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The Oxa2 Protein of *Neurospora crassa* Plays a Critical Role in the Biogenesis of Cytochrome Oxidase and Defines a Ubiquitous Subbranch of the Oxa1/YidC/Alb3 Protein Family[D]

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Proteins of the Oxa1/YidC/Alb3 family mediate the insertion of proteins into membranes of mitochondria, bacteria, and chloroplasts. Here we report the identification of a second gene of the Oxa1/YidC/Alb3 family in the genome of *Neurospora crassa*, which we have named *oxa2*. Its gene product, Oxa2, is located in the inner membrane of mitochondria. Deletion of the *oxa2* gene caused a specific defect in the biogenesis of cytochrome oxidase and resulted in induction of the alternative oxidase (AOD), which bypasses the need for complex IV of the respiratory chain. The Oxa2 protein of *N. crassa* complements Cox18-deficient yeast mutants suggesting a common function for both proteins. The *oxa2* sequence allowed the identification of a new subfamily of Oxa1/YidC/Alb3 proteins whose members appear to be ubiquitously present in mitochondria of fungi, plants, and animals including humans.

INTRODUCTION

Mitochondria are essential organelles that harbor some 10–20% of the proteins present in an eukaryotic cell (Kumar *et al.*, 2002). The vast majority of these proteins are encoded in the nuclear genome and synthesized in the cytosol as preproteins. These preproteins contain targeting information that is recognized by translocation complexes in the mitochondrial membranes and allows their import into mitochondria and sorting to the correct subcompartment. To date, four general translocation complexes have been identified (for review see Pfanner and Geissler, 2001; Hoogenraad *et al.*, 2002; Neupert and Brunner, 2002). The TOM complex (translocase of the outer membrane) serves as a general entry gate that recognizes preproteins on the mitochondrial surface and allows their transport across the outer membrane. Two TIM complexes (translocases of the inner membrane) mediate either insertion of preproteins into the inner membrane or the translocation of matrix proteins across the membrane. Finally, the Oxa1 translocase, which also resides in the inner membrane, is required for the insertion of proteins from the matrix into the lipid bilayer of the inner membrane.

The TOM and TIM translocases most likely evolved during the establishment of the endosymbiotic relationship of mitochondria and the host eukaryotic cell and do not have identifiable homologues in bacteria. On the other hand, Oxa1 belongs to a large protein family with members in bacteria and chloroplasts, often referred to as the Oxa1/YidC/Alb3 family (Luirink *et al.*, 2001; Kuhn *et al.*, 2003). The Oxa1 protein of the yeast *Saccharomyces cerevisiae* was the founding member of the family, originally identified as an essential factor for the biogenesis of respiratory chain complexes (Bauer *et al.*, 1994; Bonnefoy *et al.*, 1994a). In the absence of Oxa1, no cytochrome oxidase activity is found in mitochondria and the levels of the cytochrome bc_1 complex and the ATP synthase are drastically reduced. Oxa1 is required for the insertion of a number of mitochondrially encoded proteins into the inner membrane as well as for the integration of some nuclear encoded membrane proteins that reach the inner membrane on a conservative sorting pathway via a sorting intermediate in the matrix (He and Fox, 1997; Hell *et al.*, 1997, 1998, 2001; Herrmann *et al.*, 1997). Similarly, the Oxa1 homologue of *Escherichia coli*, YidC, plays an important role in the insertion of a number of inner membrane proteins and in the biogenesis of enzyme complexes of the respiratory chain (Samuelson *et al.*, 2000; Scotti *et al.*, 2000; van der Laan *et al.*, 2003), whereas the *Arabidopsis* homologue Alb3 is essential for the insertion of proteins into the thylakoid membrane of chloroplasts (Moore *et al.*, 2000). YidC and Alb3 appear to be functionally conserved as the plastid protein can complement an *E. coli* strain lacking YidC (Jiang *et al.*, 2002). Likewise the mitochondrial Oxa1 proteins are functionally interchangeable since the homologues of humans, plants, *Neurospora crassa*, and *Schizosaccharomyces pombe* were shown to complement yeast Δ*oxa1* mutants (Bonnefoy *et al.*, 1994b, 2000; Hamel *et al.*, 1997; Nargang *et al.*, 2002).

Oxa1 function is essential for the correct insertion of Cox2, one of the core subunits of cytochrome oxidase. Cox2 is a mitochondrially encoded protein containing two transmem-

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brane domains. Both its N- and C-termini have to be translocated across the inner membrane into the intermembrane space via mechanisms that require Oxa1 function. In two recent studies, an additional inner membrane component, Cox18, was described that also plays a crucial role in the biogenesis of the yeast cytochrome oxidase complex (Souza *et al.*, 2000; Saracco and Fox, 2002). Cox18 may be involved in the topogenesis of the C-terminal domain of Cox2 because, in the absence of Cox18, an HA epitope tag fused to the C terminus of Cox2 remains protease-inaccessible after opening of the outer membrane, whereas in wild-type mitochondria the epitope was degraded (Saracco and Fox, 2002). Cox18 shows low sequence similarity to some members of the Oxa1/YidC/Alb3 family, but because *COX18* homologues were only found in the genomes of *S. cerevisiae*, *Candida albicans* and *Kluyeromyces lactis* an evolutionary relation to other Oxa1 proteins remained unclear (Hikkel *et al.*, 1997; Saracco and Fox, 2002).

Here we report the identification of the Oxa2 protein of *N. crassa*, a novel member of the Oxa1/YidC/Alb3 protein family. The sequence of this protein allowed the identification of a new ubiquitous subbranch of this protein family, which includes Cox18 and appears to play a critical role in the biogenesis of the cytochrome oxidase complex.

MATERIALS AND METHODS

Recombinant DNA Techniques and Plasmid Constructions

Standard methods were used for DNA manipulations (Sambrook *et al.*, 1989). The *oxa2* gene was obtained by amplification of genomic DNA of *N. crassa* and subcloned into pGEM2 (Promega, Madison, WI) for in vitro transcription/translation and in pYX142 (Novagen, Madison, WI) for expression in yeast.

