We have characterized Cox16p, a new cytochrome oxidase (COX) assembly factor. This protein is encoded by COX16, corresponding to the previously uncharacterized open reading frame YJL003w of the yeast genome. COX16 was identified in studies of COX-deficient mutants previously assigned to complementation group G22 of a collection of yeast pet mutants. To determine its location, Cox16p was tagged with a Myc epitope at the C terminus. The fusion protein, when expressed from a low-copy plasmid, complements the mutant and is detected solely in mitochondria. Cox16p-myc is an integral component of the mitochondrial inner membrane, with its C terminus exposed to the intermembrane space. Cox16 homologues are found in both the human and murine genomes, although human COX16 does not complement the yeast mutant. Cox16p does not appear to be involved in maturation of subunit 2, copper recruitment, or heme A biosynthesis. Cox16p is thus a new protein in the growing family of eukaryotic COX assembly factors for which there are as yet no specific functions known. Like other recently described nuclear gene products involved in expression of cytochrome oxidase, COX16 is a candidate for screening in inherited human COX deficiencies.

Cytochrome oxidase (COX), the terminal enzyme of the mitochondrial respiratory chain, catalyzes the transfer of electrons derived from sugars, fats, and amino acids to molecular oxygen. The mammalian enzyme consists of 13 subunits and has a well characterized structure (1). The three largest subunits, which are encoded in mitochondrial DNA, form the catalytic core of the enzyme, with subunits 1 and 2 binding the known COX assembly factors have not been identified. In contrast to the wealth of information about the structure of cytochrome oxidase, the mechanism by which the holoenzyme complex is assembled remains unclear. Studies in the yeast Saccharomyces cerevisiae have provided much of the information regarding the proteins involved in the COX assembly pathway. Some of these proteins are involved in recruiting copper to mitochondria and COX (3–5). Others have been implicated in heme A biosynthesis (6, 7) and transport and maturation of the mitochondrially encoded subunits (8–10). Despite these advances, the precise functions of a number of COX assembly factors remain unknown (11–13). It is also unclear whether all of the proteins required for COX assembly have been identified. This information is important for understanding the mechanism of COX assembly and for elucidating the genetic basis of human COX deficiencies (14, 15).

Here we report the identification and characterization of Cox16p, a new COX assembly factor encoded by a previously uncharacterized yeast open reading frame (YJL003w; GenBank™ accession number Z49278). Mutations in COX16 result in a failure to complete assembly of cytochrome oxidase, and cox16 mutants are respiration-deficient. Cox16p does not appear to function in mitochondrial copper homeostasis or in the synthesis of heme A. The phenotype of cox16 mutants also argues against a role of Cox16p in processing of Cox2p or membrane insertion of the mitochondrially encoded subunits of COX. Like many of the other COX assembly factors, Cox16p is an integral component of the mitochondrial inner membrane and appears to exist in a higher molecular weight complex. Recent additions to the data base reveal that COX16 has homologues in Schizosaccharomyces pombe, as well as in the murine and human genomes. Human COX16 is thus a candidate gene for human COX deficiencies in which mutations in the known COX assembly factors have not been identified.

**MATERIALS AND METHODS**

**Strains and Media**—The genotypes of the strains used in this study are indicated in Table I. The composition of media for growth and analysis of yeast strains has been described elsewhere (16).

**Cloning and Disruption of COX16**—The yeast genomic library used to clone COX16 was constructed from partial Sau3A fragments of nuclear DNA of the respiratory-competent haploid strain S. cerevisiae D273–10B/A1. C25/U1, a mutant from complementation group G22 (8), was transformed with yeast genomic DNA as described previously (11), and the COX16 gene identified by isolating subclones capable of conferring respiratory competence on C25/U1. The COX16 gene was disrupted by insertion of a 1.1-kb URA3 fragment at the internal HindIII site and transformation of the respiratory-competent haploid strain, W303–1A, with the linear fragment containing the disrupted gene and flanking sequences. The transformation yielded the cox16 mutant aW303Δcox16.
Characterization of Cox16p

Table I

| Strain | Genotype | Source |
|--------|----------|--------|
| W303-1A | ade2-1 his3-11,15 leu2-3,112 trp-1 ura3-1 | This study |
| W303-1B | ade2-1 his3-11,15 leu2-3,112 trp-1 ura3-1 | This study |
| aW303A/C0X16 | ade2-1 his3-11,15 leu2-3,112 trp-1 ura3-1 Cox16 ura3-1 | This study |
| aW303A/C0X16/myc1 | ade2-1 his3-11,15 leu2-3,112 trp-1 ura3-1 Cox16 ura3-1 Myc1 | This study |
| aW303A/C0X15 | ade2-1 his3-11,15 leu2-3,112 trp-1 ura3-1 Cox16 ura3-1 Cox15 | This study |
| aW303A/C0X18 | ade2-1 his3-11,15 leu2-3,112 trp-1 ura3-1 Cox18 | This study |

