Missense mutation of the COQ2 gene causes defects of bioenergetics and de novo pyrimidine synthesis

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Coenzyme Q10 (CoQ10) deficiency has been associated with an increasing number of clinical phenotypes that respond to CoQ10 supplementation. In two siblings with encephalomyopathy, nephropathy and severe CoQ10 deficiency, a homozygous mutation was identified in the CoQ10 biosynthesis gene COQ2, encoding polyisoprenyl-p-hydroxybenzoate transferase. To confirm the pathogenicity of this mutation, we have demonstrated that human wild-type, but not mutant COQ2, functionally complements COQ2 defective yeast. In addition, an equivalent mutation introduced in the yeast COQ2 gene also decreases both CoQ6 concentration and growth in respiratory-chain dependent medium. Polyisoprenyl-p-hydroxybenzoate transferase activity was 33–45% of controls in COQ2 mutant fibroblasts. CoQ-dependent mitochondrial complexes activities were restored in deficient fibroblasts by CoQ10 supplementation, and growth rate was restored in these cells by either CoQ10 or uridine supplementation. This work is the first direct demonstration of the pathogenicity of a COQ2 mutation involved in human disease, and establishes yeast as a useful model to study human CoQ10 deficiency. Moreover, we demonstrate that CoQ10 deficiency in addition to the bioenergetics defect also impairs de novo pyrimidine synthesis, which may contribute to the pathogenesis of the disease.

INTRODUCTION

Coenzyme Q10 (CoQ10) is a vital molecule that transports electrons from mitochondrial respiratory chain complexes I and II to complex III (1). In addition, it functions as a cofactor for uncoupling proteins (2), as an antioxidant stabilizing plasma membrane and a regulator of the extracellularly-induced ceramide-dependent apoptotic pathway (1,3). CoQ10 also enhances survival of chemotherapy-treated cells (4) and is required for the stabilization of complex III in mitochondria (5). At least nine nuclear gene (COQ) products are involved in the CoQ biosynthesis complex pathway in yeast, which have homologous genes in all the species currently studied (6). This pathway and its regulation are still incompletely understood (1). CoQ10 deficiency (MIM 607426) has been associated with clinically heterogenous diseases, which have been delineated into five major phenotypes: (i) an encephalomyopathic form (7,8), (ii) a predominantly ataxic form with cerebellar atrophy (9,10), (iii) a multisystem infantile variant with brain and renal disease (11,12), (iv) Leigh syndrome (13) and (v) isolated myopathy (14,15). It is anticipated that mutations in COQ genes or other components of the CoQ10 biosynthetic pathway are likely to cause these diverse syndromes (1).

We have recently demonstrated that two siblings with CoQ10 deficiency harbor a homozygous c.890 A>G mutation in the COQ2 gene (MIM 609825) (16), which encodes polyisoprenyl-p-hydroxybenzoate transferase (17). This mutation changes amino acid 297 from tyrosine into cysteine, in a conserved trans-membrane domain (16). To demonstrate
pathogenicity of this mutation, we performed functional complementation in *Saccharomyces cerevisiae* using the BY4741Δcoq2 strain, which is unable to grow in non-fermentable carbon source. We have studied the role of CoQ10 supplementation on both respiratory chain complexes I + III and II + III and growth in deficient fibroblasts. Also, we have shown the requirement of uridine for maintaining growth of deficient fibroblasts demonstrating the dependence of these cells on pyrimidine biosynthesis.

**RESULTS**

**Complementation of ΔCOQ2 yeast**

Figure 1A compares the 5′-nucleotide sequences of the cloned cDNA and shows that the human gene contains four ATG initiation codons. The third ATG produces a transcript of similar length to the yeast gene. Wild-type human COQ2 (hCOQ2) and yeast COQ2 (yCOQ2) cDNA sequences were sub-cloned into the yeast expression vector pYES2.1-TOPO TA/V5-His with a GAL promoter, and transformed into BY4741Δcoq2 strain, a null mutant strain for this gene. Increased growth in glycerol was observed when either homologous yCOQ2 or heterologous third ATG-initiated hCOQ2 was expressed in BY4741Δcoq2 (Fig. 1B). Lower growth was observed with cDNA initiated at the first or second ATG, and no growth was detected with hCOQ2 starting at the fourth ATG (Fig. 1B). The reported full-length hCOQ2 has been cloned as a hybrid molecule: the 3′-sequence was obtained from mRNA while the 5′-sequence was amplified from genomic DNA due to difficulties in amplifying the 5′-region of the mRNA (17). Complementation of COQ2 null yeast with this cDNA (initiating at the first ATG) induced ~50% growth at 120 h compared with yCOQ2 complementation (18), a similar growth rate as those we have obtained with first ATG hCOQ2 (Fig. 1B).

**Localization of hCoq2p in yeast mitochondria**

To determine the localization of human Coq2p protein in yeast mitochondria, an immunoblot was developed with an antibody raised against the human Coq2 peptide (Fig. 1C). This protein was not detected in either wild-type yeast or coq2 mutant strains, but it was detected in the mitochondria of both Δcoq2:hCOQ2 and HeLa cells. The apparent molecular weight of Coq2p in HeLa cells is higher probably because of a different processing of the precursor polypeptide in yeast.

