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Evolution of Mitochondrial Oxa Proteins from Bacterial YidC

INHERITED AND ACQUIRED FUNCTIONS OF A CONSERVED PROTEIN INSERTION MACHINERY*

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Members of the Oxa1/YidC family are involved in the biogenesis of membrane proteins. In bacteria, YidC catalyzes the insertion and assembly of proteins of the inner membrane. Mitochondria of animals, fungi, and plants harbor two distant homologues of YidC, Oxa1 and Cox18/Oxa2. Oxa1 plays a pivotal role in the integration of mitochondrial translation products into the inner membrane of mitochondria. It contains a C-terminal ribosome-binding domain that physically interacts with mitochondrial ribosomes to facilitate the co-translational insertion of nascent membrane proteins. The molecular function of Cox18/Oxa2 is not well understood. Employing a functional complementation approach with mitochondria-targeted versions of YidC we show that YidC is able to functionally replace both Oxa1 and Cox18/Oxa2. However, to integrate mitochondrial translation products into the inner membrane of mitochondria, the ribosome-binding domain of Oxa1 has to be appended onto YidC. On the contrary, the fusion of the ribosome-binding domain onto YidC prevents its ability to complement COX18 mutants suggesting an indispensable post-translational activity of Cox18/Oxa2. Our observations suggest that during evolution of mitochondria from their bacterial ancestors the two descendants of YidC functionally segregated to perform two distinct activities, one co-translational and one post-translational.

The synthesis of hydrophobic membrane proteins in a hydrophilic environment bears the high risk of the formation of unproductive protein aggregates. This problem is typically avoided by a strict coupling of synthesis and membrane integration. In the cytosol of eukaryotic and prokaryotic cells, signal recognition particles recognize ribosomes that synthesize hydrophobic polypeptides and target them onto membrane-embedded translocation complexes (1–3).

Over the last years several factors were identified that are required to integrate proteins into the inner membrane of bacteria. The major component in this reaction is the Sec machinery that presumably serves as the entry gate for most inner membrane proteins (for an overview see Refs. 4–6). To integrate proteins into the lipid bilayer, the Sec translocase coop-

erates with a further component, called YidC (for review see Refs. 4, 7, and 8).

In this case, YidC might function as a chaperone that helps membrane proteins to adopt their appropriate three-dimensional folds and to assemble into protein complexes (9). Some membrane proteins, however, do not require the Sec translocase but rather are directly inserted by YidC (10, 11). Functional reconstitution experiments with purified YidC confirmed the potential of YidC to function as an “integrator” that catalyzes the insertion of certain membrane proteins into lipid bilayers independently of further components (12, 13). *In vivo*, the bacterial signal recognition particle may play a role in membrane targeting of some YidC substrates thereby promoting the co-translational insertion of nascent polypeptides (14–16). A close proximity of YidC and the bacterial ribosome during protein insertion is indeed indicated by cross-linking experiments that reveal the contact to YidC of very short nascent chains that emerge from the peptide exit tunnel of the ribosomes only by a few residues (17). A direct physical interaction of bacterial ribosomes and YidC was, however, not shown so far.

The protein insertion machinery of mitochondria derived from the bacterial system but was considerably adapted to meet the needs of the organelle during evolution (for review see Ref. 18). Mitochondria of higher eukaryotes like fungi and animals lost both the Sec machinery and signal recognition particles (19). A homologue of the bacterial YidC protein plays a pivotal role in the integration of both mitochondrially and nuclear encoded inner membrane proteins in mitochondria. This mitochondrial protein, which is called Oxa1, is ubiquitously present in mitochondria. The Oxa1 protein of the baker's yeast *Saccharomyces cerevisiae* was the first member of the conserved Oxa1/YidC protein family (20, 21). By sequence similarity, Oxa1 homologues were identified in the inner membrane of bacteria (called YidC proteins) and in the inner envelope and thylakoid membrane of chloroplasts (Alb3 and ARTEMIS).

Oxa1 is required for the insertion of mitochondrial translation products into the inner membrane. In yeast, eight proteins are encoded by the mitochondrial genome, seven of which are highly hydrophobic membrane proteins: cytochrome *b* of the *bc*₁ complex; Cox1, Cox2, and Cox3 of the cytochrome oxidase; and Atp6, Atp8, and Atp9 of the F₀F₁-ATPase. Oxa1 catalyzes the integration of these proteins into the inner membrane, and as a consequence Oxa1 deletion strains are deficient of cytochrome oxidase, and the activities of both other enzymatic complexes are strongly diminished (22, 23). Although most of these proteins still insert into the membrane (although at considerably reduced rates (23)) the membrane integration of Cox2 strictly depends on Oxa1 function (24, 25). This protein contains two transmembrane segments, and, following synthesis both termini are exported into the intermembrane space.

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The intermembrane space domains of Cox2 are highly charged, and especially the C-terminal one is of considerable length, which might explain why Cox2 strictly requires Oxa1 for its topogenesis. Cox2 is synthesized in the matrix with an N-terminal extension, which is proteolytically removed by the intermembrane space peptidase Imp1. Because translocation of the N terminus is a prerequisite for the processing in the intermembrane space, translocation-deficient strains like Oxa1 mutants accumulate Cox2 in its precursor form.

Mitochondria lack signal recognition particles, and the co-translational insertion of the hydrophobic translation products is achieved by an alternative mechanism: the mitochondrial Oxa1 protein exposes a C-terminal α -helical stretch of about 100 residues into the matrix that functions as ribosome-binding domain (26, 27). This domain attaches in close proximity to the polypeptide exit tunnel to the mitochondrial ribosome (27, 28) and thereby ensures intimate contact of the emerging nascent chain to the membrane-embedded core domain of Oxa1. Because the mitochondrial genome almost exclusively encodes hydrophobic membrane proteins, a signal recognition particle that differentiates between hydrophilic and hydrophobic nascent chains might have become dispensable during evolution. The second group of Oxa1 substrates is nuclear encoded inner membrane proteins that are inserted into the inner membrane following their complete import from the cytosol into the matrix (26, 29).

In addition to Oxa1, mitochondria of plants, fungi, and animals contain a second member of the Oxa1/YidC protein family, which was called Cox18/Oxa2. This protein lacks the C-terminal ribosome-binding domain of Oxa1 and plays a specific role for the biogenesis of the cytochrome oxidase complex (30–32). The molecular function of Oxa2 in this process is, however, not clear.