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Construction of pMGL5 and the COX16-myc Fusion—To overexpress a Cox16p-myc fusion protein, we first constructed pMGL5, a yeast/E. coli shuttle plasmid containing a Myc epitope tag in the backbone of the multi-copy vector Yep351 (17). Briefly, a 3.3-kb NarI-AorII fragment of Yep351 containing the LEU2 marker and the 2-μm origin of replication, was used to replace a 3.8-kb NarI-AalII fragment in YCPmyc111 (a gift from Dr. Troy Harkness, modified from Gietz and Rothstein, Department of Development and Human Genetics, Columbia University, New York. a

Miscellaneous Methods—Transformation of yeast cells was carried out by the method of Schiestl and Gietz (19). Yeast strains were grown to stationary phase in YPGal, and mitochondria were isolated as described previously (20). For analysis of in vivo labeled mitochondrial translation products, strains were grown in YPGal and labeled with [35S]methionine in the presence of cycloheximide (21). The labeling reaction was terminated after 15 min by addition of an excess of 80 mM cold thionine in the presence of cycloheximide (21). The labeling reaction was terminated after 15 min by addition of an excess of 80 mM cold thionine in the presence of cycloheximide (21)

Analysis of mitochondrial hemes in both the C25/U1 and cox16 null mutant revealed the presence of heme A and heme O (data not shown) in amounts seen with most other COX assembly mutants examined to date.2

Analysis of mitochondrial hemes in both the C25/U1 and cox16 null mutant revealed the presence of heme A and heme O (data not shown) in amounts seen with most other COX assembly mutants examined to date.2
transport or insertion of one of the heavy metals known to be associated with COX.

Cloning and Disruption of COX16—To identify the gene responsible for the cytochrome oxidase deficiency of G22 mutants, C25/U1 was transformed with a yeast genomic library, and uracil prototrophic clones were checked for growth on non-fermentable carbon sources. A transformant, C25/U1/T1, rescued both the uracil auxotrophy and respiratory deficiency of G22 mutants. Subcloning generated the construct pG22/ST2, yielding pG22/ST4. The respiratory-complementing plasmid containing the COX16/myc1 allele was obtained by inserting a 1-kb HindIII fragment containing the URA3 gene at an internal HindIII site of COX16 in pG22/ST2, yielding pG22/ST4. The respiratory-competent haploid strain W303–1A was transformed with a linear BglII-SphI fragment of pG22/ST4. An uracil-independent and respiration-deficient transformant (aW303ΔCOX16) was determined by Southern blot analysis of genomic DNA to have the URA3 insertion in COX16 (not shown). The biochemical phenotype of aW303ΔCOX16 was ascertained to be similar to that of the parental wild type (W303–1A), a cox18 null mutant (ΔCOX18), a cox16 null mutant (ΔCOX16), and a cox15 null mutant (COX15) were grown in YPGal and labeled with [35S]methionine in the presence of cycloheximide for 15 min as described under “Materials and Methods.” Excess methionine was added, and samples were taken after the indicated times of chase at 30°C. Mitochondrial translation products were analyzed on a 12.5% polyacrylamide gel containing 6 M urea and 6% glycerol and are identified in the margin as described in Fig. 1.

Fig. 1. Spectral analysis and steady state levels of COX subunits in cox16 mutants. A, spectra of mitochondrial cytochromes. Mitochondria were prepared from the two wild type strains D273–10B/A1 (D273) and W303–1A (W303) and from the cox16 mutants C25, E699, and aW303ΔCOX16 (ΔCOX16) and extracted at a protein concentration of 5 mg/ml with potassium deoxycholate (11). Difference spectra of the oxidized (potassium ferricyanide) versus reduced (sodium dithionite) extracts were recorded at room temperature. The position of cytochrome aa3 is marked. B, Western analysis of COX subunits. Mitochondria were isolated from the wild type W303–1A (W303), the mutant aW303ΔCOX16 (ΔCOX16), aW303ΔCOX16/myc1 (myc1) and aW303ΔCOX16/myc2 (myc2), which are the cox16 mutant transformed with the COX16-myc fusion in a high-copy and a CEN plasmid, respectively. Subunits 1, 2, and 3 of cytochrome oxidase (Cox1, Cox2, Cox3) were analyzed by separating 10 μg of mitochondrial protein on 12% polyacrylamide gels. The nuclear-encoded subunits (Cox4, Cox5, Cox6, Cox7, 7a, 8) were detected by separating 20 μg of mitochondrial protein on a 16.5% polyacrylamide/6 M urea gel. Following transfer to nitrocellulose, the blots were probed with subunit-specific antibodies and visualized using enhanced chemiluminescence. C, the wild type strains D273–10B/A1 and the mutant C25 were grown in YPGal and labeled in vivo with [35S]methionine in the presence of cycloheximide (46). Mitochondria were isolated and separated by SDS-PAGE on a 7.5–25% linear polyacrylamide gel. Following transfer to nitrocellulose, the blots were probed with subunit-specific antibodies and visualized using enhanced chemiluminescence. The identity of the labeled proteins are identified in the margin as described in Fig. 1.

