Analysis of Leigh Syndrome Mutations in the Yeast SURF1 Homolog Reveals a New Member of the Cytochrome Oxidase Assembly Factor Family

Megan Bestwick,‡ Mi-Young Jeong,‡ Oleh Khalimonchuk, Hyung Kim, and Dennis R. Winge*

University of Utah Health Sciences Center, Departments of Medicine and Biochemistry, Salt Lake City, Utah 84132

Received 25 February 2010/Returned for modification 5 April 2010/Accepted 30 June 2010

Three missense SURF1 mutations identified in patients with Leigh syndrome (LS) were evaluated in the yeast homolog Shy1 protein. Introduction of two of the Leigh mutations, F249T and Y344D, in Shy1 failed to significantly attenuate the function of Shy1 in cytochrome c oxidase (CcO) biogenesis as seen with the human mutations. In contrast, a G137E substitution in Shy1 results in a nonfunctional protein conferring a CcO deficiency. The G137E Shy1 mutant phenocopied shy1Δ cells in impaired Cox1 hemylation and low mitochondrial copper. A genetic screen for allele-specific suppressors of the G137E Shy1 mutant revealed Coa2, Cox10, and a novel factor designated Coa4. Coa2 and Cox10 are previously characterized CcO assembly factors. Coa4 is a twin CXC motif mitochondrial protein localized in the intermembrane space and associated with the inner membrane. Cells lacking Coa4 are depressed in CcO activity but show no impairment in Cox1 maturation or formation of the Shy1-stabilized Cox1 assembly intermediate. To glean insights into the functional role of Coa4 in CcO biogenesis, an unbiased suppressor screen of coa4Δ cells was conducted. Respiratory function of coa4Δ cells was restored by the overexpression of CYC1 encoding cytochrome c. Cycl is known to be important at an ill-defined step in the assembly and/or stability of CcO. This new link to Coa4 may begin to further elucidate the role of Cycl in CcO biogenesis.

Leigh syndrome (LS) is a highly progressive neurological disorder of infancy characterized by necrotizing lesions in the midbrain and brain stem (32). Humans afflicted with LS have compromised oxidative phosphorylation (OXPHOS) function due to mutations in nuclear or mitochondrial genes encoding respiratory chain components or their assembly factors. Although LS infants are born with a normal appearance, neurological lesions develop within months and dysfunction extends to other organs, resulting in a high mortality rate. LS patients typically have mutations affecting complex I or complex IV (cytochrome c oxidase [CcO]) of the OXPHOS pathway (14). Patients with a specific CcO deficiency most often have mutations in the SURF1 gene that encodes a CcO assembly factor (9, 15, 41).

SURF1 is not absolutely required for CcO biogenesis in humans, since SURF1-deficient patient fibroblasts retain 10 to 15% of residual CcO activity (32). The yeast homolog of SURF1 is Shy1 (SURF1 homolog in yeast) and has a conserved function in CcO biogenesis (24). Yeast lacking Shy1 retain residual CcO activity, but growth of the mutant strain is impaired (24). Yeast lacking Shy1 are unable to form the S2 assembly intermediate (16). Thus, it is not possible to isolate the CcO assembly intermediates (16). Therefore, it is not possible to isolate the CcO complex in shy1Δ yeast cells to identify any missing cofactors. However, Shy1 was shown to have a key role in formation of the heterobimetallic CuA:heme a3 center in yeast Cox1 (18).
the COX10 can partially restore respiratory function in assembly factors, SCO1 and SCO2, result in a cellular copper deficiency (37). Maintenance of the matrix overexpression of both sor activity, a marked synergistic effect was apparent in the muscle samples from patients with mutations to two other CcO subunits Cox5a and Cox6. CcO biosynthesis, a process linked to the role of cytochrome c in CcO assembly. We show that the respiratory defect of cells lacking Coa4 is specifically suppressed by the overexpression of the IMS electron carrier cytochrome c (CYC1). This is the first time CYC1 has been found as a suppressor of a Coa4 mutation.

**MATERIALS AND METHODS**

**Yeast strains and vectors.** The *Saccharomyces cerevisiae* strains used in this study are summarized in Table 1. The SHY1 open reading frame (ORF) was cloned into plasmids pRS413 and pRS423 under the control of its own promoter and terminator (450 base pairs upstream and downstream of the ORF). The G171E, F280T, and Y344D mutations were made in both the high- and low-copy vectors using site-directed mutagenesis. The SHY1 ORF with a 3’-13Myc tag was cloned from genomic DNA of the DY5113 strain into pRS416 and pRS426 vectors under the control of its own promoter and the CYC7 terminator. This study.

