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

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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-

brane 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 *tom20Δ*, *mas37Δ* and *GAL10-TOB55* strains were described before ((27, 29, 11), respectively). The *tom70Δ/tom71Δ* double-deletion strain, the *yme1Δ* 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 μg 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$_2$PO$_4$, 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. 1A). 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. 1B, 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. 1B, 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. 1C, 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. 1C, 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. 1D, 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. 1D, lanes 3 and 4).
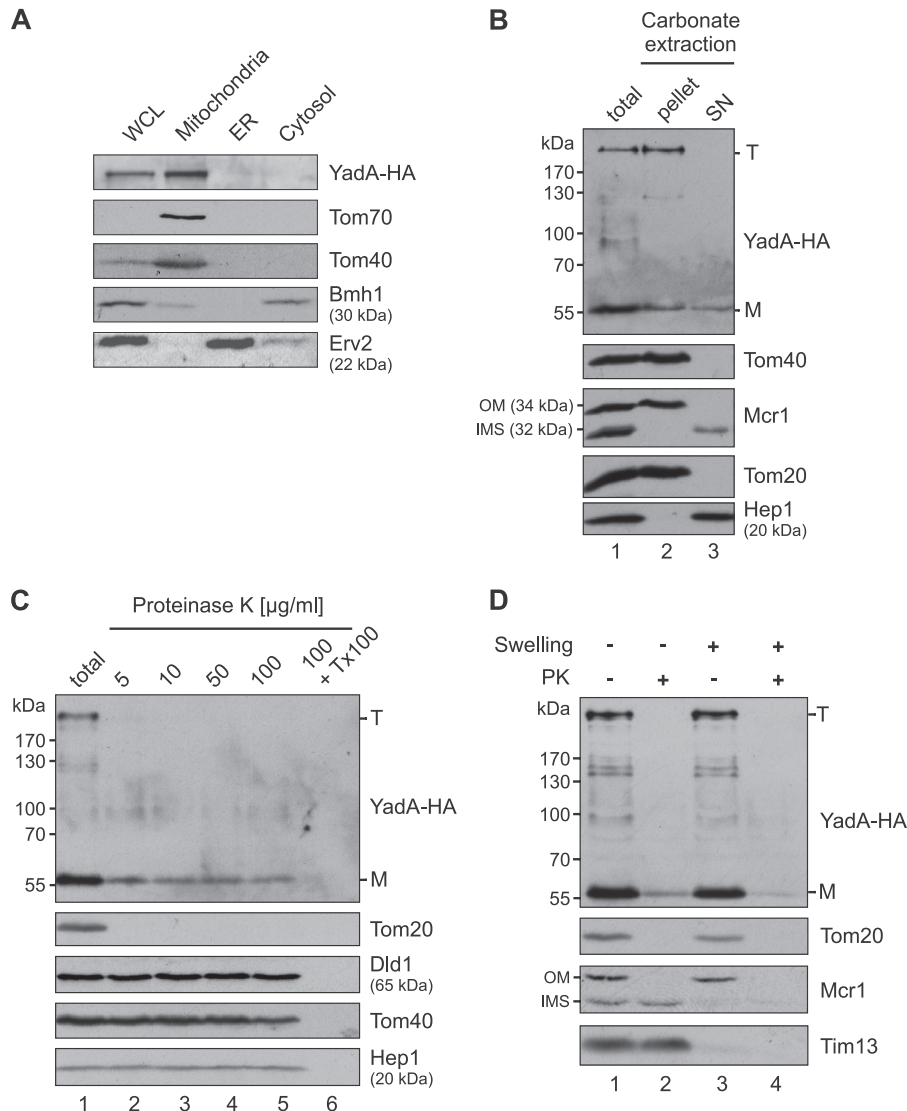
FIGURE 1. **The trimeric autotransporter YadA is targeted to the mitochondrial outer membrane.** *A*, whole cell lysate (*WCL*) of yeast cells expressing YadA-HA and fractions corresponding to mitochondria, endoplasmic reticulum (*ER*), and cytosol were analyzed by SDS-PAGE and immunodetection with antibodies 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.