Strains and Growth Conditions

Growth and handling of *N. crassa* were as described (Davis and De Serres, 1970). The starting *N. crassa* strains used in this study were HII (*thr-3, arg-12^S, pyr-3, inl, inv, mei-2 a*) and 74*A* (74-OR23-IV*A*). Strain oxa2hyg-39 (*thr-3, arg-12^S, pyr-3, inl, inv, mei-2 a*) was derived by transformation of HII with a pBR322 derivative containing the hygromycin resistance gene from plasmid pCSN43 (Staben *et al.*, 1989) and a 2.3-kb region containing the *oxa2* gene and flanking regions that was produced by PCR of *N. crassa* genomic DNA. The strain was examined by Southern analysis to confirm the existence of a single ectopic copy of the *oxa2* region. Thus, oxa2hyg-39 contains the duplication substrate for RIP mutagenesis (Selker, 1990). The oxa2^{RIP}-35 strain was a single ascospore isolated from a cross of 74*A* × oxa2hyg-39 and may contain any of the mutant genes from oxa2hyg-39. The strain was grown in media containing threonine, uridine, inositol, and glucose. The presence of RIP generated mutations in the *oxa2* regions of the oxa2^{RIP}-35 strain was confirmed by DNA sequencing of PCR generated *oxa2* specific products.

S. cerevisiae strains were isogenic to the wild-type strain W303a. For construction of Δ*cox18* and Δ*oxa1* mutant strains, the *COX18* and *OXA1* genes were deleted individually by replacement by *HIS3* gene cassettes. Yeast cultures were grown at 30°C YP medium supplemented with 2% glucose, glycerol, or galactose or on lactate medium (Herrmann *et al.*, 1994). Mitochondria were isolated as previously described for yeast (Herrmann *et al.*, 1994) and *N. crassa* (Pfanner and Neupert, 1985).

In Vitro Protein Import and Mitochondrial Subfractionation

Import into isolated mitochondria of in vitro–synthesized proteins was according to published procedures (Herrmann *et al.*, 1997). Standard import reactions were carried out in import buffer (600 mM sorbitol, 50 mM KCl, 10 mM $MgCl_2$, 2.5 mM EDTA, 2 mM potassium phosphate, 1 mg/ml bovine serum albumin, 50 mM HEPES, pH 7.4) in the presence of 2 mM NADH and 2 mM ATP. Mitochondria were converted to mitoplasts by 10-fold dilution in ice-cold 20 mM HEPES, pH 7.4, which leads to swelling of the mitochondria and rupture of the outer membrane. Protease treatment was generally performed by addition of 50 μg/ml proteinase K to the reaction and incubation for 30 min at 0°C. The efficiency of swelling and proteinase K treatment were monitored by Western blotting using Tom70, cytochrome b_2, and Mge1 as marker proteins representing the outer membrane, the intermembrane space, and the matrix, respectively.

For isolation of the Oxa1 complex, 1 mg of mitochondria isolated from strain K5-15-23-1, which expresses an hexahistidinyl-tagged version of Oxa1, were lysed in 2% dodecyl maltoside, 100 mM NaCl, 20 mM Tris, pH 8. The extract was cleared by centrifugation (8 min, 16,000 rpm, 4°C) and incubated with Ni-NTA agarose. The resin was washed and bound proteins were subjected to SDS-PAGE.

Sequence Analysis

Mitochondrial Oxa1 and Oxa2 proteins were identified by BLAST searches of the NCBI database. Phylogenetic analysis was performed using PAUP* 4.0 b10 for Macintosh and UNIX (Swofford, 1988). *Bacillus subtilis* sequences were specified as the outgroup. The sequences were aligned using ClustalX (Thompson *et al.*, 1997), with subsequent manual refinements. Robustness of internal branches was conducted through 1000 bootstrap replicates with tree bisection-reconnection branch swapping.

Miscellaneous

Antisera against the C terminus of Oxa2 were raised in rabbits by injecting the chemically synthesized peptide CGLDWEPNHTAVKK representing amino acid residues 418 through 430 coupled to keyhole limpet hemocyanin. The sera were affinity purified using the immobilized peptide as a matrix. Mitochondrial translation products were radiolabeled according to published procedures (Westermann *et al.*, 2001). Immunoprecipitation was carried out as described (Herrmann *et al.*, 2001). Enzymatic measurement of the cytochrome oxidase activity was performed essentially as described before; for these measurements, the amounts of mitochondria in the preparations were normalized by their malate dehydrogenase activities (Hell *et al.*, 2000). Cytochrome spectra (Bertrand and Pittenger, 1969) and respiration measurements (Tanton *et al.*, 2003) were determined as described.

RESULTS

The Genome of N. crassa Encodes a Second Oxa1 Homologue Present in Mitochondria

The completely sequenced genome of *N. crassa* contains two open reading frames encoding putative proteins with homology to members of the Oxa1/YidC/Alb3 family. One of these genes, *oxa1*, produces the essential 43-kDa Oxa1 protein, which is an orthologue of the Oxa1 protein of yeast (Nargang *et al.*, 2002). The second gene (accession number XP_327064), which we named *oxa2*, encodes a product of 48.1 kDa. The protein sequence contains a hydrophobic central domain that shows sequence identities of 25.7 and 24.5% to the core domains of Oxa1 proteins of *S. cerevisiae* and *Homo sapiens*, respectively (Figure 1A). Prediction of transmembrane domains revealed five hydrophobic stretches that are at positions identical to the five transmembrane segments generally present in members of the Oxa1/YidC/Alb3 family (underlined regions in Figure 1A). The protein sequence contains a classical N-terminal mitochondrial presequence of 57 amino acid residues (Claros and Vincens, 1996), suggesting that the mature Oxa2 of *N. crassa* is a mitochondrial protein of 42.2 kDa.

To assess the size of the *oxa2* gene product we expressed the Oxa2 protein in an in vitro transcription/translation system in reticulocyte lysate. This resulted in a radiolabeled protein of an apparent size of 48 kDa (Figure 1B, lane 1). To detect the size of the endogenous Oxa2 protein, a C-terminal peptide of the Oxa2 protein sequence was used to develop antisera that recognized a protein in *N. crassa* mitochondria of an apparent size of 43 kDa on immunoblots (Figure 1B, lane 2), consistent with the calculated size of the mature Oxa2 protein.