In this study, we followed a genetic complementation approach to explore the functional similarities and differences between the bacterial YidC protein and its mitochondrial homologues Oxa1 and Cox18/Oxa2. We found that YidC can functionally replace both mitochondrial homologues. However, to achieve complementation of *oxa1* mutants, the C-terminal ribosome-binding domain of Oxa1 had to be appended to YidC to allow coupling of YidC to the mitochondrial translation machinery. On the contrary, for functional replacement of Cox18 the attachment of the ribosome-binding domain was not required but rather abolished the ability of YidC to complement. From this we conclude that during evolution of mitochondria, the YidC gene was duplicated to generate two functionally different descendants: Oxa1, which primarily functions as a protein insertase, and Cox18/Oxa2, which is specialized to fulfill its function post-translationally.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Media—All strains used in this study were isogenic to the wild type strain W303-1A. For generation of the *oxa1* and *cox18* deletion mutants, the entire coding regions of the *OXA1* and *COX18* genes, respectively, were replaced by *HIS3* cassettes (29, 32). For expression of mitochondria-targeted YidC, the sequence encoding the promoter and the N-terminal 119 amino acid residues was amplified by PCR using the primers GS01 (5'-GGGCTCGAGGCATCGGACATTTTCCCG-3') and GS02 (5'-GGGCGCGCCGCTCCAAGACGTGTTG-3') and cloned into the XhoI/NotI sites of pRS426 (33). Into the NotI and SacI sites of the resulting plasmid the sequence encoding the conserved domain of YidC was inserted; the sequence was amplified by PCR using the primers GS03 (5'-GGGCGCGCCGATGGATCCATAGCTTTGTG-3') and GS04 (5'-GGGAGCTCTGACCGCCCTTATTTAGCG-3'). For expression of mtYidC-RBD,¹ the sequence encoding mtYidC was amplified using the primers GS01 and MP38 (5'-CCCTCTA-

GAGGATTTTTCTCTCGCGGC-3'), digested with XhoI and XbaI, and subcloned into pBluescript (Stratagene, La Jolla, CA). The sequence encoding the ribosome-binding domain of Oxa1 was amplified from yeast genomic DNA using the primers MP39 (5'-GGGTCTAGACTACAGACAATGATTTTGAGAAAC-3') and MP40 (5'-CCCGAGCTTCATTTTTTGTATTATAATGAA-3'), digested with XbaI and SacI, and cloned into the pBluescript construct. Then the mtYidC-RBD insert was released by digestion with XhoI and SacI and cloned into pRS426. For construction of overexpression plasmids, the mtYidC and mtYidC-RBD coding regions were cloned into pYX142 expression vector (Novagen, La Jolla, CA) under control of the strong *TPI1* promoter. Yeast cultures were grown at 30 °C in lactate medium, YP medium (1% yeast extract, 2% peptone) supplemented with 2% galactose, or minimal medium supplemented with 20 μ g/ml adenine, histidine, and tryptophan, and 30 μ g/ml of leucine and lysine (34, 35). Mitochondria were isolated as described previously (35).

Labeling of Mitochondrial Translation Products in Vivo—Cells were grown in minimal medium containing 0.17% (w/v) yeast nitrogen base, 0.5% (w/v) ammonium sulfate, 2% raffinose and supplemented with appropriate amino acids, adenine or uracil as required (34). The labeling reaction was performed essentially as described (36): a cell suspension of an optical density (A_{578}) of 1.0 was treated with cycloheximide (150 μ g/ml) to block the synthesis of nuclear encoded proteins. After incubation for 5 min at 30 °C, 5 μ l of [³⁵S]methionine (10 mCi/ml) were added. The samples were incubated under agitation at 30 °C for 30 min. The labeling was stopped by the addition of 1 mM unlabeled methionine and 12 μ g/ml puromycin. Aliquots equivalent to an optical density of 0.5 were taken 0, 30, 60, 120, and 180 min after the reaction was stopped. The cells were then collected by centrifugation, washed twice in water, and lysed in the presence of 235 mM NaOH, 130 mM β -mercaptoethanol, and 1.5 mM phenylmethylsulfonyl fluoride. The samples were incubated 10 min at 0 °C. The proteins were then precipitated by trichloroacetic acid, resolved by SDS-PAGE, and visualized by autoradiography.

Labeling of Mitochondrial Translation Products in Isolated Mitochondria (in Organello)—Translation products were labeled in mitochondria as described previously (35, 36). Mitochondria (40 μ g protein) were incubated in translation buffer (0.6 M sorbitol, 159 mM KCl, 15 mM KH_2PO_4 , 13 mM MgSO_4 , 0.15 mg/ml of all amino acids except methionine, 4 mM ATP, 0.5 mM GTP, 5 mM α -ketoglutarate, 5 mM phosphoenolpyruvate, 3 mg/ml fatty acid-free bovine serum albumin, 20 mM Tris/HCl, pH 7.4) containing 0.6 units of pyruvate kinase and 10 μ Ci of [³⁵S]methionine. Typically, samples were incubated for 30 min at 24 °C, and labeling was stopped by the addition of 25 mM unlabeled methionine. The samples were further incubated for 5 min to complete synthesis, and mitochondria were isolated by centrifugation, washed in 1 ml of 0.6 M sorbitol, 20 mM Hepes/HCl, pH 7.4, and lysed in 25 μ l of sample buffer (2% sodium dodecyl sulfate, 10% glycerol, 2.5% β -mercaptoethanol, 0.02% bromophenol blue, 60 mM Tris/HCl, pH 6.8). Samples were shaken at 4 °C for 10 min prior to loading on the gel.

Protein Import into Isolated Mitochondria—Precursor proteins were synthesized in reticulocyte lysate (Promega) in the presence of [³⁵S]methionine and imported into isolated mitochondria essentially as described previously (29). 2 mM NADH, 2 mM ATP, and an ATP-regenerating system containing 2.5 mM malate, 2.5 mM succinate, 1 mM creatine phosphate, and 0.1 mg/ml creatine kinase was added during the import reaction to generate a highly energized state of the mitochondria. Non-imported precursor protein was removed by incubation with proteinase K (100 μ g/ml) for 30 min on ice. The protease was inactivated by the addition of 2 mM phenylmethylsulfonyl fluoride.

Chemical Cross-linking—Cross-linking and immunoprecipitation were carried out essentially as described (37). Mitochondrial gene products were radiolabeled in isolated mitochondria in a translation buffer with bovine serum albumin omitted and Tris/HCl replaced by Hepes/KOH to prevent quenching by the amino group-specific cross-linking reagents. Stock solutions (24 mM) of the cross-linking reagent dithio-bis(succinimidyl propionate) was freshly prepared in dimethyl sulfoxide and diluted 1/100 into the labeling reaction. To mock-treated samples was added an equal concentration of dimethyl sulfoxide. Following cross-linking, 25 mM glycine and cold methionine were added to quench unreacted cross-linkers.