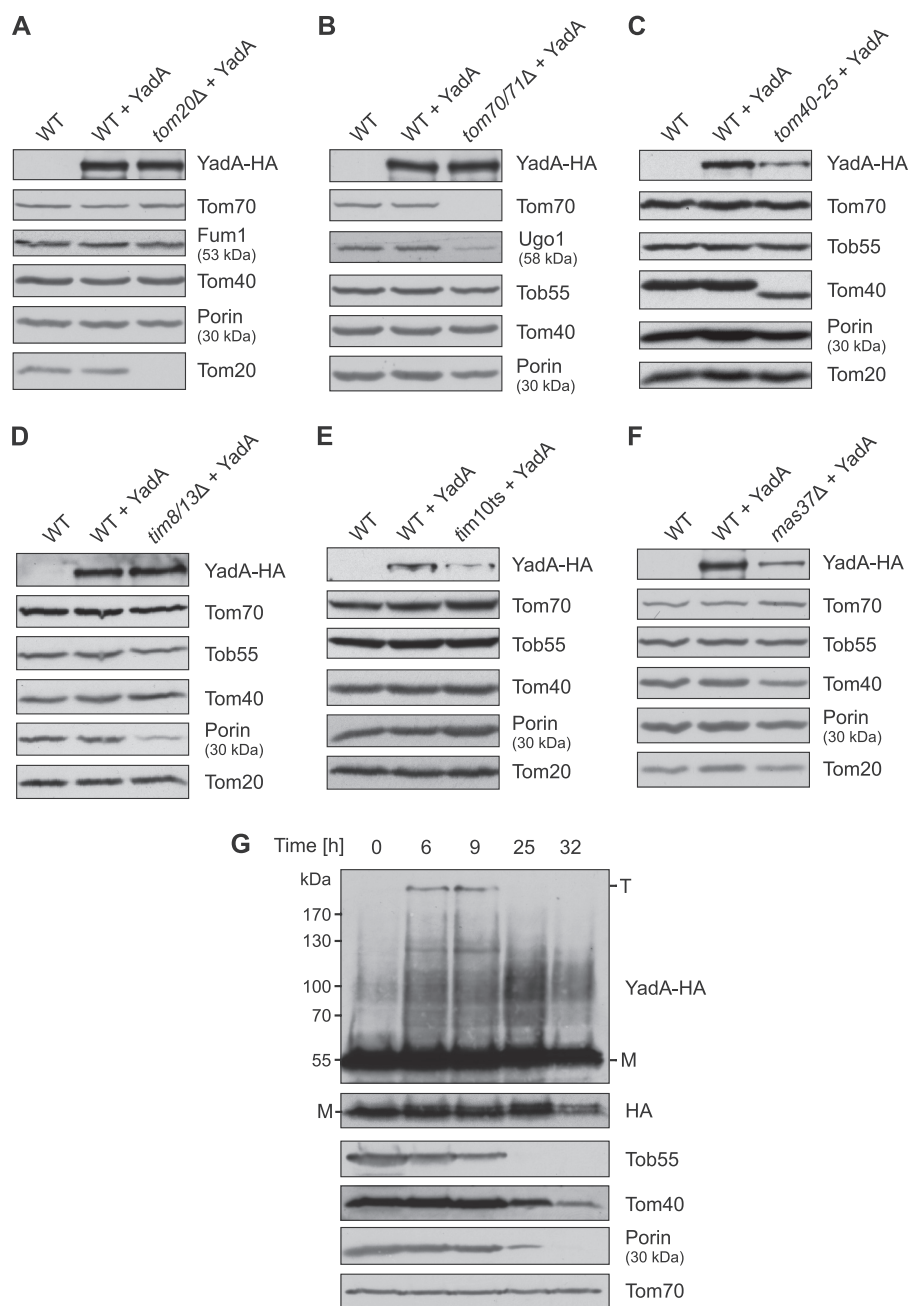To discriminate between these two alternatives we asked whether the import receptors of the TOM complex play a role

FIGURE 2. **The assembly of YadA into the mitochondrial OM depends on the TOM and TOB complexes.** Mitochondria were isolated from *A, tom20Δ, B, tom70Δ/tom71Δ, C, tom40–25, D, tim8Δ/tim13Δ, E, tim10ts,* and *F, mas37Δ* 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 hetero-hexameric 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 *mas37Δ* 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

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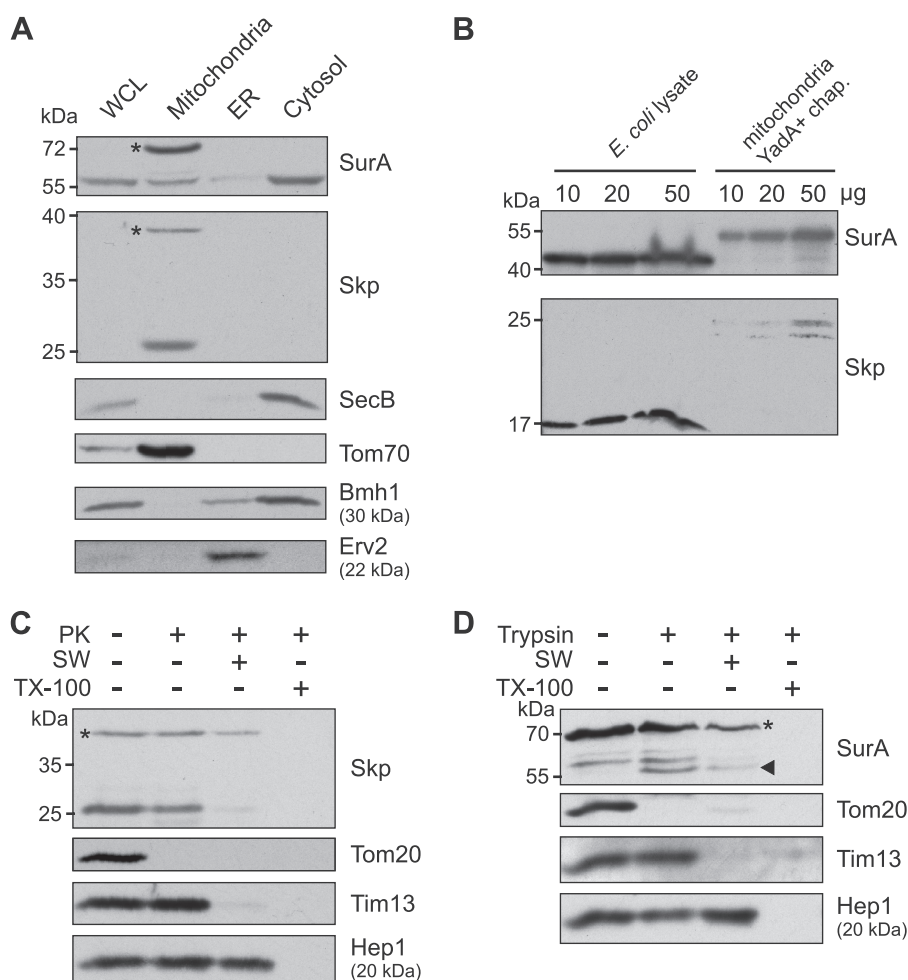[4] T. Ulrich and D. Rapaport, unpublished results.