**TABLE 1. Yeast strains used in this work**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>W303</td>
<td>MATa ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1</td>
<td>24</td>
</tr>
<tr>
<td><em>shy1Δ</em> mutant</td>
<td>MATa ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 shy1Δ:URA3</td>
<td>This study</td>
</tr>
<tr>
<td><em>coa4Δ</em> mutant</td>
<td>MATa ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coa4Δ::KanMX4</td>
<td>5</td>
</tr>
<tr>
<td><em>coa4Δ shy1Δ</em> mutant</td>
<td>MATa ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coa4Δ:KanMX4 shy1Δ:URA3</td>
<td>This study</td>
</tr>
<tr>
<td><em>cyc1Δ cyc7Δ</em> mutant</td>
<td>MATa ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cyc1Δ::CYC13 ey2Δ:TRP1</td>
<td></td>
</tr>
<tr>
<td>BY4743</td>
<td>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><em>cox11Δ</em> mutant</td>
<td>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 coa1Δ::kanMX4</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><em>cox11Δ shy1Δ</em> mutant</td>
<td>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 coa1Δ::kanMX4 shy1Δ::URA3</td>
<td>30</td>
</tr>
<tr>
<td>BY4743</td>
<td>MATa his3Δ1 his3Δ1 leu2Δ0/met15Δ0/MET15 ura3Δ0/ura3Δ0 lys2Δ0/LYS2</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><em>shy1Δ</em> mutant</td>
<td>MATa his3Δ1 his3Δ1 leu2Δ0/met15Δ0/MET15 ura3Δ0/ura3Δ0 lys2Δ0/LYS2</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><em>coa4Δ</em> mutant</td>
<td>MATa his3Δ1 his3Δ1 leu2Δ0/met15Δ0/MET15 ura3Δ0/ura3Δ0 lys2Δ0/LYS2</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>DY5113</td>
<td>MATa ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1</td>
<td>30</td>
</tr>
<tr>
<td><em>SHY1-13Myc mutant</em></td>
<td>MATa ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 SHY1-13Myc::TRP1</td>
<td>This study</td>
</tr>
<tr>
<td><em>SHY1-13Myc coa4Δ</em> mutant</td>
<td>MATa ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 SHY1-13Myc::TRP1 coa4Δ::KanMX4</td>
<td></td>
</tr>
<tr>
<td><em>COA4-13Myc mutant</em></td>
<td>MATa ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 COA4-13Myc::KanMX4</td>
<td>This study</td>
</tr>
</tbody>
</table>

Furthermore, it was recently shown that Surf1 in bacteria is a heme-binding protein (10), although these findings have yet to be confirmed in eukaryotes.

Additional insights into the function of SURF1/Shy1 came from the isolation of genetic suppressors of *shy1Δ* respiratory deficiency in yeast (3). Respiratory function can be partially restored in *shy1Δ* cells by enhancing Cox1 translation through the overexpression of *MSS51* (6), a dual-function protein that acts as a *COX1* translational activator in addition to binding to the newly synthesized Cox1 polypeptide. Suppression of the *shy1Δ* respiratory defect is also observed with enhanced expression levels of the two CcO subunits Cox5a and Cox6 corresponding to the human S2-containing subunits CoxIV and Va (15). Overexpression of *COA2*, a recently identified CcO assembly factor shown to interact with Shy1, can also suppress the *shy1Δ* respiratory defect (30). Finally, overexpression of the *COX10* gene that encodes the hydroxyfarnesyl transferase, which generates heme a as the first step in heme a biosynthesis, can partially restore respiratory function in *shy1Δ* cells. Although overexpression of *COX10* has only very weak suppressor activity, a marked synergistic effect was apparent in the overexpression of both *MSS51* and *COX10* (29).

Shy1 has a secondary function in yeast in the maintenance of the conserved mitochondrial copper storage pool that is used in the copper metallation of Cox1 and Cox2 during CcO biogenesis. Yeast cells lacking Shy1 contain mitochondria with a partially depleted matrix copper storage pool, and the respiratory defect of *shy1Δ* cells can be partially reversed by growth in the presence of exogenous copper (29). Similarly, liver and muscle samples from patients with SURF1 mutations exhibit a cellular copper deficiency (37). Maintenance of the matrix copper pool is postulated to be linked to active CcO biogenesis in general, as patient tissue with mutations to two other CcO assembly factors, SCO1 and SCO2, result in a cellular copper deficiency as well (22).

Human SURF1 and yeast Shy1 are both mitochondrial proteins tethered to the inner membrane (IM) by two transmembrane (TM) helices with a large central domain projecting into the intermembrane space (IMS). Most LS patients with SURF1 mutations have gene deletions or rearrangements. Missense mutations in SURF1 are quite rare, with only a limited number being reported. These mutations tend to be associated with a mild clinical phenotype, and patient survival is prolonged (28). We selected a subset of known missense mutations, two of which lie within the IMS globular domain and a third that maps to the second TM domain. In an attempt to gain further insights into which functional step of SURF1 was compromised by the missense mutations, we engineered and characterized the corresponding mutations in conserved residues of yeast *SHY1*. In doing so, we have additionally identified a new member of the CcO assembly factor family, Coa4, that may be linked to the role of cytochrome c in CcO assembly. We show that the respiratory defect of cells lacking Coa4 is specifically suppressed by the overexpression of the IMS electron carrier cytochrome c (CYC1). This is the first time CYC1 has been found as a suppressor of a Coa4 assembly mutant.
in the vectors created. The COA2 and COX10 vectors used in this study, as well as the construction of the high-copy-number library, have all been described previously (19, 29, 30). Yeast strains were transformed using lithium acetate. Culture conditions for the yeast strains were either rich (YPD) medium or synthetic complete (SC) medium lacking the appropriate nutrients for plasmid selection.

**Supersensor screens.** For screening of the SHY1(G137E)-dependent high-copy-number suppressor, the BY4743 shy1Δ cells containing a single-copy pRS413-SHY1(G137E) were transformed with the coaΔ genomic DNA library (19, 29, 30). Transformants were plated onto the minimal selective plates containing 2% glycerol-lactate as a carbon source and incubated for 6 days at 30°C.

To confirm the dependence of the selected respiratory-competent clones on the mutant form of SHY1, the colonies were grown in the medium supplemented with histidine to eliminate pRS413-SHY1(G137E). Plasmids were rescued from 35 transformants, showing strong dependence on SHY1(G137E), and further analyzed by DNA sequencing.