Immunoprecipitation and Co-immunoprecipitation—For immunoprecipitation, mitochondria were lysed in 25 μ l of 1% SDS, 50 mM Tris-HCl, pH 7.5, cleared by centrifugation at 10 min at 16,000 \times g, and diluted in 40 volumes of 0.1% Triton X-100, 300 mM KCl, 5 mM EDTA, 10 mM Tris/HCl, pH 7.4. For co-immunoprecipitation reactions, the SDS buffer was omitted and the mitochondria were lysed directly in Triton buffer. After a clarifying spin for 10 min at 16,000 \times g at 4 °C, the extract was added to 2 mg of protein A-Sepharose (Amersham Bio-

¹ The abbreviations used are: RBD, ribosome-binding domain; wt, wild type.

sciences) and 4–6 μ l of rabbit antisera. After incubation of 1 h at 4 °C, the beads were washed twice in lysis buffer and once in 20 mM Tris/HCl, pH 7.4. Precipitated proteins were dissolved in sample buffer, separated by SDS-PAGE, and exposed to x-ray film.

Mitochondrial Subfractionation—For hypotonic swelling to selectively rupture the outer membrane, mitochondria were incubated in the presence of 60 mM sorbitol for 30 min on ice. Efficient swelling was monitored by Western blotting using antibodies against epitopes of the matrix and the intermembrane space.

Ribosomal pellet fractions were essentially isolated as described (26). Mitochondria (200 μ g) were washed in 80 mM KCl, 0.6 M sorbitol, 20 mM Hepes, pH 7.4, and lysed in 2 ml of 1% Triton X-100, 25 mM KCl, 5 mM EDTA, 20 mM Hepes, pH 7.4. After a clarifying spin for 15 min at 30,000 $\times g$ at 2 °C, the extract was split. One-half was directly loaded onto a cushion of 1.6 M sucrose, 20 mM Hepes, pH 7.4, and centrifuged for 6 h at 45,000 rpm at 4 °C in a SW60 rotor (Beckman). Proteins of the supernatant and of the second half were precipitated by the addition of 12% trichloroacetic acid. Proteins of the ribosomal pellet fraction were directly dissolved in lysis buffer.

Miscellaneous—Enzymatic measurements of respiratory chain complexes were performed essentially as described before (38).

RESULTS

Mitochondrial and Bacterial Members of the Oxa1/YidC Protein Family Differ in Their C Termini—Members of the Oxa1/YidC protein family are characterized by moderately conserved hydrophobic core domains each comprising five transmembrane spans (Fig. 1A). The core domains of bacterial and mitochondrial homologues are flanked by non-related regions. In particular, YidC proteins of bacteria typically contain an additional transmembrane domain at the very N terminus that plays a role in targeting of YidC to the inner membrane (39, 40). Mitochondrial Oxa1 proteins lack this transmembrane span. The N terminus of Oxa1 represents a mitochondrial targeting signal that directs this nuclear gene product to mitochondria and that is proteolytically removed following import (41). In addition, Oxa1 proteins consistently contain C-terminal extensions that protrude into the matrix and function as ribosome-binding domains (26, 27). These domains are absent in the bacterial homologues (Fig. 1B). Despite the significant similarity in the secondary structure and topology of YidC and Oxa1 proteins (*cf.* Fig. 1C), the primary sequences even of the core domains display only a few invariant amino acid residues, and the similarity among bacterial and mitochondrial members ranges only between 15 and 20%.

The Bacterial YidC Protein Can Be Targeted to the Inner Membrane of Mitochondria—To test whether, despite their limited sequence identity, YidC and Oxa1 proteins are functionally conserved, we followed a genetic complementation approach in the yeast *S. cerevisiae*. To this end, we constructed a chimeric protein of the mitochondrial targeting domain of Oxa1 fused to the hydrophobic core domain of YidC, which we named mitochondria-targeted YidC or mtYidC (Fig. 2A). In addition, we generated a second variant to which the ribosome-binding domain of Oxa1 was appended (mtYidC-RBD).

The core domain of YidC is significantly more hydrophobic than that of Oxa1. Because highly hydrophobic sequences in preproteins can interfere with their import competence (42) we first tested whether these constructs can be imported into mitochondria. We synthesized mtYidC-RBD in the presence of [³⁵S]methionine in reticulocyte lysate and incubated the radiolabeled protein with isolated mitochondria (Fig. 2B). During the incubation, the precursor form of mtYidC-RBD (Fig. 2B, *pre*) was converted into a proteolytically matured species (Fig. 2B, *m*). This processed form was inaccessible to added protease indicating its complete translocation across the outer membrane. When the outer membrane of the mitochondria was ruptured prior to the protease treatment, two prominent fragments of mtYidC-RBD of apparent masses of about 19 and 10 kDa were generated