Subfractionation of cellular constituents confirmed that Oxa2 was enriched in mitochondrial preparations and absent in both the cytosolic fraction and a postmitochondrial high-speed pellet (Figure 1C). Thus, Oxa2 is a second Oxa1/YidC/Alb3 family member present in mitochondria of *N. crassa*.

To assess whether Oxa2 might be associated with Oxa1 in the inner membrane, we isolated Oxa1 from a *N. crassa* strain that expresses Oxa1 with a C-terminal hexahistidinyl tag (Oxa1^{His}, Nargang *et al.*, 2002). Oxa2 was not copurified with Oxa1^{His} after lysis with the mild detergent dodecyl malto-

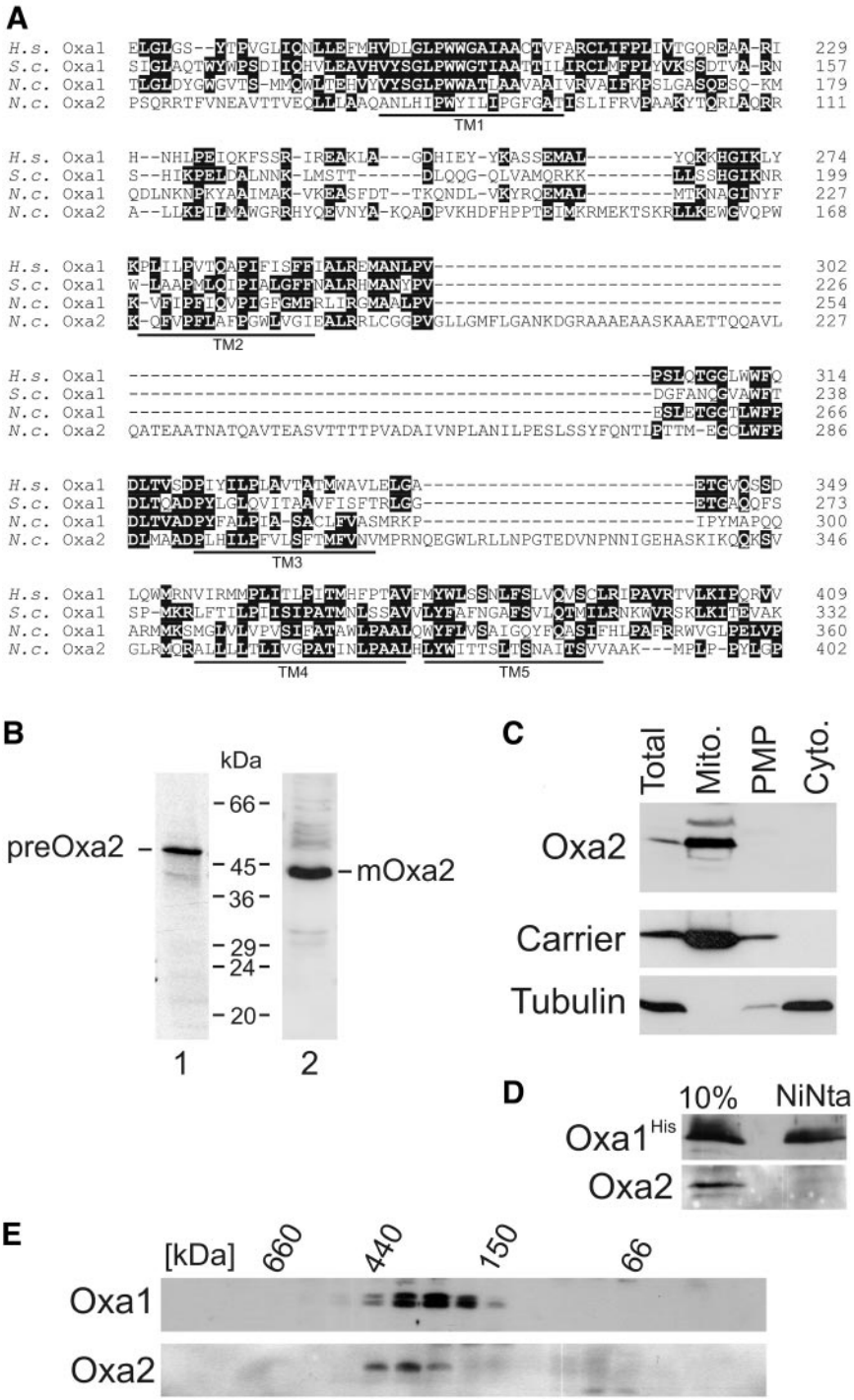

Figure 1. *N. crassa* mitochondria harbor a second Oxa1 homologue. (A) Alignment of the conserved core domains of Oxa2 from *N. crassa* and Oxa1 sequences from *N. crassa*, *S. cerevisiae,* and *H. sapiens*. Solid bars under the sequence indicate predicted membrane spanning domains. Shading indicates residues identical in at least two of the proteins. (B) The *oxa2* gene product is found in *N. crassa* mitochondria. Radiolabeled Oxa2 was synthesized in reticulocyte lysate, subjected to SDS-PAGE and autoradiographed (lane 1). Lane 2 shows a Western blot of isolated *N. crassa* mitochondrial immunodecorated with Oxa2-specific antibodies. The precursor and mature forms of Oxa2 are depicted as preOxa2 and mOxa2, respectively. (C) Oxa2 is a mitochondrial protein. The distribution of Oxa2 in *N. crassa* cells was analyzed by Western blotting of the subcellular fractions: total; mitochondria (Mito.); postmitochondrial membrane pellet (PMP) and cytosol (Cyto.). Mitochondrial ATP/ADP carrier and the cytosolic tubulin proteins are shown as controls. (D) Oxa2 does not copurify with the Oxa1 complex. Mitochondria containing a hexahistidinyl-tagged version of Oxa1 ($Oxa1^{His}$) were lysed with dodecyl maltoside. $Oxa1^{His}$ was purified by affinity chromatography on NiNTA and the presence of Oxa2 in the $Oxa1^{His}$ fraction was assessed by Western blotting. The left lane shows 10% of the extract used for the purification. (E) Oxa2 is part of a high-molecular-weight complex. Mitochondrial protein was fractionated on a Superose 6 column as described (Nargang *et al.,* 2002) and the migration of Oxa1 and Oxa2 was determined by Western blotting. The positions of molecular-weight standards are indicated.

side (Figure 1D). Oxa1 forms a homooligomeric complex in the inner membrane, which fractionates at ~200–300 kDa after lysis with dodecyl maltoside (Nargang *et al.,* 2002). Similarly, Oxa2 migrated in a high-molecular-weight complex slightly larger than the Oxa1 complex (Figure 1E). Monomeric Oxa2 was not found under these conditions. From these data we conclude that Oxa2 is part of a high-molecular-weight complex in the inner membrane that is distinct from the Oxa1 complex. A transient or weak interaction between both complexes, however, cannot be excluded.