For screening of the high-copy-number suppressor of the coaΔ allele, a genomic DNA library generated from coa2Δ cells was transformed into BY4743 coaΔ cells. About 56,000 transformants were obtained, plated, and resuspended in isotonic buffer, and incubated on ice for 20 min in the presence of an indicated amount of NaCl for 20 min on ice (13). The treated organelles were collected by centrifugation, HEPES, pH 7.4) in the presence or absence of an indicated amount of NaCl for 20 min on ice. The treated organelles were collected by centrifugation, resuspended in isotonic buffer, and incubated on ice for 20 min in the presence or absence of proteinase K (0.1 mg/ml). Following centrifugation, the pellets were loaded onto SDS-PAGE, and proteins were detected in the pellet by immunoblotting.

**BN-PAGE.** Blue native PAGE (BN-PAGE) was performed essentially as described previously (46), with 1% digitonin or 1% immunoblotting. After incubation on ice for 15 min and centrifugation (20,000 × g for 15 min at 2°C), supernatants were mixed with sample buffer (5% Coomassie brilliant blue G250; peroxidase staining for heme detection of Cyc1 [TMBZ]/H2O2 procedure (40).

**RESULTS**

**Characterization of human SURF1 missense mutations in the yeast Shy1 protein.** Missense mutations in SURF1 are very rare, with only a limited number being reported (28). We selected three known missense mutations in SURF1 that result in G124E, F249T, and Y274D substitutions (31, 39). Gly124 and Tyr274 are conserved residues in SURF1 orthologs, whereas Ile246 is a conserved hydrophobic residue. Gly124 and Ile246 map to the IMS globular domain of SURF1, whereas Tyr274 is predicted to exist within the second TM domain (Fig. 1A and B).

We introduced the corresponding mutations at the conserved residues of the yeast Shy1 protein, yielding G137E, F249T, and Y274D mutations (Fig. 1). Expression of the G137E Shy1 mutant in shy1Δ cells in two distinct genetic backgrounds (BY4743 and W303) resulted in a CoO deficiency that precluded growth on nonfermentable carbon sources (Fig. 1C). In contrast, the respiratory deficiency of shy1Δ cells in the W303 and BY4743 backgrounds was reversed by the introduction of vector-borne ShY1 with F249T and Y274D codon mutations, although BY4743 mutant cells with the Y274D Shy1 mutant were partially impaired in growth on glycerol-lactate medium (Fig. 1C). None of the mutant Shy1 proteins had any dominant negative effects when expressed in wild-type cells (data not shown). The three mutant Shy1 proteins were stably expressed, although the G137E mutant protein was partially attenuated in abundance (Fig. 1A). Cells harboring the F249T and Y274D mutant Shy1 proteins exhibited normal oxygen consumption (data not shown) and CoO activity (Fig. 1D). In contrast, cells expressing the G137E Shy1 mutant showed low CoO activity (Fig. 1D) and low oxygen consumption (data not shown), consistent with the lack of growth on glycerol-lactate medium.

Furthermore, G137E Shy1-containing cells showed reduced steady-state Cox2 levels (Fig. 2A). In line with these data, BN-PAGE analysis showed that shy1Δ cells expressing the G137E Shy1 mutant as well as the vector control cells lacked the bc1-CoO supercomplex, yet the dimeric bc1 complex was normal (Fig. 2B). Cells expressing either the F249T or Y274D Shy1 mutant showed no major difference in supercomplex formation compared to wild-type cells grown at either 30°C or a more stringent 37°C (Fig. 2B and data not shown).

As mentioned, Shy1 mediates formation of the heme a$_2$Cu$_{5}$ center in Cox1 (18). This function of Shy1 can be assessed through a hydrogen peroxide assay previously described (19). Cells depleted of Cox1 or Sco1 stall CoO assembly and lead to the accumulation of a pro-oxidant intermediate consisting of Cox1 with reactive heme a$_2$. The hydrogen peroxide sensitivity is reversed by the subsequent depletion of Shy1 (19), cox1Δ shy1Δ double null cells are resistant to hydrogen peroxide treatment because the heme a$_2$Cu$_{5}$ site is not stably formed.
The reintroduction of SHY1 restores hydrogen peroxide sensitivity expected of cox11/H9004 cells (Fig. 2D). Introduction of the F249T and Y344D Shy1 mutants into the cox11/H9004 shy1/H9004 null strain restored hydrogen peroxide sensitivity in the cells, consistent with these mutants being nearly wild type in function (Fig. 2D). In contrast, transformation of the double null cells with the G137E Shy1 mutant failed to restore hydrogen peroxide sensitivity, consistent with it being a nonfunctional variant of Shy1.

Shy1-mediated formation of the heme a3:CuB center in Cox1 occurs within a Cox1 assembly intermediate containing Shy1 (18). This complex is visualized on BN-PAGE as a 450-kDa band (25, 29, 30) (Fig. 2C). This complex was not detected in cells containing the G137E Shy1 mutant, and cells expressing the F249T and Y344D Shy1-Myc mutant showed a reduction in the abundance of this complex compared to wild-type Shy1 (Fig. 2C).

Cells lacking Shy1 have attenuated levels of mitochondrial copper, and supplementation of the growth medium with exogenous copper yielded a partial reversal of the growth defect on nonfermentable carbon sources (29). Respiratory growth of cells expressing the G137E Shy1 mutant was partially restored by exogenous copper as with the shy1/H9004 cells (data not shown). The remaining candidates required the presence of the mutant SHY1.