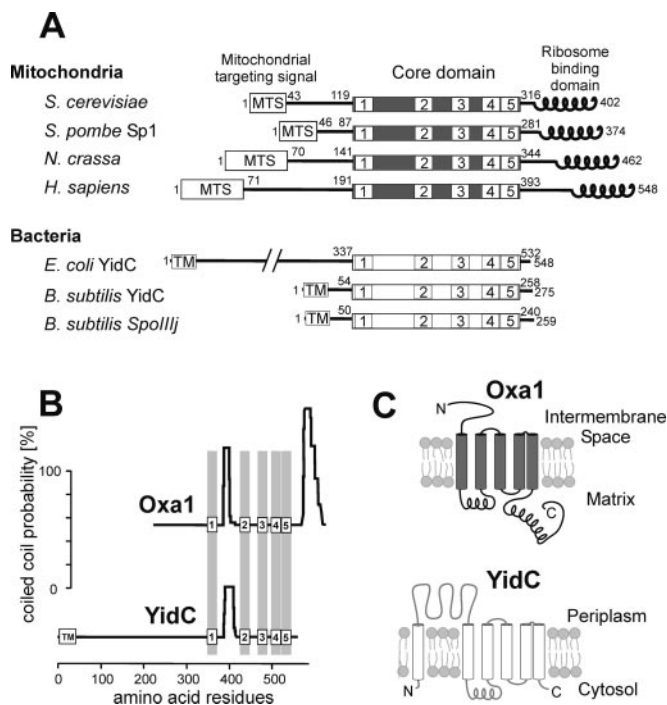


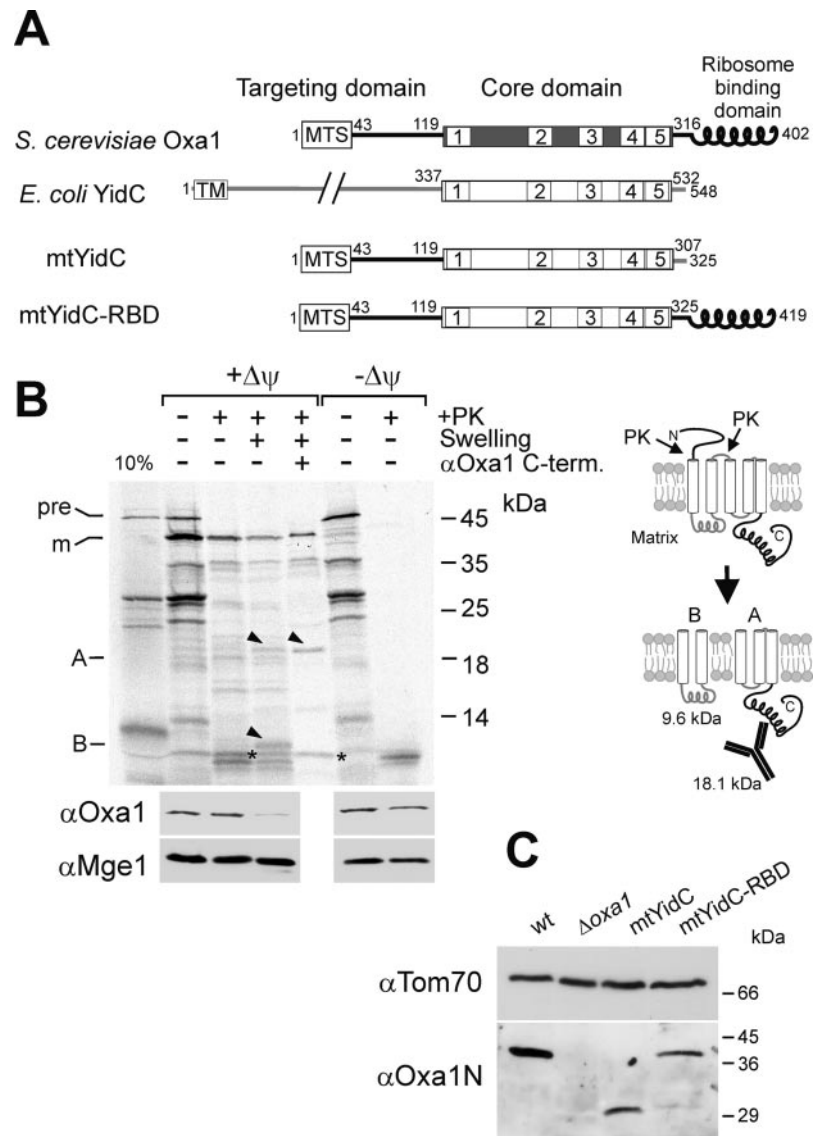
FIG. 1. Mitochondrial Oxa1 proteins contain C-terminal ribosome-binding domains that are absent in the bacterial homologues. A, schematic representation of mitochondrial Oxa1 proteins and bacterial YidC proteins for which experimental data were published. The positions of the five transmembrane spans in the conserved domain are indicated by boxed numbers. The mitochondrial targeting signals of Oxa1 homologues are indicated. The N-terminal transmembrane domains (TM) in the signal-anchor sequences of the bacterial YidC proteins are shown. The predicted C-terminal coiled coil structures in Oxa1 proteins, which are assumed to function as ribosome-binding domains, are presented as helices. The numbers refer to the amino acid residues in the respective sequences. *S. pombe*, *Schizosaccharomyces pombe*; *N. crassa*; *H. sapiens*, human; *B. subtilis*, *Bacillus subtilis*. B, coiled coil prediction (52) for Oxa1 of *S. cerevisiae* and YidC of *Escherichia coli*. The graph represents the coiled coil prediction using a window size of 14 residues, but the C-terminal coiled coil domain is also predicted with high propensity with window sizes of 21 and 28 residues (data not shown). The positions of the transmembrane domains are indicated and depicted as in A. C, model of the topologies of Oxa1 and YidC proteins. The regions for which coiled coil domains are predicted are shown as helices.

(Fig. 2B, black arrowheads). These masses matched the masses expected for the properly inserted mtYidC-RBD protein (Fig. 2B, model). The larger fragment could be isolated by immunoprecipitation with a C-terminal Oxa1 antibody verifying that the ribosome-binding domain of the construct was indeed correctly localized to the mitochondrial matrix. Thus, YidC can be imported into mitochondria despite its significant hydrophobicity.

Next we constructed plasmids for expression of mtYidC and mtYidC-RBD under control of the yeast *OXA1* promoter. These plasmids were transformed into a yeast mutant lacking the endogenous *OXA1* gene. Then we isolated mitochondria from these strains and assessed the steady state levels of the mitochondria-targeted YidC proteins by Western blotting using antibodies raised against the N terminus of Oxa1 (Fig. 2C). The mature Oxa1 protein has a calculated mass of 40 kDa but migrates at 36 kDa (41). This form was absent in the mtYidC and mtYidC-RBD mutant in which proteins of about 30 and 40 kDa, respectively, were detected with the N-terminal Oxa1 antibody. These molecular masses approximately match the expected molecular masses of mtYidC and mtYidC-RBD, which are 32 and 43 kDa, respectively. The steady state levels of both mutant forms were somewhat lower than that of endogenous Oxa1.

FIG. 2. The conserved core domain of YidC can be targeted to mitochondria.

