.J., Gumbart, J.C., Lukacik, P., Chang, H., Easley, N.C., Lithgow, T., Buchanan, S.K., 2013. Structural insight into the biogenesis of β-barrel membrane proteins. Nature 501, 385-390.

Paetzel, M., 2014. Structure and mechanism of Escherichia coli type I signal peptidase. Biochim. Biophys. Acta. 1843, 1497-1508.

Papanikou, E., Karamanou, S., Economou, A., 2007. Bacterial protein secretion through the translocase nanomachine. Nat. Rev. Microbiol. 5, 839-851.

Paschen, S.A., Neupert, W., Rapaport, D., 2005. Biogenesis of β-barrel membrane proteins of mitochondria. Trends Biochem. Sci. 30, 575-582.

Paschen, S.A., Waizenegger, T., Stan, T., Preuss, M., Cyrklaff, M., Hell, K., Rapaport, D., Neupert, W., 2003. Evolutionary conservation of biogenesis of β-barrel membrane proteins. Nature 426, 862-866.

Patel, R., Hsu, S.C., Bedard, J., Inoue, K., Jarvis, P., 2008. The Omp85-related chloroplast outer envelope protein OEP80 is essential for viability in Arabidopsis. Plant Physiol. 148, 235-245.

Pfanner, N., Wiedemann, N., Meisinger, C., Lithgow, T., 2004. Assembling the mitochondrial outer membrane. Nat. Struct. Mol. Biol. 11, 1044-1048.

Randall, L.L., Hardy, S.J., 2002. SecB, one small chaperone in the complex milieu of the cell. Cell Mol. Life Sci. 59, 1617-1623.

Rapaport, D., Mayer, A., Neupert, W., Lill, R., 1998. Cis and trans sites of the TOM complex in unfolding and initial translocation of preproteins. J. Biol. Chem. 273, 8806-8813.

Rouviere, P.E., Gross, C.A., 1996. SurA, a periplasmic protein with peptidyl-prolyl isomerase activity, participates in the assembly of outer membrane porins. Genes Dev. 10, 3170-3182.

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

Schatz, G., 1997. Just follow the acid chain. Nature 388, 121-122.

Schleiff, E., Becker, T., 2011. Common ground for protein translocation: access control for mitochondria and chloroplasts. Nat. Rev. Mol. Cell Biol. 12, 48-59.

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

Sklar, J.G., Wu, T., Gronenberg, L.S., Malinverni, J.C., Kahne, D., Silhavy, T.J., 2007a. Lipoprotein SmpA is a component of the YaeT complex that assembles outer membrane proteins in Escherichia coli. Proc. Natl. Acad. Sci. U. S. A. 104, 6400-6405.

Sklar, J.G., Wu, T., Kahne, D., Silhavy, T.J., 2007b. Defining the roles of the periplasmic chaperones SurA, Skp, and DegP in Escherichia coli. Genes Dev. 21, 2473-2484.

Soll, J., Schleiff, E., 2004. Protein import into chloroplasts. Nat. Rev. Mol. Cell Biol. 5, 198-208.

Sommer, M.S., Daum, B., Gross, L.E., Weis, B.L., Mirus, O., Abram, L., Maier, U.G., Kuhlbrandt, W., Schleiff, E., 2011. Chloroplast Omp85 proteins change orientation during evolution. Proc. Natl. Acad. Sci. U. S. A. 108, 13841-13846.

Tranel, P.J., Keegstra, K., 1996. A novel, bipartite transit peptide targets OEP75 to the outer membrane of the chloroplastic envelope. Plant Cell 8, 2093-2104.

Ullers, R.S., Luirink, J., Harms, N., Schwager, F., Georgopoulos, C., Genevaux, P., 2004. SecB is a bona fide generalized chaperone in Escherichia coli. Proc. Natl. Acad. Sci. U. S. A. 101, 7583-7588.

Ulrich, T., Gross, L.E., Sommer, M.S., Schleiff, E., Rapaport, D., 2012. Chloroplast β-barrel proteins are assembled into the mitochondrial outer membrane in a process that depends on the TOM and TOB complexes. J. Biol. Chem. 287, 27467-27479.