FIG. 1. Human SURF1 and yeast Shy1. (A) Alignment of the yeast (Sce) Shy1 and human (Hsp) SURF1 amino acid sequences (ClustalW). Bars indicate the predicted transmembrane domains. The asterisks indicate identically conserved residues; colons and periods represent similarly conserved residues. The arrowheads indicate amino acid residues mutated in Leigh syndrome patients. (B) A schematic view of Shy1. Two predicted transmembrane domains (TM1 and TM2) are indicated along with the IMS (250 residues) and the N (70-residue) and C (25-residue) terminus. Marked are the substitutions in Shy1 corresponding to pathogenic mutations in SURF1. OM, outer membrane. (C) W303 or BY4743 shy1Δ cells expressing either Myc-tagged wild-type Shy1 or its G137E, F249T, or Y344D mutant derivatives were precultured in selective media containing 2% raffinose-0.2% glucose, serially diluted, and spotted onto the plates containing 2% glucose or 2% glyceral-2% lactate as a carbon source. Pictures were taken after 2 days and 4 days of incubation at 30°C for glucose and glyceral-lactate, respectively. (D) Mitochondria isolated from the BY4743 transformants mentioned above were assayed (n = 3) for CcO activity (change in the OD550 [ΔOD550/min/10 mg protein]).

Suppressors of the G137E Shy1 respiratory defect. Gly137 is a highly conserved residue in Shy1 proteins from various species, suggesting that it may be important for Shy1 activity. Since the G137E Shy1 mutant is a nonfunctional variant, we screened for extragenic allele-specific suppressors of the respiratory defect of the G137E Shy1 mutant to identify additional proteins that may be relevant for Shy1 function. Yeast cells harboring the nonfunctional G137E Shy1 mutant were transformed with high-copy DNA library, and suppressors of the respiratory defect were isolated on SC-glycerol-lactate medium. Respiratory growth was confirmed to be dependent on the library vector by 5-FOA, which resulted in 58 candidates. The vector expressing the G137E Shy1 mutant was shed as well, showing that 23 of the candidates were able to grow on glyceral-lactate without the Shy1 mutant. These candidates were not pursued, since we expected many of these clones to be vector-encoded SHY1 or the well-known MSS51 suppressor (6, 27). The suppressors of the G137E Shy1 respiratory defect were identified by screening the library for the ability to grow on SC-glycerol-lactate medium in the absence of the Shy1 mutant. The remaining candidates required the presence of the mutant SHY1.
vector for respiratory growth, and library vectors were isolated and sequenced. Multiple copies of COA2 and COX10 were recovered as suppressors of the respiratory defect (Fig. 3A). Both Coa2 and Cox10 are known CcO assembly factors and have been indicated as weak suppressors of the shy1/H9004 cell respiratory defect individually on YP-glycerol-lactate (30). The suppressive activity of either COA2 or COX10 in shy1/H9004 cells is enhanced in the presence of the G137E Shy1 variant when grown on YP- or SC-glycerol-lactate.

An additional suppressor candidate was determined to contain a library plasmid, encoding the YLR218c ORF that we designated COA4 (cytochrome oxidase assembly 4) (Fig. 3A). Expression of this gene alone does not suppress the respiratory defect of shy1Δ cells; however, in the presence of the G137E Shy1 allele, growth is observed on YP-glycerol-lactate (Fig. 3A). In a recent systematic analysis of yeast proteins with a conserved twin CX9C motif, Coa4 (Cmc3) was identified and shown to be a mitochondrial IMS protein (23) (Fig. 3B). This motif is common to a group of IMS-localized proteins, several of which are involved in CcO assembly (20). Coa4 is important for CcO assembly.

To confirm the IMS localization of Coa4 (23), we tested the mitochondrial localization of a vector-borne Myc-tagged Coa4 allele in coa4/H9004 cells (Fig. 4). Coa4 was found to be predominantly associated with gradient-purified mitochondria (Fig. 4A) and to be associated with the membrane fraction regardless of high-salt washes (Fig. 4B). The protein was solubilized by sodium carbonate washing and degraded by proteinase K in

FIG. 2. Characterization of the SURF1/Shy1 mutants in yeast. (A) Immunoblot of the mitochondria (30 μg) isolated from the BY4743 (BY) transformants shown in panel C of Fig. 1. Organelles were separated by SDS-PAGE, and steady-state levels of the Cox2, Cyt1, and Cyc1 were assessed with the respective antibodies. Steady-state levels of the WT Shy1 and the three Shy1 mutants were determined using anti-Myc antibodies. The levels of Por1 served as a loading control. (B) Purified mitochondria (100 μg) were subjected to BN-PAGE. The distribution of respiratory complexes was analyzed by Western blotting with antibodies against Cox2 and Cyt1. (C) The Shy1 high-molecular-weight complex was assessed by BN-PAGE as described above for anti-Myc antibodies. The monomeric form of complex V (loading control) was visualized with anti-F1 serum. (D) BY4741 cox11Δ shy1Δ cells, transformed with the episomal vectors expressing wild-type Cox11, Shy1, or the respective mutant forms of the latter were grown to the mid-exponential phase and incubated with or without indicated concentration of H2O2 for 2 h at 30°C. Serial dilutions were spotted onto the plates containing 2% glucose and incubated for 36 to 48 h at 30°C.
isolated mitoplasts (Fig. 4C and D). These results confirm the IMS localization of Coa4 and identify an association with the IM. This is consistent with Coa4 lacking any predicted transmembrane helices.