Oxa2 Is Required for the Biogenesis of Cytochrome Oxidase

To examine the phenotype of mutants lacking Oxa2, the gene was inactivated by repeat induced point mutation (RIP). The RIP mutants were found to be viable and Western blot analysis of mitochondrial proteins revealed that the mutant was devoid of Oxa2 (Figure 2A). Thus, Oxa2 is not an essential gene in *N. crassa* though the $oxa2^{RIP}$ mutant has a reduced growth rate in medium containing glucose (Fig-

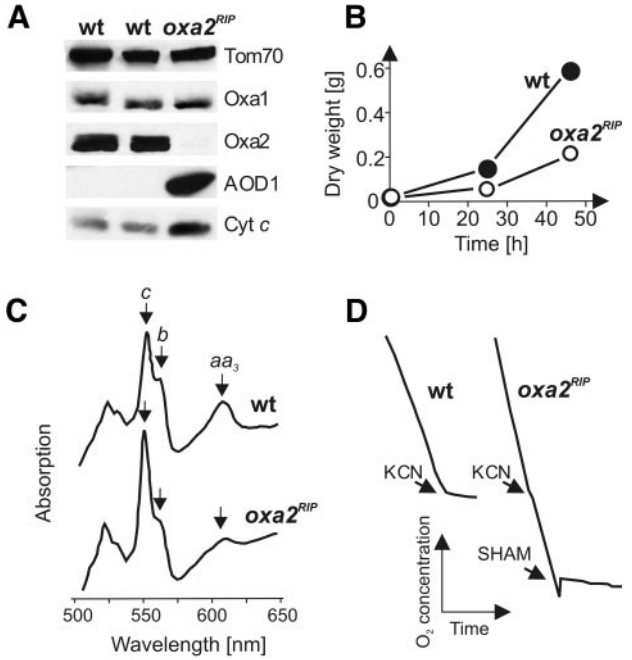

Figure 2. Oxa2 is required for the biogenesis of cytochrome oxidase in *N. crassa*. (A) Mitochondria were isolated from the wild-type strains used in the RIP cross, 74*A* (wt, left lane), oxa2hyg-39 (wt, middle lane), and the RIPed strain oxa2^{RIP}-35 (*oxa2^{RIP}*). Mitochondria were solubilized in sample buffer and subjected to SDS-PAGE. The gel was blotted to nitrocellulose and immunodecorated with antisera to the indicated proteins (AOD1, alternative oxidase; Cyt *c*, cytochrome *c*). (B) Strain oxa2hyg-39 harboring a wild-type allele of *oxa2* (wt) and the *oxa2*-deficient mutant oxa2^{RIP}-35 (*oxa2^{RIP}*) were grown in liquid medium at 22°C with shaking for the indicated times. Mycelia were harvested, dried overnight at 37°C, and weighed. (C) Mitochondria were isolated from the strains used in A and cytochrome spectra were obtained. The absorbance maxima for cytochromes aa_3, *b*, and *c* are indicated. (D) Mycelia from the strains used in A were analyzed for oxygen consumption. The tracings show the decrease in oxygen concentration as it is consumed by the cells over time. KCN was added to inhibit cytochrome oxidase, and thereby respiration via the normal, cytochrome-mediated electron transport chain. Salicylhydroxamic acid (SHAM) was added to inhibit the alternative oxidase.

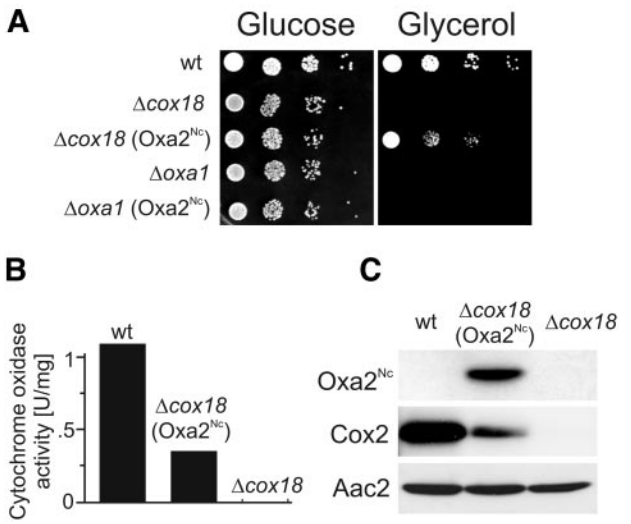

Figure 3. Oxa2 can take over the function of yeast Cox18. (A) Yeast wild-type (wt), Δ*cox18,* and Δ*oxa1* mutants with or without plasmids expressing *N. crassa* Oxa2 (Oxa2^{Nc}) were grown to log phase. Tenfold serial dilutions of the cultures were spotted on YP plates containing 2% glucose or 2% glycerol. The plates were incubated at 30°C for 2 (glucose) or 3 days (glycerol). (B) Levels of cytochrome oxidase activity in isolated mitochondria from wild-type, or from Δ*cox18* mutants with or without an Oxa2-expressing plasmid. (C) Mitochondrial extracts (100 μg mitochondrial protein) of the yeast strains indicated were subjected to SDS-PAGE, transferred to nitrocellulose, and decorated with antibodies against Oxa2, Cox2, and the ATP/ADP carrier (Aac2) as a loading control.