Ulrich, T., Oberhettinger, P., Schütz, M., Holzer, K., Ramms, A.S., Linke, D., Autenrieth, I.B., Rapaport, D., 2014. Evolutionary Conservation in Biogenesis of β-Barrel Proteins Allows Mitochondria to Assemble a Functional Bacterial Trimeric Autotransporter Protein. J. Biol. Chem., in press.

Valent, Q.A., Kendall, D.A., High, S., Kusters, R., Oudega, B., Luirink, J., 1995. Early events in preprotein recognition in E. coli: interaction of SRP and trigger factor with nascent polypeptides. EMBO 14, 5494-5505.

Van den Berg, B., Clemons, W.M., Jr., Collinson, I., Modis, Y., Hartmann, E., Harrison, S.C., Rapoport, T.A., 2004. X-ray structure of a protein-conducting channel. Nature 427, 36-44.

Vertommen, D., Ruiz, N., Leverrier, P., Silhavy, T.J., Collet, J.F., 2009. Characterization of the role of the Escherichia coli periplasmic chaperone SurA using differential proteomics. Proteomics 9, 2432-2443.

Volokhina, E.B., Grijpstra, J., Stork, M., Schilders, I., Tommassen, J., Bos, M.P., 2011. Role of the periplasmic chaperones Skp, SurA, and DegQ in outer membrane protein biogenesis in Neisseria meningitidis. J. Bacteriol. 193, 1612-1621.

Voulhoux, R., Bos, M.P., Geurtsen, J., Mols, M., Tommassen, J., 2003. Role of a highly conserved bacterial protein in outer membrane protein assembly. Science 299, 262-265.

Vuong, P., Bennion, D., Mantei, J., Frost, D., Misra, R., 2008. Analysis of YfgL and YaeT interactions through bioinformatics, mutagenesis, and biochemistry. J. Bacteriol. 190, 1507-1517.

Waizenegger, T., Habib, S.J., Lech, M., Mokranjac, D., Paschen, S.A., Hell, K., Neupert, W., Rapaport, D., 2004. Tob38, a novel essential component in the biogenesis of β-barrel proteins of mitochondria. EMBO Rep. 5, 704-709.

Walther, D.M., Bos, M.P., Rapaport, D., Tommassen, J., 2010. The mitochondrial porin, VDAC, has retained the ability to be assembled in the bacterial outer membrane. Mol. Biol. Evol. 27, 887-895.

Walther, D.M., Papic, D., Bos, M.P., Tommassen, J., Rapaport, D., 2009a. Signals in bacterial β-barrel proteins are functional in eukaryotic cells for targeting to and assembly in mitochondria. Proc. Natl. Acad. Sci. USA 106, 2531-2536.

Walther, D.M., Rapaport, D., 2009. Biogenesis of mitochondrial outer membrane proteins. Biochim. Biophys. Acta 1793, 42-51.

Walther, D.M., Rapaport, D., Tommassen, J., 2009b. Biogenesis of beta-barrel membrane proteins in bacteria and eukaryotes: evolutionary conservation and divergence. Cell Mol. Life Sci. 66, 2789-2804.

Wiedemann, N., Kozjak, V., Chacinska, A., Schönfish, B., Rospert, S., Ryan, M.T., Pfanner, N., Meisinger, C., 2003. Machinery for protein sorting and assembly in the mitochondrial outer membrane. Nature 424, 565-571.

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

Wimley, W.C., 2003. The versatile β-barrel membrane protein. Curr. Opin. Struct. Biol. 13, 404-411.

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

Xie, K., Hessa, T., Seppala, S., Rapp, M., von Heijne, G., Dalbey, R.E., 2007. Features of transmembrane segments that promote the lateral release from the translocase into the lipid phase. Biochemistry 46, 15153-15161.

Zimmer, J., Nam, Y., Rapoport, T.A., 2008. Structure of a complex of the ATPase SecA and the protein-translocation channel. Nature 455, 936-943.