Cells lacking Coa4 in the BY4743 background exhibit a respiratory defect when plated on SC-glycerol-lactate medium at room temperature, but the growth defect was less pronounced at elevated temperatures (Fig. 5A and 6A) (23). No marked growth defect was observed for \( \textit{coa4} / \textit{H9004} \) cells with the \( \textit{W303} \) background at 23 or 30°C (data not shown), yet CcO activity was depressed to \( \frac{1}{2} \) of wild-type activity in this background (Fig. 5B). CcO activity in \( \textit{coa4} / \textit{H9004} \) cells in the \( \textit{BY4743} \) background was reduced to \( \frac{1}{2} \) of that of the wild type (Fig. 5B). Yeast typically propagate well on glycerol-lactate medium at 30°C when CcO activity is \( \frac{1}{2} \) of that of the wild type. The \( \textit{bc} \) reductase activity was unchanged in mutant cells of either background (Fig. 5B and data not shown). Consistent with the attenuated CcO activity, the steady-state levels of Cox1, Cox2, and Cox3 were reduced in the \( \textit{coa4} \) null mutant (Fig. 5C). The abundance of the \( \textit{bc}:\textit{CcO} \) supercomplex was also markedly diminished (Fig. 5D) without any change in the ATP synthase monomeric or dimeric complexes. Solubilization of the IM with dodecyl maltoside further confirmed the attenuated levels of CcO (Fig. 5D). Although Coa4 appears to have a role in the assembly or stability of the CcO complex, it is not associated with the CcO supercomplex (Fig. 5G), as Coa4 fractionates in low-mass fractions upon sucrose gradient centrifugation.

Deletions of genes encoding CcO assembly factors often lead to diminished Cox1 translation by trapping Mss51 in Cox1

**FIG. 4. Localization and topology of Coa4.** (A) BY4743 \( \textit{coa4} \Delta \) cells expressing \( \textit{COA4-Myc} \) were lysed and fractionated. Cytosolic and mitochondrial fractions were subjected to SDS-PAGE and analyzed by immunoblotting with anti-Myc, anti-Por1, and anti-Pgk1 antibodies. T, total cell lysate; C, cytosolic fraction; M, mitochondrial fraction. (B) Isolated organelles were resuspended in 20 mM HEPES (pH 7.4) and NaCl of the indicated concentrations and sonicated. The soluble and insoluble fractions were fractionated and analyzed by immunoblotting with anti-Myc, anti-Sod2, and anti-Cyt1 antibodies. (C) Purified mitochondria (30 \( \mu \)g) were incubated on ice for 30 min with 20 mM HEPES (pH 7.4) for swelling or with 0.1 M sodium bicarbonate (pH 11.5) for carbonate extraction (ext.) in the presence of 2 mM PMSF (phenylmethylsulfonyl fluoride). Following incubation, the soluble and insoluble fractions were separated by high-speed centrifugation and analyzed by immunoblotting. The soluble matrix protein Sod2 and integral membrane protein Cox2 were detected with the respective antibodies. T, total mitochondria; S, supernatant; P, pellet; ext., extraction. (D) Intact (SW−) or osmotically shocked mitochondria (SW+) were incubated with (PK+) or without (PK−) 0.1 mg/ml of proteinase K (PK) for 30 min on ice. Followed centrifugation, the treated organelles were separated by SDS-PAGE and analyzed by immunoblotting with anti-Myc, anti-Cyt2, and anti-Aco1.

isolated mitoplasts (Fig. 4C and D). These results confirm the IMS localization of Coa4 and identify an association with the IM. This is consistent with Coa4 lacking any predicted transmembrane helices.

Cells lacking Coa4 in the BY4743 background exhibit a respiratory defect when plated on SC-glycerol-lactate medium at room temperature, but the growth defect was less pronounced at elevated temperatures (Fig. 5A and 6A) (23). No marked growth defect was observed for \( \textit{coa4} / \textit{H9004} \) cells with the \( \textit{W303} \) background at 23 or 30°C (data not shown), yet CcO activity was depressed to \( \frac{1}{2} \) of wild-type activity in this background (Fig. 5B). CcO activity in \( \textit{coa4} / \textit{H9004} \) cells in the \( \textit{BY4743} \) background was reduced to \( \frac{1}{2} \) of that of the wild type (Fig. 5B). Yeast typically propagate well on glycerol-lactate medium at 30°C when CcO activity is \( \frac{1}{2} \) of that of the wild type. The \( \textit{bc} \) reductase activity was unchanged in mutant cells of either background (Fig. 5B and data not shown). Consistent with the attenuated CcO activity, the steady-state levels of Cox1, Cox2, and Cox3 were reduced in the \( \textit{coa4} \) null mutant (Fig. 5C). The abundance of the \( \textit{bc}:\textit{CcO} \) supercomplex was also markedly diminished (Fig. 5D) without any change in the ATP synthase monomeric or dimeric complexes. Solubilization of the IM with dodecyl maltoside further confirmed the attenuated levels of CcO (Fig. 5D). Although Coa4 appears to have a role in the assembly or stability of the CcO complex, it is not associated with the CcO supercomplex (Fig. 5G), as Coa4 fractionates in low-mass fractions upon sucrose gradient centrifugation.