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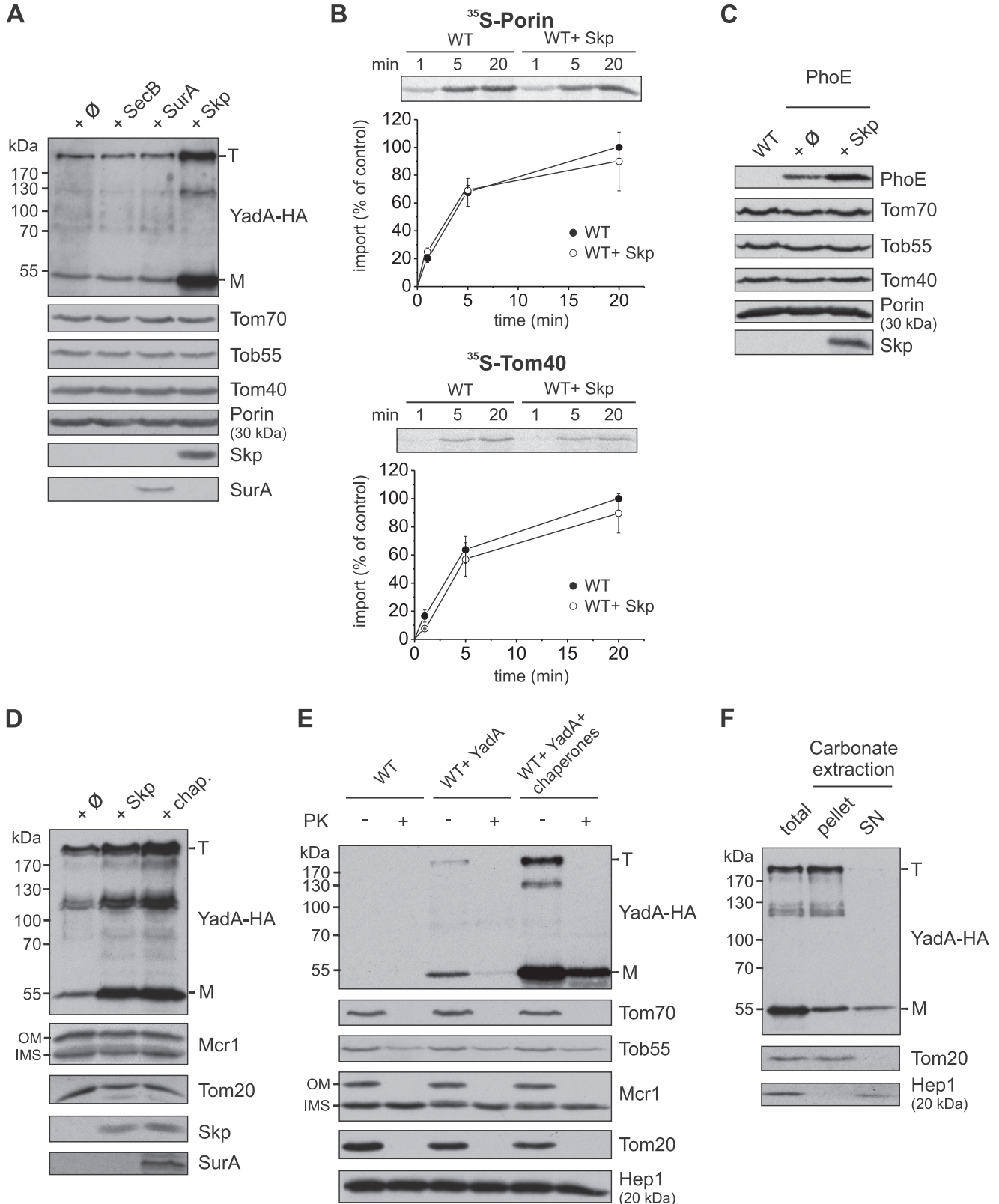
latter by at least 2-fold (Fig. 4*C*), suggesting a more general effect of Skp.

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 proteo-

lytic activity is Yme1 which is known to degrade proteins in the mitochondrial IMS (55). Thus, we next investigated the lifespan 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.