Fig. 2. Turnover of in vivo labeled mitochondrial translation products in wild type and cox16 mutants. The parental wild type (W303–1A), a cox18 null mutant (ΔCOX18), a cox16 null mutant (ΔCOX16), and a cox15 null mutant (COX15) were grown in YPGal and labeled with [35S]methionine in the presence of cycloheximide for 15 min as described under “Materials and Methods.” Excess methionine was added, and samples were taken after the indicated times of chase at 30°C. Mitochondrial translation products were analyzed on a 12.5% polyacrylamide gel containing 6 M urea and 6% glycerol and are identified in the margin as described in Fig. 1.

Fig. 3. The COX16 gene encodes a small, acidic protein. A, a hydrophathy plot of Cox16p predicts a single membrane-spanning segment. B, the primary amino acid sequence of Cox16p is shown with the potential mitochondrial targeting sequence (as determined by the PSORT program) denoted by the bar above the sequence. The predicted transmembrane domain is indicated by the bar beneath the sequence.

Characterization of Cox16p
Characterization of Cox16p

Fig. 4. Cox16p is a mitochondrial inner membrane protein. A. Western analysis of mitochondria (Mit) and postmitochondrial supernatant (PMS) fractions from the wild type strain W303–1A (W303), the cox16 mutant aW303ΔCOX16 (ΔCOX16), and the transformants aW303ΔCOX16/myc1 (myc1) and aW303ΔCOX16/myc2 (myc2), expressing a Myc-tagged Cox16p from a high copy plasmid and from a CEN plasmid, respectively. Mitochondrial (20 μg) or postmitochondrial supernatant fractions (40 μg) were separated on 15% gels and analyzed as described in Materials and methods. The concentrations of the three mitochondrial encoded subunits and subunit 5 in the transformants are similar to the wild type levels, regardless of the vector used to express the Myc-tagged Cox16p (Fig. 4B). N-terminal signal sequence, the Myc epitope was added at the C terminus. Growth of transformants, expressing the tagged protein from either a high copy episomal (aW303ΔCOX16/myc1) or low copy CEN plasmid (aW303ΔCOX16/myc2) on non-fermentable carbon sources (ethanol/glycerol), was indistinguishable from that of transformants expressing an untagged version of Cox16p. Restoration of growth on these substrates correlated with the recovery of cytochrome oxidase. This was evident from the spectra (not shown) and Western analysis of COX subunits (Fig. 1B). The concentrations of the three mitochondrially encoded subunits and subunit 5 in the transformants are similar to the wild type levels, regardless of the vector used to express the Myc-tagged Cox16p (Fig. 4B).

A low molecular weight protein in mitochondria from aW303ΔCOX16/myc1 and aW303ΔCOX16/myc2 is detected with the Myc antibody (Fig. 4A). This band is absent in the post-mitochondrial supernatant fraction from these strains and is also undetectable in wild type and mutant mitochondria. Based on its migration, this protein has an apparent mass of 21 kDa, which is 6 kDa larger than the combined size of native Cox16p and the additional 1 kDa contributed by the Myc epitope. The reason for the discrepancy, which may be even larger if Cox16p has a cleavable signal, is not clear but is probably related to anomalous binding of SDS by this acidic protein.

Almost all of the COX assembly factors identified to date are constituents of the mitochondrial inner membrane, as would be expected from their involvement in assembly of a hydrophobic multimeric complex of this membrane. The prediction of a membrane-spanning domain in Cox16p suggested that it might be an integral membrane protein. This is confirmed by the solubility properties of the protein. Titration of aW303ΔCOX16/myc1 mitochondria with deoxycholate in the presence of 0.5 M NaCl showed that extraction of Cox16p-myc requires a minimum of 0.25% detergent (Fig. 4B).