Deletions of genes encoding CcO assembly factors often lead to diminished Cox1 translation by trapping Mss51 in Cox1
In vivo radiolabeling of mitochondrial translation products revealed that normal levels of Cox1 were synthesized during the pulse and short-chase phase of the translation assay (Fig. 5E), suggesting that coa4Δ cells are not subjected to the downregulation of the Cox1 synthesis seen for shy1Δ cells and several CcO assembly mutants (6). Deletion of COA4 in a shy1Δ background retains the attenuated level of Cox1 synthesis seen with shy1Δ cells. Thus, Coa4 is downstream of the Shy1 effect on Cox1 synthesis. To confirm that the Cox1 assembly intermediates, including those stabilized by Mss51 and Shy1 are normal in coa4Δ cells, we performed BN-PAGE on Myc epitope-tagged Shy1 (Fig. 5F). The levels of abundance of the high-mass Shy1-Cox1 complex were similar in wild-type and coa4Δ cells, substantiating the conclusion that Cox1 synthesis and early maturation steps are normal in the mutant cells. Furthermore, no interaction was observed between Coa4 and Shy1 by direct coimmunoprecipitation (data not shown).

Since shy1Δ cells are impaired in mitochondrial copper, we quantified the copper content of mitochondria from coa4Δ cells. Mitochondrial copper was reduced to 50% of wild-type levels in both shy1Δ and coa4Δ cells (data not shown), but supplemental copper failed to enhance the respiratory deficiency of coa4Δ cells.
of which are involved in the early assembly stages of Cox1 maturation. This further implies, along with the in vivo translation data, that Coa4 is important in the assembly of CcO after the maturation of Cox1.

To identify a high-copy-number suppressor that restores respiratory growth of coa4/H9004 cells, we transformed a high-copy-number DNA library into the null strain and recovered respiratory-competent clones from 56,000 transformants. Twenty clones contained a DNA fragment encompassing the gene CYC1 encoding the soluble electron carrier cytochrome c. CYC1 as well as CYC7 were subcloned into pRS425 and transformed into coa4/H9004 cells. Cyc1 is the dominant cytochrome c present in aerobic yeast, whereas Cyc7 is an isoform expressed in hypoxic cells (11). Overexpression of either Cyc1 or Cyc7 in coa4/H9004 cells restored respiratory growth at room temperature and 30°C (Fig. 6A), as well as CcO activity (Fig. 6B). Restoration of the CcO:bc1 supercomplexes was also observed with overexpression of CYC1 or CYC7 in coa4Δ cells (Fig. 6C).

Cytochrome c is known to be important for the assembly and stability of CcO in yeast and mammals (5, 44), although the mechanism remains to be elucidated. To assess whether Cyc1 is deficient in coa4Δ cells, mitochondria isolated from wild-type and coa4Δ cells were subjected to SDS-PAGE and subjected to the in-gel heme staining. A total of 1 µg of horse heart cytochrome c (Cyth) served as a control. Coomassie staining was used to show that the same amount of the total mitochondrial protein is loaded.

FIG. 6. Overexpression of cytochrome c suppresses the respiratory defect in coa4Δ cells. (A) Respiratory growth of the BY4743 wild-type and coa4Δ cells transformed with episomal vectors expressing CYC1 or CYC7 was analyzed as described in the legend to Fig. 5A. RT, room temperature. (B) BN-PAGE analysis of the respiratory complexes as previously described in the legend to Fig. 2. (D) Steady-state levels of Cox1, Cyc1, and Cyt1 analyzed by immunoblotting. (E) Isolated mitochondria (75 µg) were separated by SDS-PAGE and subjected to the in-gel heme staining. A total of 1 µg of horse heart cytochrome c (Cyth) served as a control. Coomassie staining was used to show that the same amount of the total mitochondrial protein is loaded.
bc₁ complex, oppositely charged residue pairs contribute to long-range coulombic interactions (34). A series of Lys→Glu charge reversal mutations were engineered in Cycl to test whether attenuation in this interaction alters the suppressor activity of Cycl. Two different double mutants carrying K₇⁸E K₉⁹E and K₉⁹E K₉⁹E and a quadruple mutant containing the four Lys→Glu substitutions were tested for suppressor activity in coa₄Δ cells as well as Cycl function in cyc1Δ cyc7Δ double null cells (Fig. 7A). Addition of the quad Cycl mutant to the cyc1Δ cyc7Δ double mutant failed to restore respiratory growth, but suppressor activity in coa₄Δ cells was only partially impaired (Fig. 7A). The different Cycl mutants were all expressed as stable proteins and could be observed by steady-state immunoblotting (Fig. 7C). Expression of a second Cycl mutant (the W₆₅S mutant) that impairs Cycl function (5, 44) in coa₄Δ cells failed to restore respiratory growth (data not shown), although interpretation of this result is complicated by the reported inefficient mitochondrial import of this mutant protein (5, 44). Whereas Cycl and Cyc7 were efficient suppressors of the respiratory defect of coa₄Δ cells, neither protein alone upon overexpression facilitated respiratory growth of the Shy1 G¹³⁷E mutant cells (Fig. 7B).

To gain insights into the mechanism by which Cycl restores CcO function in coa₄Δ cells, we sought to investigate the membrane association of Cycl and stability of CcO in WT versus coa₄Δ cells. Cycl is associated with the inner membrane by forming transient encounter complexes with bc₁ and CcO, primarily by electrostatic interactions in addition to hydrophobic contacts with the lipid bilayer that involve cardiolipin (36, 42, 43). First, mitoplasts isolated from each cell were treated with increasing salt concentrations or proteinase K, and membrane association of Cycl was assessed by immunoblotting with increasing salt concentrations or proteinase K, and membrane association of Cycl and stability of CcO in WT and coa₄Δ cells was analyzed as described in the legend to Fig. 1. (B) Respiratory growth of the BY4743 wild-type and shy1Δ cells transformed with episomal vectors expressing wild-type CYC1 and CYC7. (C) Expression of the wild-type and mutant forms of Cycl in whole-cell extracts assayed by immunoblotting. Whole-cell proteins were precipitated with 20% trichloroacetic acid (TCA) and resolved on 15% SDS-PAGE. Pgk1 was used as a loading control.