A, schematic representation of Oxa1, YidC, and the two mitochondria-targeted YidC versions, mtYidC and mtYidC-RBD. The labeling is as in Fig. 1A. **B**, mtYidC-RBD can be imported into isolated mitochondria. mtYidC-RBD precursor was synthesized in the presence of [³⁵S]methionine and incubated with isolated wild type yeast mitochondria for 30 min at 25 °C. The sample was divided into four aliquots. One aliquot was mock-treated; the others were incubated with proteinase K (PK) either without or after hypotonic swelling of the mitochondria. From one aliquot, C-terminal protease fragments of mtYidC-RBD were isolated by immunoprecipitation using an antibody raised against the ribosome-binding domain of Oxa1 (see sketch on the right). For samples shown in the two right lanes ($-\Delta\psi$), 2 μ M valinomycin was added during the import reaction to deplete the membrane potential and to prevent protein import. Positions of molecular mass standards are shown on the right. Precursor and mature forms of mtYidC-RBD are labeled as *pre* and *m*, respectively. Arrowheads indicate proteolytic fragments of mtYidC-RBD (A and B, see sketch on the right). The asterisk depicts a C-terminal fragment derived from non-imported mtYidC-RBD precursor because this fragment also appears in the absence of a membrane potential. Western blotting using antibodies against the intermembrane space domain of Oxa1 and the matrix protein Mge1 served as a control for mitoplasting efficiency by swelling. A model of the predicted topology of mtYidC-RBD in the inner membrane is shown on the right. The expected proteolytic fragments and their predicted molecular masses are indicated. **C**, the expression of mtYidC and mtYidC-RBD was confirmed by Western blotting of extracts of wild type (wt), Δ oxa1, mtYidC, and mtYidC-RBD mitochondria. For detection of Oxa1 and the mitochondria-targeted versions of YidC, antibodies recognizing the N terminus of Oxa1 were used. Signals of the mitochondrial protein Tom70 are shown as loading control.



YidC Requires an Additional Ribosome-binding Domain to Complement an oxa1 Deletion Mutant—To assess whether the fusion proteins can take over the function of yeast Oxa1, we tested the ability of the strains to grow on the non-fermentable carbon source glycerol (Fig. 3A). In contrast to wild type cells, *oxa1* deletion strains are unable to grow under these conditions because of their lack of a functional respiratory chain (Fig. 3A, first and second rows). This respiration-negative phenotype was not reversed upon expression of mtYidC, and hence the bacterial YidC protein was unable to take over the function of Oxa1. This observed inability of mtYidC to complement the Oxa1-deficient strain was not caused by the lower amounts of mtYidC compared with Oxa1 because overexpression of mtYidC did likewise not suppress the respiration-deficient phenotype of *OXA1* deletion mutants (data not shown). Notably, the addition of the ribosome-binding domain of Oxa1 onto the mtYidC protein partially restored the growth on glycerol. We conclude that YidC is able to take over the function of Oxa1 in protein insertion but only when equipped with the C terminus of Oxa1.

This complementation of *oxa1* deletion mutants by mtYidC-RBD is further supported by the enzyme activities of the respiratory chain complexes found in the various strains (Fig. 3B). The deletion of *OXA1* severely reduces the activities of the *bc*₁

complex and the F₀F₁-ATPase and causes the completely absence of cytochrome oxidase (22, 43). Whereas the expression of mtYidC did not considerably mitigate this defect, the presence of mtYidC-RBD either completely restored (*bc*₁ complex) or partially restored (cytochrome oxidase, ATPase) the activities of the respiratory chain enzymes. In consistence, expression of mtYidC-RBD restored the steady state levels of subunits of these three respiratory chain complexes, which are destabilized in the absence of Oxa1 (Fig. 3C). Even subunit 2 of the cytochrome oxidase (Cox2), a strictly Oxa1-dependent inner membrane protein, was partially regained by expression of mtYidC-RBD; however, it did not reach wild type levels.

To assess the biogenesis of the cytochrome oxidase in more detail, mitochondrial translation products were radiolabeled in isolated mitochondria, and the Cox2 protein synthesized was isolated by immunoprecipitation (Fig. 3D). In wild type mitochondria, Cox2 accumulates in its mature form indicating that its N terminus was translocated across the inner membrane and processed by the intermembrane space protease Imp1 (Fig. 3D). In contrast, in mitochondria lacking Oxa1, Cox2 accumulated in the matrix and failed to be matured. Whereas expression of mtYidC did not significantly cure the defect of the *oxa1* deletion mutant, expression of mtYidC-RBD resulted in a partial processing of Cox2. In summary, we conclude that the

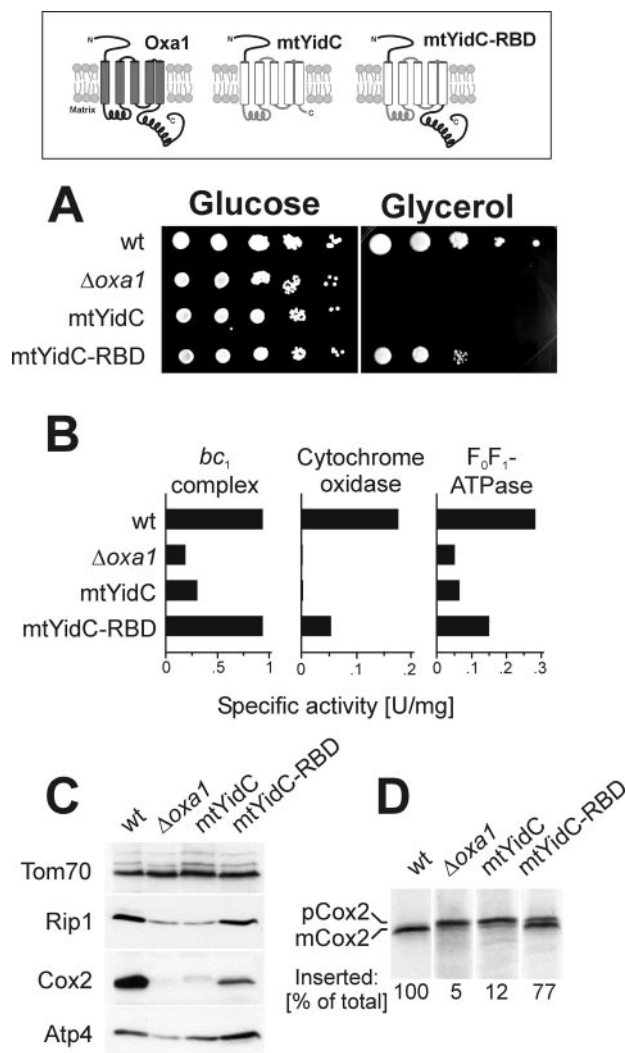


FIG. 3. The expression of mtYidC-RBD partially complements an *oxa1* deletion mutant. The topologies of Oxa1, mtYidC, and mtYidC-RBD are depicted in the *scheme*. **A**, yeast wild type cells (wt) and $\Delta oxa1$ mutants carrying either an empty pRS426 plasmid (–) or expression constructs for mtYidC and mtYidC-RBD were grown to log phase. 10-fold serial dilutions of the cultures were spotted on YP plates (1% yeast extract, 2% peptone, 1% agar) containing 2% glucose or 2% glycerol as indicated. The plates were incubated at 30 °C for 2 days (glucose) or 3 days (glycerol). **B**, the enzymatic activities of the bc_1 complex (complex III), the cytochrome oxidase complex (complex IV), and the F_0F_1 -ATPase were measured in the strains indicated. **C**, endogenous steady state levels of the Rieske iron sulfur protein (Rip1, a subunit of the bc_1 complex), Cox2, and Atp4 (a subunit of the hydrophobic F_0 sector of the ATPase) were assessed by Western blotting of mitochondrial extracts (50 μ g) of the strains indicated. The levels of Tom70 are shown for control. **D**, translation products were radiolabeled in mitochondria isolated from strains indicated for 20 min at 25 °C. The Cox2 protein synthesized was isolated by immunoprecipitation, resolved by SDS-PAGE, and visualized by autoradiography. The percentage of matured Cox2 was calculated following quantification and correction for the respective methionine content of the two Cox2 species. *pCox2*, precursor form of Cox2; *mCox2*, proteolytically matured form of Cox2.