ure 2B). Examination of mitochondrial cytochromes revealed that the growth rate defect is likely due to a deficiency of cytochrome aa_3 in the mutant (Figure 2C). As shown previously for other *N. crassa* mutants deficient in cytochrome aa_3, the level of cytochrome *c* is elevated (Bertrand and Pittenger, 1972; Bertrand *et al.,* 1977). As expected for an *N. crassa* cytochrome deficient mutant, the *oxa2*^{RIP} strain also contains alternative oxidase (Figure 2A, AOD1), which transfers electrons directly from ubiquinone to molecular oxygen, thus bypassing the last two complexes of the standard electron transport chain (Vanlerberghe and McIntosh, 1997). Alternative oxidase is resistant to KCN and inhibited by salicylhydroxamic acid (SHAM). Alternative oxidase is normally not expressed in wild-type cells (Lambowitz and Slayman, 1971) and thus we did not detect AOD1 in wild-type mitochondria (Figure 2A). As a consequence, respiratory activity of wild-type cells can be blocked by KCN, which inhibits cytochrome oxidase (Figure 2D). In the *oxa2*^{RIP} strain, respiration was not affected by KCN. However, inhibition of alternative oxidase function with SHAM blocked respiration in the mutant. Taken together, the above data show that Oxa2 is required for biogenesis or stability of cytochrome oxidase.

Oxa2 Complements Yeast cox18 Mutants

Next we tested whether the Oxa2 protein of *N. crassa* can fulfil the function of Oxa1 or Cox18 in yeast mitochondria. Expression of *N. crassa* Oxa2 was unable to rescue the respiration-defective phenotype of yeast Δ*oxa1* mutants, but did restore growth to a *cox18* deletion mutant on nonfermentable carbon sources (Figure 3A). The ability of Oxa2 to take over the function of Cox18 was further supported by the finding that the levels of the cytochrome oxidase activity in the rescued mutant were partially restored (Figure 3B) and by the presence of Cox2 in the complemented strain (Figure 3C). The ability of Oxa2 to restore the defects in mitochondria of the *cox18* mutant suggests that Oxa2 represents a functional orthologue of yeast Cox18.

Oxa2 Interacts with Unassembled Cox2 and Cox3 Proteins

*Oxa*I is required for membrane integration of Cox2. Thus, in Oxa1-deficient mutants the leader sequence of Cox2 does not reach the Imp1 protease in the intermembrane space and Cox2 accumulates in its precursor form. To assess whether Oxa2/Cox18 is likewise necessary for membrane integration of Cox2, we radiolabeled translation products in isolated mitochondria of yeast mutants lacking either Cox18 or Oxa1 (Figure 4A). This revealed normal processing of Cox2 in the absence of Cox18 function, indicating that this protein is not required for membrane integration of the N terminus of Cox2.

To determine if Oxa2 associates with mitochondrially encoded proteins, translation products were radiolabeled in isolated mitochondria of yeast strains that did or did not express

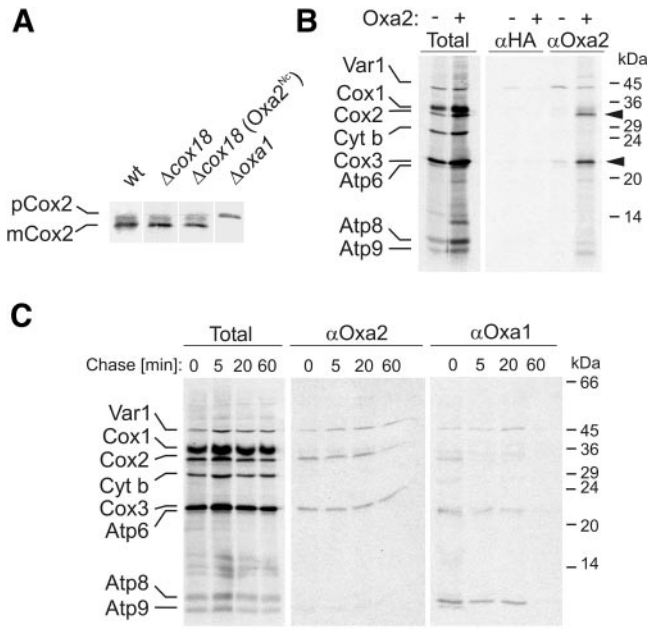

Figure 4. Oxa2 binds to newly synthesized Cox2 and Cox3. (A) Translation products were radiolabeled for 20 min in isolated mitochondria of wild-type or Δ*oxa1* cells or of Δ*cox18* mutants lacking or containing an *N. crassa* Oxa2 expression plasmid as indicated. The mitochondria were lysed, Cox2 was isolated by immunoprecipitation with Cox2-specific antibodies, and the labeled protein was visualized by autoradiography. (B) Translation products were radiolabeled in mitochondria of wild-type (Oxa2−) or Δ*cox18* cells expressing *N. crassa* Oxa2 (Oxa2+). Mitochondria were either directly applied to SDS-PAGE (Total) or subjected to immunoprecipitation with HA- or Oxa2-specific antibodies. The HA serum served as control. The Oxa2 antiserum unspecifically recognized Var1 as this was purified also from wild-type extract that did not contain any Oxa2 protein. The positions of Cox2 and Cox3 coimmunoprecipitated with the Oxa2 antiserum are indicated by arrowheads. (C) Proteins were radiolabeled in Δ*cox18* ($Oxa2^{Nc}$) mitochondria for 20 min. Labeling was stopped by addition of an excess of cold methionine and the samples further incubated for the times indicated (chase). Mitochondria were either directly subjected to SDS-PAGE (Total) or subjected to coimmunoprecipitation with Oxa2- and Oxa1-specific antibodies. In B and C, the "Total" lanes correspond to 10% of the material used for immunoprecipitation reactions.

N. crassa Oxa2. After lysis of the organelles, we immunoprecipitated Oxa2 and analyzed coimmunoprecipitated proteins by autoradiography (Figure 4B). A specific interaction of Oxa2 with newly synthesized Cox2 and Cox3 (Figure 4B, arrows) and minor amounts of Cox1 was observed. Other translation products were not specifically coimmunoprecipitated with Oxa2. The interaction of Oxa2 with Cox2 and Cox3 was relatively long lived, because when the reaction was chased after the labeling period by addition of cold methionine, Cox2 and Cox3 remained bound to Oxa2 for at least 1 h (Figure 4C, middle panel). This is in contrast to Oxa1, which interacts only loosely and transiently with translation products (Figure 4C, right panel; Hell *et al.*, 1998). From this we conclude that Oxa2 functions downstream of Oxa1 at later stages of the biogenesis of the cytochrome oxidase complex.