**FIG. 7.** Charge reversal mutations in cytochrome c retain residual suppressor activity for coa₄Δ cells but not cyc1Δ cyc7Δ mutant cells. (A) Respiratory growth of the BY4743 wild-type and coa₄Δ cells transformed with episomal vectors expressing wild-type and mutant forms of CYC1 or wild-type CYC1 was analyzed as described in the legend to Fig. 1. (B) Respiratory growth of the BY4743 wild-type and shy1Δ cells transformed with episomal vectors expressing wild-type CYC1 and CYC7. (C) Expression of the wild-type and mutant forms of Cycl in whole-cell extracts assayed by immunoblotting. Whole-cell proteins were precipitated with 20% trichloroacetic acid (TCA) and resolved on 15% SDS-PAGE. Pgk1 was used as a loading control.

**DISCUSSION**

Studies of three missense SURF1 mutations identified in patients with LS were evaluated with the yeast Shy1 protein. The G¹³⁷E substitution in Shy1 results in a nonfunctional protein conferring a CcO deficiency. The G¹³⁷E Shy1 mutation also recapitulates other SHY1 deletion phenotypes, including inefficient heme a insertion and low mitochondrial copper. The inefficient heme a insertion activity likely arises from the destabilization of bc₁;CcO supercomplexes. However, the attenuation in membrane association of Cycl in coa₄Δ cells suggests that Coa4 may have a role in the membrane stabilization of Cycl, which is likely important for its additional role in CcO assembly.

Studies of three missense SURF1 mutations identified in patients with LS were evaluated with the yeast Shy1 protein. The G¹³⁷E substitution in Shy1 results in a nonfunctional protein conferring a CcO deficiency. The G¹³⁷E Shy1 mutation also recapitulates other SHY1 deletion phenotypes, including inefficient heme a insertion and low mitochondrial copper. The inefficient heme a insertion activity likely arises from the destabilization of the ~450-kDa Shy1/Cox1 assembly intermediate. The lack of a robust CcO deficiency of the F²⁴⁹T Shy1 mutant shows near normal CcO assembly in yeast, the steady-state levels of the Shy1/Cox1 assembly intermediate are partially attenuated. This attenuation of the complex may have a more significant impact on CcO assembly in human LS patients.

The loss of function in the G¹³⁷E Shy1 mutant created an opportunity for a genetic suppressor screen focusing on allele-specific suppressors that would shed further insights on the Shy1 function in CcO biogenesis and potentially provide information on protein interactions. An abnormal protein can at times be restored to greater functionality by changes in an
interacting partner. Presently, COA2, COA4, and COXI0 were found to be allelic suppressors of the G^{137}E Shy1 mutant.

Coa4 is a newly identified CoO assembly factor. Its initial characterization was recently reported in a comprehensive study of the 14 twin CX9C motif proteins in yeast (23). As with the other twin CX9C proteins, Coa4 is an IMS protein whose import is dependent on the Mia40 machinery (23). Cells lacking Coa4 in two distinct genetic backgrounds have reduced CoO activity and steady-state protein levels but do not show a reproducible change in the bc1 complex. The respiratory defect is most pronounced at room temperature and minimal at 37°C. Mitochondrial translation of Cox1-Cox3 appears normal in coa4Δ cells. Many CoO assembly mutants are impaired in Cox1 synthesis, but coa4Δ cells do not fall into that group. This is consistent with the lack of significant suppression of the respiratory defect by overexpression of MSS51 or other factors (COX10 and COA1) that stabilize newly synthesized Cox1. In addition, the Shy1/Cox1 assembly intermediate is wild type in coa4Δ cells. Thus, the CoO assembly/stability impairment in coa4Δ cells is not related to a defect in Cox1 maturation. No interaction of Coa4 and Shy1 or the bc1:CcO supercomplex was evident.

Cells lacking Coa4 resemble shy1Δ cells in exhibiting a reduced mitochondrial copper content. The matrix copper content was similarly reduced in these deletion strains as well as coa1Δ cells (~50% of that of the wild type). Whereas copper supplementation enhanced respiratory growth of coa1Δ and shy1Δ cells, it was less effective in promoting respiratory growth of coa4Δ cells. We postulated previously that Coa1 and Shy1 may, in addition to their roles in Cox1 maturation, modulate Cu(I) import into the matrix or export to the IMS for CcO biogenesis (29). Coa4 may likewise be part of the Cu(I) routing pathway.

A major clue to the function of Coa4 was the isolation of Cyc1 as an extragenic suppressor of the respiratory defect of coa4Δ cells. Cyc1 is important for the assembly and/or stability of CcO (5, 44), and this is the first time that Cyc1 has been found as a suppressor of a CoO mutant. High-copy-number CYC1 is an efficient suppressor of coa4Δ cells but not Shy1 G^{137}E mutant cells. Elevated Cyc1 was effective despite the lack of diminution in chromosomally encoded Cyc1 levels in coa4Δ cells. The suppressor activity of Cyc1 was attenuated but not eliminated by a quadraple Cyc1 mutant with four Lys→Glu substitutions in the docking pocket that affects interactions with Cox2. However, quadraple mutant Cyc1 fails to support respiratory growth in cells lacking WT Cyc1 and Cyc7.