Western analysis of intact mitochondria and mitoplasts from aW303ΔCOX16/myc1 revealed Cox16p to be a constituent of the mitochondrial inner membrane as it is only found in the pellet, mitoplast fraction (Fig. 4C). Treatment with proteinase K caused Cox16p to be degraded in mitoplasts but not mitochondria. The decreased signal seen in the proteinase K-treated mitochondria is probably due to a subpopulation of mitochondria with damaged outer membranes. Similar results were obtained when mitochondria and mitoplasts were probed with antibody against Sco1p, an inner membrane protein protruding into the intermembrane space (32). Under these conditions, subunit 5 is protected from digestion by proteinase K in mitoplasts (data not shown).

Some of the COX assembly factors characterized to date

of G22 mutants. It displays a selective absence of cytochromes aa3 and a loss of cytochrome oxidase activity. The cytochrome oxidase deficiency, combined with the lack of complementation of aW303ΔCOX16 by C25 and E699, constitutes strong evidence that the mutations in these strains are allelic with the cox16 disruption.

Cox16p consists of 118 amino acid residues with a predicted mass of 14.1 kDa. The sequence includes one potential transmembrane domain (Fig. 3). The presence of a mitochondrial targeting sequence at the N terminus (Fig. 3B) is predicted by the P-Sort program (http://sort威尔.nibb.ac.jp/helpw2.2.html). The amino acid composition of Cox16p is biased toward acidic residues, and analysis of the putative mature protein reveals a PI of 5.0 (http://ca.expasy.org/tools/pi_tool.html). Cox16p does not reveal any homology to proteins of known function in the most recent databases, nor does it appear to have any identifiable functional domains that might provide clues about its function.

Expression and Mitochondrial Localization of a cox16-myc Fusion Protein—The location of Cox16p was studied with a Myc-tagged protein. Because of the presence of a potential cleavable

Fig. 5. Cox16p has murine and human homologs. An alignment of S. cerevisiae (Sc) and S. pombe (Sp) Cox16ps with the murine (Mm) and human (Hs) Cox16 homologs. The alignment was generated by the ClustalW program (http://www.ebi.ac.uk/clustalw/) and shaded with the Boxshade Program (http://www.ch.embnet.org/software/BOX_form.html). Identical residues are shaded in black, and conservative replacements are shaded in gray.

The location of Cox16p was studied with a Myc-tagged Cox16p from a high copy plasmid and from a CEN plasmid, respectively. Mitochondrial (20 μg) or postmitochondrial supernatant fractions (40 μg) were separated on 15% gels and analyzed as described in Materials and methods. The concentrations of the three mitochondrial encoded subunits and subunit 5 in the transformants are similar to the wild type levels, regardless of the vector used to express the Myc-tagged Cox16p (Fig. 4B).
appear to be part of higher molecular weight homo- or hetero
ligomeric complexes (11, 21, 33, 34). The native molecular mass of Cox16p-myc was estimated, by sedimentation of a 1% deoxy-
cholate extract of mitochondria in a 7 to 20% linear sucrose
gradient, to be —84 kDa for both aW303ΔCOX16/myc1 and
aW303ΔCOX16/myc2 (data not shown).

Cox16p Has Mamalian Homologs—Searches of current
protein and expressed sequence tag databases indicate that
Cox16p appears to have a human (HSCP203; accession number
NP_057552) and a murine (accession number NP_079737) ho-
mologue. The human cDNA was originally identified in a
screen for novel proteins expressed in CD4+ hematopoietic
stem/progenitor cells (35). Human COX16 is located on the long
arm of chromosome 14, in the interval 14q22.1–14q24.3 (LOC51241). Fig. 5 presents an alignment of the Cox16 pro-
teins from yeast, humans, and mice. This analysis reveals that
the highest sequence conservation is in the region of the trans-
membrane domain and the C-terminal half. The four Cox16
proteins shown in Fig. 5 share 24% identity and 40% conserved residues.

To test if the human gene can complement the yeast cox16
mutant the cDNA for human COX16 was amplified by PCR
from a HeLa cell cDNA library. The cDNA was cloned into a yeast
expression vector containing the ADH1 promoter and termina-
or in a YEp351 backbone, allowing constitutive ex-

aW303ΔCOX16 mutants. It is unclear whether this observation is relevant to the
human COX16 but may be due to the heterologous context. The inability of a mammalian gene to exert its function in a yeast
background is not without precedent. Only about one-third of the human COX assembly factors identified to date comple-
ment corresponding yeast mutants (38, 40). We tend to think,
therefore, that human COX16 has the same function as yeast
Cox16p. The discovery of a new COX assembly factor provides
another candidate gene for sequencing in patients with auto-
somal recessively inherited COX deficiencies without identified
mutations.

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