Oxa2 Is Sorted into the Inner Membrane on a Conservative Sorting Pathway

Oxa1 reaches its location in the inner membrane by a two-step process: the protein is first imported into the matrix and then inserted in an export-like step into the membrane (Herrmann *et al.*, 1997). This insertion reaction depends on a functional Oxa1 translocase in the membrane. To determine if Oxa2 also reaches the inner membrane via a matrix-localized sorting intermediate, radiolabeled Oxa2 protein was imported into isolated yeast mitochondria for different time periods and the location of the protein was determined (Figure 5A). Isolated yeast mitochondria were used for these studies because, in contrast to mitochondria of *N. crassa*, the outer membrane of yeast mitochondria can be easily opened by hypoosmostic swelling so that the submitochondrial location of proteins can be easily assessed by protease-protection experiments. The Oxa2 precursor (Figure 5A, pre) was efficiently imported into the mitochondria and converted to a processed mature form of 43 kDa (Figure 5A, m). In mitochondria, this mature Oxa2 form was inaccessible to added protease (Figure 5A, middle panel). In contrast, after opening the outer membrane by swelling, protease treatment resulted in partial degradation of Oxa2 into two fragments with apparent sizes of 16 and 17 kDa (Figure 5A, right panel). These fragments are consistent with an Oxa1-like topology of the Oxa2 protein which upon digestion of intermembrane space domains should lead to fragments with predicted molecular weights of 16.6 and 17.8 kDa (Figure 5B). Interestingly, the Oxa2 fragments only appeared after longer import times and initially Oxa2 was found to be predominantly protease-inaccessible in the matrix. To assess this in more detail, Oxa2 was imported for different time periods and the amounts of the matrix-localized and inserted species were quantified (Figure 5C). This again revealed initial accumulation of a sorting intermediate in the matrix and a subsequent increase of the membrane-inserted Oxa2 protein. This suggests that Oxa2, like Oxa1, reaches the inner membrane via a matrix-localized sorting intermediate and inserts into the inner membrane in an export-like reaction.

To assess whether integration of Oxa2 from the matrix into the inner membrane depends on the Oxa1 translocase, Oxa2 precursor was imported into mitochondria of a Δ*oxa1* mutant strain. Even after long incubation times no protease-accessible species of Oxa2 was found in the inner membrane of these mutants as judged by the lack of the characteristic cleavage fragments seen after import into wild-type mitochondria (Figure 5D). This indicates that Oxa1 is required for insertion of Oxa2 into the inner membrane. Thus, the matrix-localized sorting intermediate of Oxa2 appears to be inserted into the inner membrane by the Oxa1 translocase. Because Δ*oxa1* mitochondria have significantly reduced membrane potential levels, we can, however, not formally exclude the possibility that the reduced insertion efficiency is due to a diminished energetic state of the mitochondria.

Oxa2 Defines a New Subfamily of Oxa1/YidC/Alb3 Proteins

The functional conservation of Oxa2 of *N. crassa* and Cox18 of yeast suggests that the proteins are evolutionary conserved. To assess the conservation of both proteins in other species, we screened the genome databases for further homologues of Oxa2. BLAST searches with the *N. crassa* Oxa2 sequence revealed a large number of homologous sequences encoded in the genomes of fungi, plants and animals. In the human genome, a homologue of Oxa2 is encoded on chromosome 4 (accession number NP_776188), for which a number of expressed sequence tags are present in the databases, indicating expression of the human Oxa2 gene. The Oxa2 proteins form a separate branch in the phylogenetic tree of the Oxa1/YidC/Alb3 protein family and, thus, apparently