C-terminal ribosome-binding domain appears to be functionally transplantable from Oxa1 to mtYidC. Once equipped with this domain, the mtYidC can catalyze the insertion of mitochondrially encoded proteins, although not with the same efficiency as Oxa1.

The Ribosome-binding Domain Is Required for Contact of mtYidC to Mitochondrial Translation Products—The inability of the RBD-less version of mtYidC to promote membrane integration of newly synthesized Cox2 suggests that mtYidC on its

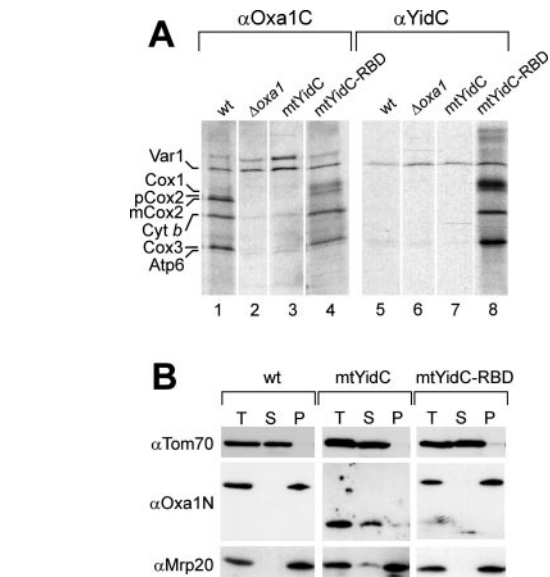


FIG. 4. mtYidC requires the ribosome-binding domain to contact mitochondrial translation products and ribosomes. **A**, cross-linking of mitochondrial translation products. Mitochondrial translation products were radiolabeled for 15 min and treated with 200 μ M of the cross-linking agent dithiobis(succinimidyl propionate) for 30 min. Cross-linking and translation was stopped by the addition of glycine and unlabeled methionine. The mitochondria were reisolated, washed, and incubated in 1% SDS for 30 s at 96 °C. The extract was centrifuged for 5 min at 15,000 \times g and used for immunoprecipitation with antisera against the C terminus of Oxa1 and YidC as indicated. The names of the proteins synthesized are indicated on the *left*. Var1 is unspecifically isolated because of its affinity to Sepharose (44). **B**, mtYidC-RBD co-sediments with ribosomes. Mitochondria of the strains indicated were lysed with 1% Triton X-100. The resulting extract was cleared by centrifugation and either directly applied to SDS-PAGE (*T*, total) or fractionated by centrifugation through a high density sucrose cushion. Proteins from the resulting supernatant (*S*) and the ribosome-containing pellet (*P*) fractions were subjected to SDS-PAGE and blotted on nitrocellulose. The membranes were probed with antibodies against the membrane protein Tom70, the ribosomal protein Mrp20, and the N terminus of Oxa1.

own fails to recruit mitochondrial ribosomes and therefore does not get into proximity to the nascent chains. To test whether mtYidC is in contact with mitochondrial translation products, we employed a cross-linking approach. Mitochondrial translation products were radiolabeled in isolated mitochondria in the presence of the cleavable chemical cross-linking reagent dithiobis(succinimidyl propionate) to introduce covalent bonds between nascent chains and proteins in their direct proximity. Then mitochondria were lysed under denaturing conditions, and the extract was immunoprecipitated with antibodies raised against the C terminus of Oxa1 or against YidC (Fig. 4A). Before applying the samples onto SDS-PAGE, the cross-links were cleaved. In wild type and mtYidC-RBD mitochondria, the mitochondrially encoded proteins Cox1, Cox2, Cox3, and cytochrome *b* were found in the immunoprecipitates (Fig. 4A, lanes 1 and 4). These proteins were absent in the samples isolated from Oxa1-deficient mitochondria as from mtYidC mitochondria because both strains lack the C-terminal Oxa1 epitope. The Var1 protein, a ribosomal subunit encoded by the mitochondrial genome, was pulled down unspecifically in all samples because of its ability to bind to Sepharose (44). Upon immunoprecipitation with a YidC-specific antiserum, Cox1, Cox2, Cox3, and cytochrome *b* were isolated from mtYidC-RBD mitochondria (Fig. 4A, lane 8) but not when mtYidC was expressed without the ribosome-binding domain. This indicates that the ribosome-binding domain of Oxa1 is required to allow contact of mtYidC to mitochondrial translation products.

To directly assess an interaction of mtYidC with mitochon-

drial ribosomes, we isolated ribosomal fractions by centrifugation of mitochondrial extracts through high density sucrose cushions and analyzed the co-sedimentation of Oxa1, mtYidC, and mtYidC-RBD by Western blotting (Fig. 4B). In wild type mitochondria, we found all the endogenous Oxa1 pool in the ribosomal pellet fraction reflecting the tight physical interaction of the Oxa1 complex with the translation machinery (Fig. 4B, second lane). Other membrane proteins that were used for control, like Tom70, were exclusively found in the ribosome-free supernatant. In mtYidC mitochondria, the mitochondria-targeted YidC protein remained entirely in the supernatant, indicating that (in contrast to its mitochondrial Oxa1 homologue) the bacterial YidC protein does not bind mitochondrial ribosomes with detectable affinity. In contrast, when provided with the ribosome-binding domain of Oxa1, the mitochondria-targeted mtYidC proteins were found in the ribosomal fraction. This suggests that YidC on its own is unable to interact both functionally and physically with mitochondrial ribosomes; however, when equipped with the ribosome-binding domain of Oxa1, a coordinated synthesis and membrane insertion of translation products is restored in mitochondria.