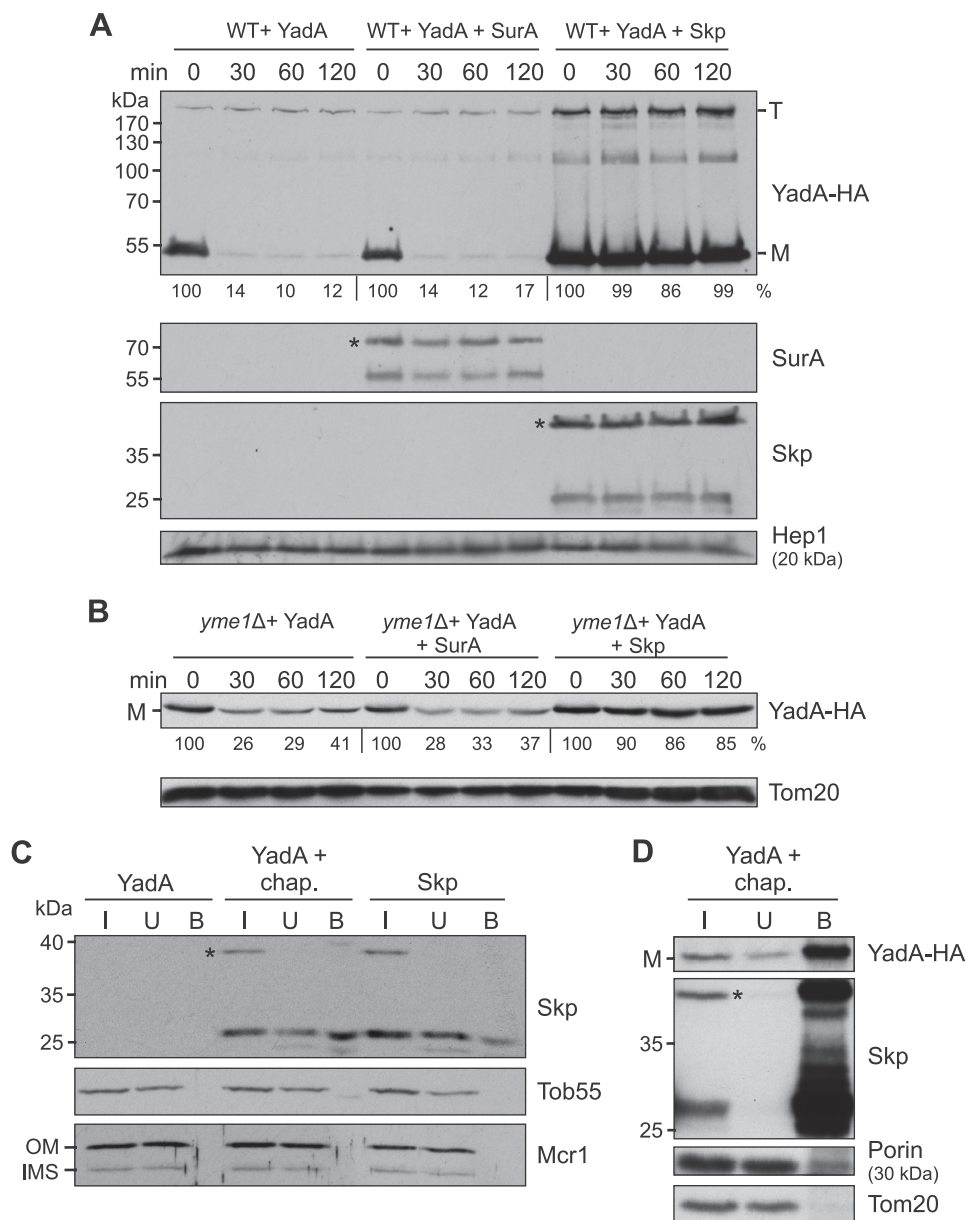
ASBMB

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. 6C). 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-
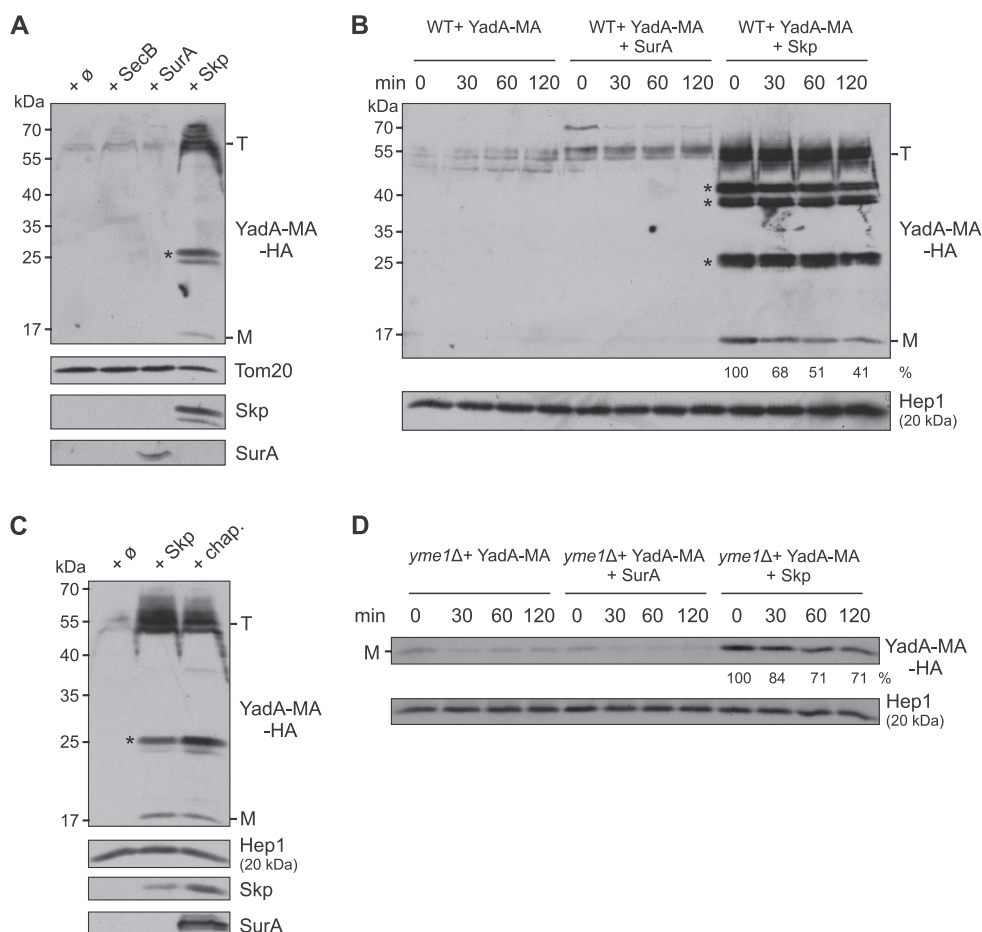
FIGURE 6. **Skp enhances the steady-state levels of YadA-MA.** *A*, mitochondria were isolated from cells co-transformed with a plasmid expressing YadA-MA together with an empty plasmid, or together with a plasmid encoding SecB, mtSurA, or mtSkp. Mitochondrial proteins were analyzed by SDS-PAGE and immunodetection 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