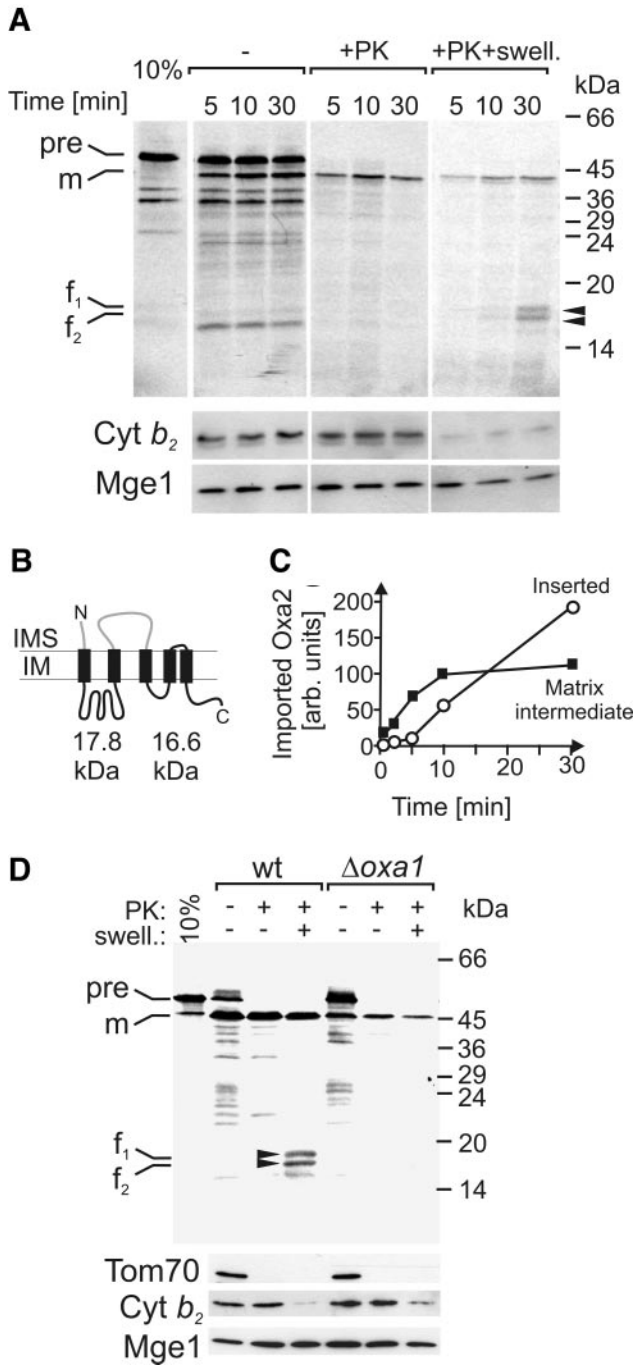

Figure 5. Oxa2 reaches the inner membrane via a conservative sorting pathway. (A) Radiolabeled Oxa2 precursor was incubated with isolated wild-type yeast mitochondria for 5, 10, or 30 min as indicated. The samples were divided into three aliquots. One aliquot was mock treated (−), the others were incubated with proteinase K (PK) either without (+PK) or after hypotonic swelling (+PK + swell.) of the mitochondria. Positions of molecular weight standards are shown on the right. Precursor and mature forms of Oxa2 are depicted as pre and m. Arrowheads indicate proteolytic fragments of Oxa2 (f_1 and f_2). Western blotting using antibodies against the intermembrane space protein cytochrome b_2 (cyt b_2) and the matrix protein Mge1 served as a control for mitoplasting efficiency by swelling. (B) Model of the predicted Oxa2 topology in the inner membrane (IM). The model is based on the topology shown for other Oxa1 homologues. Domains exposed to the intermembrane space (IMS) are shown in gray. Putative protease fragments are shown in black and their predicted molecular weights are indicated. (C) Oxa2 precursor was imported for different time periods into mitochondria as described for A. The levels of protease-inaccessible Oxa2 in the matrix and protease-generated fragments of membrane-inserted Oxa2 were quantified. Values were corrected for the numbers of methionines present in the Oxa2 species. (D) Oxa2 precursor was imported into mitochondria isolated from wild-type and Δ*oxa1* mutant cells for 25 min. The samples were further treated as for A. The Oxa2 fragments generated in wild-type mitochondria are indicated by arrowheads.

developed at an early stage of mitochondrial evolution (Figure 6A). Supplementary Table 1 lists the predicted mitochondrial members of the Oxa1/YidC/Alb3 family present in the fully sequenced eukaryotic genomes. All of these proteins are predicted to contain typical mitochondrial targeting presequences (see Supplementary Table 1) and a consistent pattern of five transmembrane domains (Figure 6B). In contrast to the Oxa1 subbranch, Oxa2 proteins (which include the proteins referred to as Cox18) typically lack a C-terminal matrix domain for which a coiled coil-structure can be predicted (see Supplementary Table 1; Lupas, 1997). This domain has been shown to allow the binding of Oxa1 to mitochondrial ribosomes (Jia *et al.*, 2003; Szyrach *et al.*, 2003). In summary, the data presented here suggest that mitochondria of plants, fungi, and animals consistently contain at least two members of the Oxa1/YidC/Alb3 family of similar structure but presumably of discrete function.

DISCUSSION

Mitochondria of plants, fungi, and animals contain two members of the Oxa1/YidC/Alb3 family. On the basis of their sequence, these proteins can be classified into two subfamilies that we have named the Oxa1 and Oxa2 subbranches. Members of both groups share a conserved core region of ~200 amino acid residues that contains five predicted transmembrane domains. This hydrophobic core region is also present in bacterial and plastid members of the Oxa1/YidC/Alb3 family and most likely represents the active center of the proteins that mediates the integration and translocation of substrate polypeptides into and across the membrane. Oxa1 proteins typically contain a C-terminal tail domain of ~100 amino acid residues that protrudes into the mitochondrial matrix and that is predicted to form a coiled coil structure (Nargang *et al.*, 2002). Studies in yeast revealed that this domain allows the binding of Oxa1 to mitochondrial ribosomes (Jia *et al.*, 2003; Szyrach *et al.*, 2003). In contrast to Oxa1 proteins, members of the Oxa2 subfamily typically contain significantly shorter C termini for which no coiled coil structures are predicted (see Supplementary Table 1).

The presence of two Oxa1/YidC/Alb3 members in mitochondria suggests that they have distinct functions that made it necessary to retain both paralogues during evolution. Like Oxa1, Oxa2 is part of a high-molecular-weight complex that is, however, distinct from the Oxa1 complex. Oxa1 has been shown to be required for the biogenesis of complexes I, III, IV, and V of the respiratory chain (Altamura *et al.*, 1996; Bonnefoy *et al.*, 2000; Hell *et al.*, 2001; Nargang *et al.*, 2002). Oxa1 interacts directly with translocation intermediates and facilitates their insertion into the inner membrane (He and Fox, 1997; Hell *et al.*, 1997, 1998). This interaction is transient and not very stable (Preuss *et al.*, 2001). In contrast to Oxa1, Oxa2 is not essential in *N. crassa* and only reduces the level of cytochrome aa_3, and by extension, cytochrome oxidase activity. The presence of alternative oxidase in the