YidC Cannot Efficiently Catalyze the Insertion of Nuclear Encoded Inner Membrane Proteins—In bacteria, membrane insertion of subunit *c* of the ATPase is catalyzed by YidC in a strictly co-translational process, and YidC is not able to insert completely synthesized subunit *c* (13, 14). In mitochondria of animals and many fungi, the homologue of the bacterial subunit *c*, Atp9, is nuclear encoded. Following import into the mitochondrial matrix, this Atp9 protein is integrated into the inner membrane in a reaction that is strongly stimulated by Oxa1 (29, 45). Hence, Oxa1 catalyzes the insertion of these nuclear encoded proteins in a post-translational reaction. The mtYidC mutants allowed us now to test whether YidC when targeted to mitochondria is able to catalyze the membrane insertion of a nuclear encoded subunit *c* homologue. To this end, we performed an *in vitro* import experiment using the radiolabeled fusion protein preSu9(1–112)-DHFR, which consists of the N-terminal 112 amino acid residues of the precursor form of Atp9 of *Neurospora crassa* fused to mouse dihydrofolate reductase (45). When incubated with wild type mitochondria, the presequence of this protein is proteolytically removed. This species then inserts into the inner membrane, exposing the N terminus into the intermembrane space. Upon selective opening of the outer membrane by hypotonic swelling, protease treatment generates a typical fragment (Fig. 5A, frag.). Thereby the matrix-localized protein-inaccessible mature fraction (Fig. 5A, *m*) can be experimentally distinguished from the membrane-inserted protein-accessible fraction of Atp9. Whereas the fusion protein inserts efficiently into the inner membrane of wild type mitochondria, the membrane integration is strongly diminished in the absence of Oxa1. When the fusion protein is imported into mtYidC and mtYidC-RBD mitochondria, it still largely accumulated in the matrix and failed to be inserted into the inner membrane. This indicates that YidC cannot efficiently take over the ability of Oxa1 in membrane integration of Atp9.

Next we tested membrane insertion of newly imported Oxa1, which is also significantly stimulated in the presence of Oxa1 (Fig. 5B). The expression of mtYidC and mtYidC-RBD slightly enhanced the insertion of this protein. It can, however, not be completely excluded that this improved insertion is caused by a slightly increased membrane potential in these mutants. Thus, the mitochondria-targeted YidC protein was unable to catalyze the efficient membrane integration of at least the two nuclear encoded Oxa1 substrates that we analyzed.

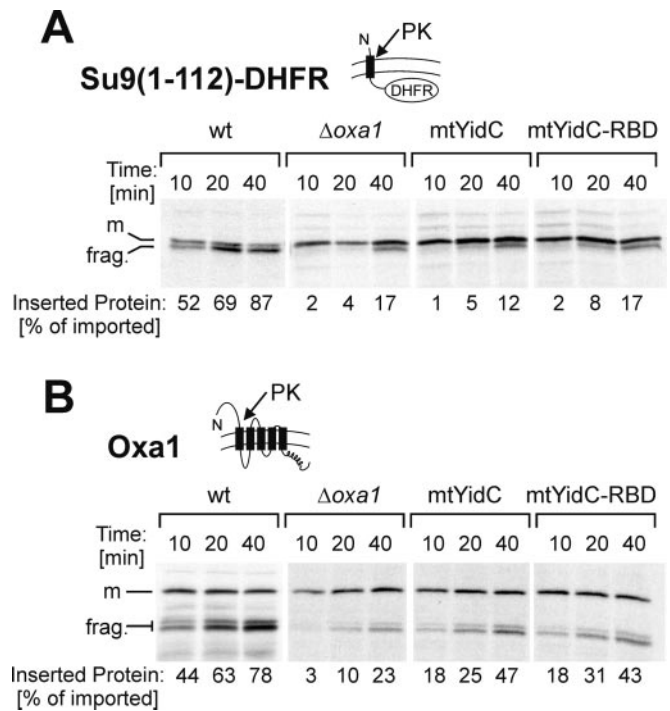


FIG. 5. The expression of mtYidC and mtYidC-RBD does not restore the insertion of nuclear encoded proteins. A, radiolabeled preSu9(1–112)-DHFR precursor was imported for the times indicated into 100 μ g mitochondria of wild type (wt) or Δ oxa1 cells lacking or expressing mtYidC or mtYidC-RBD. The mitochondria were reisolated, and the outer membrane was ruptured by hypotonic swelling. Then 50 μ g/ml proteinase K was added to the samples. After 30 min on ice, the mitochondria were reisolated, and proteins were resolved on SDS-PAGE and detected by autoradiography. The ratio of protease accessible, i.e. inserted, to total imported protein was quantified by densitometry. The numbers were corrected for the methionine content of the proteins. Efficient swelling of the mitochondria was controlled by immunoblotting. frag., protease fragment of the inserted protein; m, matrix-localized protein-inaccessible mature fraction. The topology of the correctly inserted Oxa1 protein is depicted. B, the insertion of preOxa1 precursor was analyzed as described in A.

The Second Mitochondrial Oxa1 Homologue, Cox18/Oxa2, Can Be Functionally Replaced by YidC—The inner membrane of mitochondria harbors besides Oxa1 a second member of the Oxa1/YidC protein family called Cox18 or Oxa2 (30–32). Cox18/Oxa2 proteins lack C-terminal ribosome-binding domains and are specifically required for the biogenesis of the cytochrome oxidase complex. We tested whether mitochondria-targeted YidC can take over the function of Cox18/Oxa2. Expression of mtYidC in a COX18 deletion mutant partially restored the ability to grow on non-fermentable carbon sources indicating that YidC indeed can fulfill the function of Cox18 to some degree (Fig. 6A). Astonishingly, the fusion of the ribosome-binding domain onto mtYidC did not complement the cox18 deletion mutant suggesting that the contact of mtYidC to the mitochondrial translation machinery compromised its ability to take over the function of Cox18/Oxa2. Thus, whereas the presence of the ribosome-binding domain was essential for complementation of an oxa1 mutant, it prevented the complementation of a cox18 deletion strain.

Cox18/Oxa2 is required for the stable assembly of Cox2 into the cytochrome oxidase complex. The deletion of COX18 leads to the destabilization of newly synthesized Cox2 protein, whereas other mitochondrial translation products like cytochrome *b* are not affected (Fig. 6B). The expression of mtYidC in COX18-deficient cells again restored the accumulation of newly synthesized Cox2 indicating that mtYidC can take over the role of Cox18/Oxa2 in stabilization of Cox2.