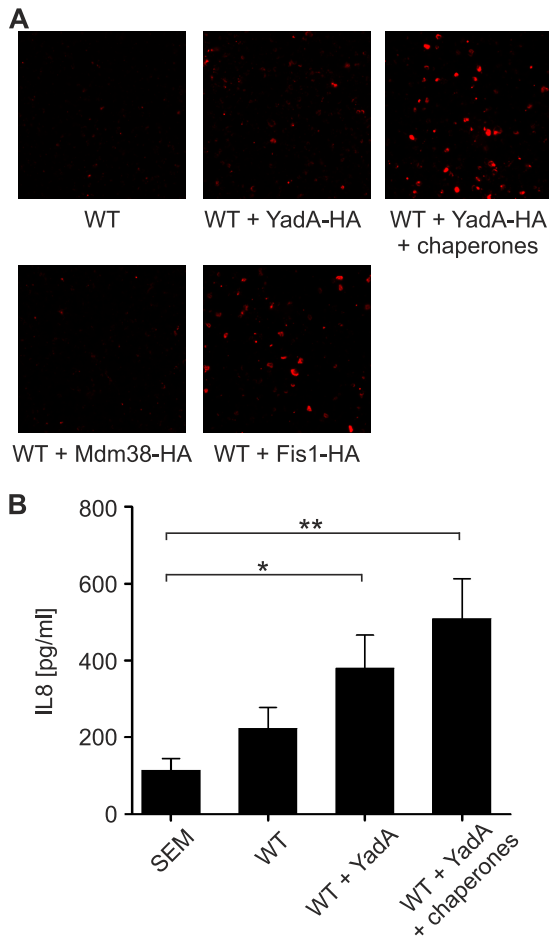
FIGURE 7. **YadA is assembled at the mitochondrial OM in its native functional conformation.** *A*, mitochondria were isolated from control cells (*WT*), cells expressing YadA-HA alone, or cells expressing YadA-HA together with the three bacterial chaperones. Mitochondria isolated from cells expressing 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.

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