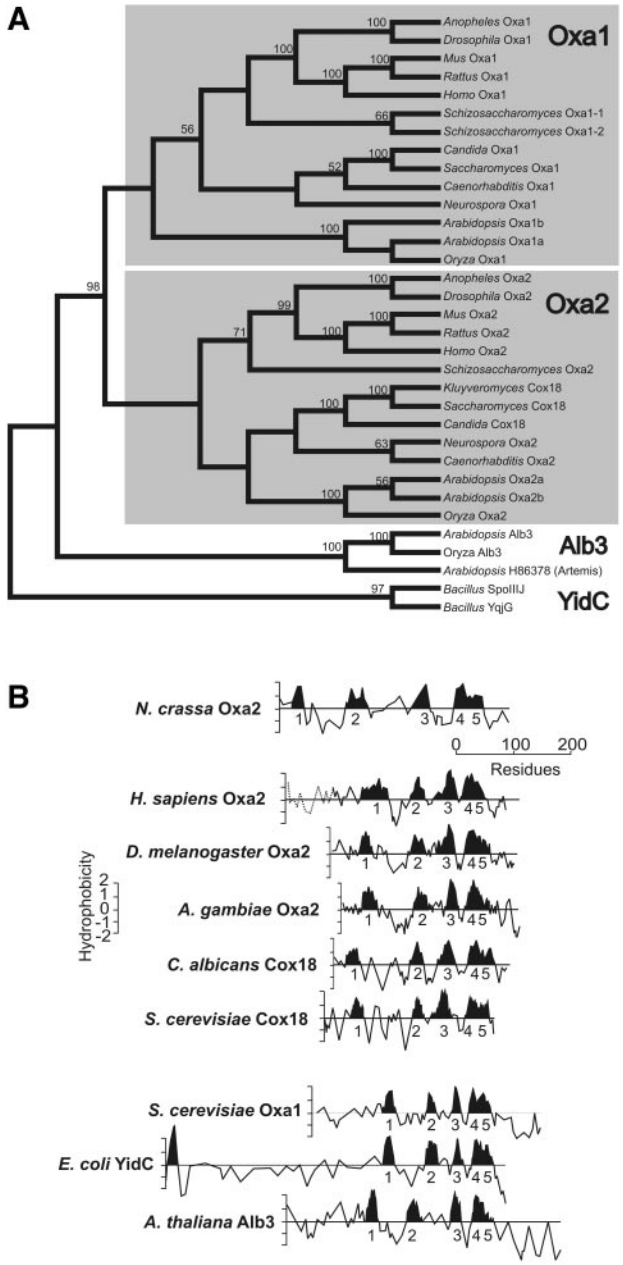

Figure 6. Oxa2 defines a subbranch of the Oxa1/YidC/Alb3 protein family. (A) Neighbor joining analysis of members of the Oxa1/YidC/Alb3 protein family. Bootstrap support larger than 50% is indicated above the branches. The Oxa1 and Oxa2 subbranches of the family are shown in shaded boxes. For accession numbers see Table 1 of the Supplementary Material. (B) Hydrophobicity profiles of Oxa2 proteins and other Oxa1/YidC/Alb3 family members. Note that Oxa2 proteins share a core domain of similar topology with other members of the Oxa1/YidC/Alb3 family but typically have shorter flanking regions. Only the portions predicted to be in the mature protein sequences are shown. The N terminus of the human protein which is not represented by EST sequences is indicated as a dotted line.

oxa2^{RIP} mutant of *N. crassa* further indicates a defective electron flow through complex IV in the mutant. Similarly, mutations in the Oxa2 homologue, Cox18, cause specific defects in the biogenesis of cytochrome oxidase in yeast (Hikkel *et al.*, 1997; Souza *et al.*, 2000; Saracco and Fox, 2002). This suggests that members of the Oxa2 subgroup may play a specific role in the biogenesis of cytochrome oxidase. We observe a physical interaction of Oxa2 with Cox2 and Cox3, which form an initial subcomplex in the assembly pathway of cytochrome oxidase (Wielburski and Nelson, 1983). The interaction is rather persistent, at least in our system using isolated mitochondria where the cytochrome oxidase subunits do not assemble into a complete enzyme complex. The observation that the Oxa1 catalyzed membrane integration step of Cox2 still occurs in mitochondria lacking Oxa2 indicates that Oxa2 functions downstream of Oxa1. It was suggested that Cox18 facilitates translocation of the C-terminal domain of Cox2 across the inner membrane into the intermembrane space (Saracco and Fox, 2002). This domain is rather large and strongly charged and its transport across the membrane might therefore require the function of a specialized translocation component. Because the export of the C-terminal domain of Cox2 occurs after the Oxa1-dependent insertion of the N terminus (Herrmann, 1995), this would be consistent with our observations. On the other hand, the stability of the binding of Oxa2 to Cox2 and Cox3 might indicate that Oxa2 also helps to assemble the different cytochrome oxidase subunits into a functional complex.

Interestingly, we identified genes encoding putative Oxa2 homologues in all completely or almost completely sequenced eukaryotic genomes, except those of *Chlamydomonas rheinhardtii* and *Plasmodium falciparum*. In both of these organisms the gene for Cox2 is located in the nucleus, and Cox2 reaches the inner membrane after import from the cytosol (Perez-Martinez *et al.*, 2001; Funes *et al.*, 2002; Gardner *et al.*, 2002). Thus, topogenesis of Cox2 in these organisms presumably does not require export of the C terminus from the matrix into the intermembrane space, which might have allowed the loss of the Oxa2 gene during evolution.

A duplication of members of the Oxa1/YidC/Alb3 family has also been reported for other organisms: *B. subtilis* contains two YidC homologues (Murakami *et al.*, 2002), mitochondria of *S. pombe* contain two functional Oxa1 homologues (Bonnefoy *et al.*, 2000) and chloroplasts of *C. reinhardtii* contain two Alb3 isoforms (Bellafiore *et al.*, 2002). However, the separation of the Oxa1 and Oxa2 subbranches most likely occurred early during the evolution of mitochondria and apparently arose independently from the other duplications observed.

Oxa2 is transported to the inner membrane via a conservative sorting pathway and integrated into the inner membrane in an Oxa1-dependent reaction as described previously for Oxa1 (Herrmann *et al.*, 1997) and subunit 9 of the ATPase of *N. crassa* (Rojo *et al.*, 1995). The dependence on Oxa1 rather than on Cox18 supports the notion that Oxa1 serves as a general insertion component for inner membrane proteins, whereas Cox18/Oxa2 plays a more specific function.

The assembly of the enzymes of the respiratory chain is a complex process that requires the function of a large number of different factors. Most of these components were identified by genetic screens for respiratory-deficient yeast mutants (Tzagoloff and Dieckmann, 1990). The classification of mitochondrial Oxa1/YidC/Alb3 proteins into two subbranches of distinct function serves as a good example for showing that the accumulation of sequence information from different model organisms can be relevant to the identification of potential functions of human proteins. Indeed, the human Oxa2/Cox18 protein can complement *S. cerevisiae* and *S. pombe cox18* mutants (N. Bonnefoy, personal communication). It will be a major goal in the future to identify the precise molecular function of Oxa2/Cox18 proteins in the biogenesis of cytochrome oxidase.

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