For the suppression of the coa4Δ cell respiratory phenotype by elevated Cyc1 may relate to an observed decrease in membrane association of Cyc1. Membrane association of Cyc1 occurs by electrostatic interactions of encounter complexes with bc1, CcO, and the lipid bilayer as well as hydrophobic effects with the bilayer (36, 42, 43). Cardiolipin contributes to the membrane anchoring of Cyc1 (36). The observed suppressive effect by Cyc1 may indicate that the phospholipid domain encompassing CcO is altered in composition in coa4Δ cells. Alternatively, other membrane proteins have been suggested to mediate the membrane association of Cyc1 with CcO (35). Either membrane lipid composition or a putative protein mediating Cyc1 docking may contribute to the known role of Cyc1 in CcO biogenesis and/or stability. There is no indication that Coa4 contributes directly to binding Cyc1, as no coimmunoprecipitation was observed between these two proteins. The attenuated CcO observed in coa4Δ cells, therefore, may arise from impaired stability of the complex that could have a marked impact on supercomplexes with bc1. Cardiolipin is important for Cyc1 membrane association as well as

FIG. 8. Association of cytochrome c with the inner mitochondrial membrane and CcO stability are decreased in coa4Δ mitochondria. (A) Mitochondria isolated from wild-type and coa4Δ cells were osmotically shocked (SW+) or left intact (SW−). Swollen mitochondria were incubated with NaCl at indicated concentrations for 20 min on ice. Then, all samples were incubated with or without PK as described in the legend to Fig. 4D. Following the incubations, organelles were fractionated, and obtained pellets subjected to SDS-PAGE and analyzed by Western blotting using antisera against Cyc1, Cox1, Cox2, Cox3, and Cox4. Note that the exposure time for the immunoblots with coa4Δ cells was longer due to the reduced steady-state levels of CoO subunits. (B) Mitochondria (50 μg) from BY4743 wild-type, coa4Δ, coa1Δ, and coa4Δ cells were treated as described above and subjected to SDS-PAGE followed by immunoblotting with anti-Cyc1 and anti-Aco1. The bar graph showing the abundances of the membrane-bound Cyc1, retained after the salt treatment of wild-type, coa4Δ, coa1Δ, or coa1ΔA mitoplasts. Quantiﬁcations were done using Image J software; the error bars indicate SD; n = 2.
supercomplex formation, so if Coa4 had a role in the membrane lipid composition, the observed phenotypes of attenuated CcO and Cycl membrane association may be explained. Future studies will address whether Coa4 affects the membrane lipid composition of the IM in a way that would be important for CcO assembly.

In addition to COA4, COA2 and COX10 were also found to be allelic suppressors of the G137E Shy1 mutant. COA2 and COX10 were shown previously to be weak suppressors of the shyΔ respiratory defect (30). Their suppression activity is enhanced by the presence of the G137E Shy1 mutant. Coa2 appears to have a role concurrent with Shy1 in the assembly of the Cu₃:heme a₉ redox center (7). Although Coa2 and Shy1 transiently interact (30), the mechanism of suppression by Coa2 of the G137E Shy1 mutant is unclear. Coa2 is membrane associated on the matrix side of the IM, whereas the Gly137 residue exists within the IMS domain of Shy1. Since the G137E Shy1 mutant fails to stably form the ~450-kDa Cox1 assembly intermediate visualized by BN-PAGE, high levels of Coa2 may enhance respiratory growth by stabilizing this important assembly intermediate, permitting Cu₃:heme a₉ site formation. Alternatively, the G137E Shy1 mutant may stabilize Coa2, enhancing its Cox1 chaperone activity.

The allelic suppression by COX10 may occur somewhat differently, as no evidence exists for a physical interaction between Cox10 and Shy1. Cox10 functions in the first step in heme biosynthesis and Shy1 functions in the hemylation of Cox1, so the intersection of Cox10 and Shy1 is rational. High levels of Cox10 may enhance the hemylation of Cox1 that may be compromised in the G137E Shy1 mutant cells. The catalytic function of Cox10 appears linked to its homo-oligomerization (7). We demonstrated that a N198K substitution in Cox10 by-passed the inefficient hemylation of Cox1 in cells lacking Coa2 through enhanced stability of the Cox10 oligomer (7). The G137E Shy1 mutant may likewise contribute in some way to Cox10 oligomerization, a process that is markedly impaired in shyΔ cells. Another potential mechanism may relate to the recent observation that bacterial Surf1 is a heme-binding protein (10). An H140A Surf1 mutant had reduced heme binding. Mutations of either of the two conserved His residues (H141 and H341) in yeast Shy1 resulted in no defect in Shy1 function, and cells harboring a double His substitution showed only a modest growth impairment on glycerol-lactate (data not shown). Thus, it is unlikely that the G137E Shy1 suppression by Cox10 relates to heme binding to Shy1.

In summary, characterization and allelic suppression studies of the Shy1 G137E mutation have led to a better understanding of the CcO assembly pathway and the factors involved in this process. A novel assembly factor, Coa4, was identified through these studies and found to be genetically linked to the CcO assembly function of Cycl.

ACKNOWLEDGMENTS

This work was supported by grant from the National Institutes of Health ES03817 to D.R.W. M.B. was supported by predoctoral training grant T32 DK07115 and a University of Utah Graduate Research Fellowship. H.K. is supported by postdoctoral grant T32 DK07115.

We acknowledge the assistance of Pam Smith for ICP measurements of mitochondrial copper.

REFERENCES


24. Maskhevik, G., B. Repetto, D. M. Glerum, C. Jin, and A. Tzagoloff. 1997. SHY1, the yeast homolog of the mammalian SURF1 gene, encodes a