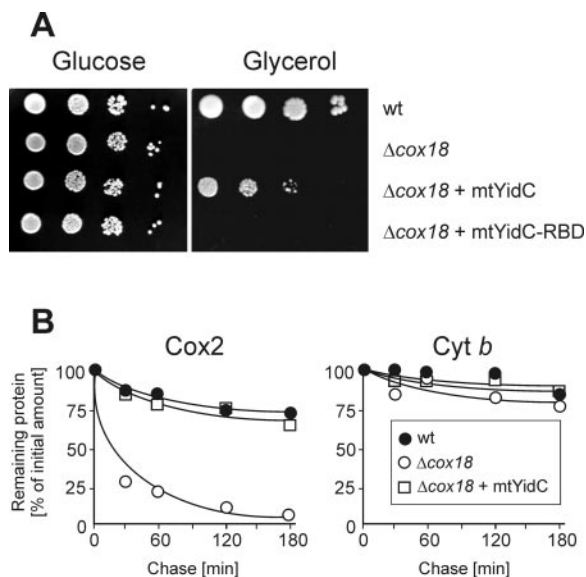


FIG. 6. The expression of mtYidC partially complements a *cox18* deletion mutant. *A*, the ability to grow on fermentable (glucose) and non-fermentable (glycerol) medium of wild type cells (wt) and of *cox18* deletion mutants lacking (Δcox18) or carrying plasmids expressing the constructs for mtYidC and mtYidC-RBD was assessed as described for Fig. 3*A*. *B*, the strains indicated were grown and treated as described under “Experimental Procedures” to specifically label mitochondrial encoded proteins. After stopping the reaction, samples were taken after the indicated times of chase at 30 °C. Proteins were separated on a 17.5% gel. The radioactivity associated with Cox2 (*left*) and cytochrome *b* (*Cyt b*, *right*) was quantified. The results are normalized to the values obtained at time 0 of the chase.

DISCUSSION

Oxa1/YidC proteins form a large family of membrane proteins that are involved in the biogenesis of membrane-embedded protein complexes in bacteria, mitochondria, and chloroplasts. On primary sequence level the members of this protein family show only low conservation with sequence identities that mostly do not exceed 20%. Despite this limited structural conservation, complementation studies revealed a functional exchangeability of different mitochondrial Oxa1 homologues (46–48), of the Alb3 protein of plastids with YidC in bacteria (40), and of the chloroplast inner envelope protein ARTEMIS with yeast Oxa1 (49). In this study, we now show that the bacterial YidC protein can partially take over functions of its mitochondrial homologues Oxa1 and Cox18. For complementation of Oxa1, however, a C-terminal ribosome-binding domain has to be appended to YidC to allow its physical and functional interaction with the mitochondrial translation apparatus. This functional conservation is surprising given the significant differences of the bacterial and mitochondrial systems. In bacteria, YidC is unable to catalyze membrane integration of most proteins without the activity of the Sec machinery (10, 50, 51). Mitochondria on the other hand lack a Sec machinery, and thus Oxa1 generally has to operate in a Sec-independent manner.

The expression of YidC restored the respiration competence of *oxa1* and *cox18* deletion mutants only to some extent, and both complemented mutants still showed reduced growth rates on glycerol. This suggests that Oxa1 and Cox18/Oxa2 are not simply mitochondrial YidC isoforms that contain or lack a ribosome-binding domain but are adapted to the specific situation in mitochondria. For example, Oxa1 and mtYidC showed considerable differences in their substrate specificity: Whereas mtYidC-RBD catalyzed the membrane integration of the mitochondrially encoded protein Cox2 (although with reduced efficiency) the insertion of nuclear encoded Oxa1 substrates was hardly stimulated upon expression of mtYidC. Interestingly,

experiments with reconstituted YidC revealed that YidC can only promote a co-translational insertion of subunit *c* of the ATPase, the bacterial homologue of Atp9 (13). Thus, YidC and Oxa1 apparently differ in their ability to mediate the post-translational insertion of this nuclear encoded protein. The transfer of genes from the mitochondrial to the nuclear genome during evolution might have required specific adaptations of Oxa1 to optimize its ability to promote the post-translational insertion of conservatively sorted inner membrane proteins in mitochondria. This idea is further supported by the observations in the accompanying study by van Bloois *et al.* (53). Oxa1, when targeted to the bacterial inner membrane, is able to functionally replace YidC in bacteria. However, whereas Oxa1 promotes the insertion of Sec-independent membrane proteins, it does not properly cooperate with the bacterial Sec machinery. This again indicates that Oxa1 and YidC still exhibit a common central function, but Oxa1 apparently lost prokaryote-specific features during evolution that were not required in the mitochondrial context.

Despite the wide exchangeability among members of the Oxa1/YidC family, mitochondria consistently contain two homologues, Oxa1 and Cox18/Oxa2 (32). The phylogenetic analysis of Oxa1/YidC proteins revealed an early duplication of the Oxa1 and Cox18/Oxa2 subbranches, both of which were maintained in plants, fungi, and animals (32). In yeast, Oxa1 and Cox18/Oxa2 do not complement each other indicating that both proteins are of distinct function (30). Here we showed that YidC, however, can complement to some degree both *oxa1* and *cox18* mutants suggesting that both mitochondrial factors are in principle of similar catalytic activity. One major difference between both components appears rather to reside in whether they contain or lack a ribosome-binding domain. To fulfill its role in the insertion of mitochondrial translation products Oxa1 has to interact with ribosomes (26, 27). Surprisingly, presence of the ribosome-binding domain interfered with a Cox18/Oxa2-like activity of YidC. This indicates that the fusion of the ribosome-binding domain to YidC does not only add a novel capacity to the protein but at the same time has negative effects on a function that the non-fused YidC had. Cox18/Oxa2 was shown to interact with Cox2 and Cox3 following their synthesis in mitochondria (32). Our results suggest that Cox18/Oxa2 might not only be able to deal with completely synthesized substrates but plays an indispensable post-translational role. This post-translational function of Cox18/Oxa2 might be the export of the C terminus of Cox2 after complete synthesis of this protein (30) or/and the assembly of fully synthesized Cox2 with other subunits of cytochrome oxidase complex.

Our observations suggest that during evolution of mitochondria a gene duplication event allowed the specialization of Oxa proteins. One homologue, Oxa1, acquired a ribosome-binding domain to facilitate its co-translational interaction with mitochondrial translation products. The other homologue, Cox18/Oxa2, however, still remained important for a post-translational step in the biogenesis of cytochrome oxidase. Because both the co-translational Oxa1 function and the post-translational Cox18/Oxa2 function are important for the biogenesis of respiratory chain complexes both homologues had to be conserved throughout evolution explaining why they are consistently found in mitochondria of plants, fungi, and animals.

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