**Complementation efficiency of mutated COQ2**

To determine whether the mutation in hCOQ2 gene (16) caused a loss of function of the encoded protein affecting both CoQ biosynthesis and respiration, we carried out complementation experiments transforming the yeast yCOQ2 null mutant strain with cDNA cloned from patients, and the yeast gene engineered to harbor the c.783 A>G mutation (equivalent to hCOQ2 c.890 A>G mutation). Cells expressing the mutant yeast gene displayed a lower growth rate than those expressing the wild-type gene (Fig. 2B), demonstrating that functional complementation is sufficiently sensitive to study the effect of a missense mutation in a CoQ biosynthetic gene. Also, transformation of BY4741 Δcoq2 yeast strain with mutated hCOQ2 showed a considerably lower respiration-dependent growth than with wild-type hCOQ2, which was, however, still significantly increased compared with the deleted strain (Fig. 2C).

**Effect on CoQ biosynthesis**

Growth results correlated with the content of Coenzyme Q₆ (CoQ₆) in the different transformed strains (Table 1). The expression of yCOQ2 caused an accumulation of CoQ₆ in transformed yeasts in the range of the wild-type strain, and strains harboring yCOQ2 (c.783 A>G) attained CoQ₆ levels ~59% of wild-type. These results demonstrate the deleterious effect of the mutation on the Coq2 protein, which, however, apparently retains some residual enzymatic activity. The expression of wild-type hCOQ2 gene increased CoQ₆ levels to ~64% of control, while expression of the mutated sequence...
produced CoQ6 levels that were only 11% of transformed cells with the human wild-type allele.

Analysis of demetoxy-Q6 (DMQ) levels, an intermediate of coenzyme Q biosynthesis, showed an increase of DMQ relative to CoQ6, in strains transformed with the mutated genes (Table 1), indicating an inhibition of the biosynthetic process also downstream of CoQ2p.

**COQ2 expression in patient’s fibroblasts**

We then analyzed the expression of COQ2 in patient’s fibroblasts by real-time-PCR (Fig. 3A). mRNA levels of this gene were significantly increased in patients harboring COQ2 mutations (16) compared with controls and other CoQ10-deficient fibroblasts isolated from ataxic patients, which do not harbor mutations in this gene (10). Furthermore, immunoblot analysis of Coq2p in fibroblasts demonstrated that cells from both P1 and P2 patients contained higher amounts of protein than both control and P3 patient fibroblasts (Fig. 3B). We also measured polyprenyl-pHB transferase activity using a sensitive radioactive method (19). This activity was significantly lower in fibroblasts of patients with hCOQ2 (c.890 A>G) gene (Fig. 3C) relative to controls and CoQ10-deficient fibroblasts from ataxic patients (10). Increased expression of both mRNA and protein appears to be a compensatory mechanism for the enzymatic deficiency in the patient’s fibroblasts.

**Effect of CoQ10 on mitochondrial complexes activities**

It has been shown that CoQ added to human cells reaches the inner mitochondrial membrane and acts on the respiratory chain (20). To demonstrate whether CoQ10 deficiency was responsible for mitochondria defects observed in CoQ10-deficient patients (12), fibroblasts were incubated for 24 h with CoQ10, and both complexes I + III and II + III activities were determined (Table 2). Both activities were significantly increased in deficient fibroblasts, which were not affected in control fibroblasts.

**Effects of CoQ10 and uridine on fibroblasts growth rates**

CoQ10 deficient fibroblasts showed slow rates of growth. To demonstrate that this phenotype was due to CoQ10 deficiency, cells (100 ± 20 cells/cm²) were seeded and grown in a medium supplemented with serum-solubilized CoQ10. Growth was significantly increased in deficient fibroblasts incubated with a supplement of CoQ10 (Fig. 4A). CoQ10 is required for the biosynthesis of pyrimidine nucleotides because it is an essential co-factor for dihydro-orotate dehydrogenase, an enzyme located in the inner mitochondrial membrane (21). To demonstrate that the deficiency of CoQ10 affects pyrimidine supply, and consequently cell growth, we incubated CoQ10-deficient fibroblasts with uridine and observed that growth was also increased compared with both control and respiratory chain-deficient fibroblasts from a patient with mitochondrial encephalomyopathy without CoQ10 deficiency (Fig. 4B). No cumulative effect of uridine and CoQ10 was noted (data not shown).

**DISCUSSION**

Despite the fact that CoQ10 deficiency has been described more than 15 years ago, its genetic bases have remained elusive until this year, when mutations were identified in two genes involved in CoQ10 biosynthesis, COQ2 and PDSS2 (16,22). The product of COQ2 catalyzes the transfer of para-hydroxybenzoate to the polyprenyl chain, one of the initial steps of CoQ biosynthesis. The aim of this work was to demonstrate the pathogenicity of the missense COQ2 mutation found in our patients using a functional complementation approach in S. cerevisiae, and to analyze its consequences on the mitochondrial electron transport chain and cell proliferation.
Human COQ2 contains four possible ATG codons on exon 1. The reported hCOQ2 sequence starts from ATG number 1. We observed complementation with constructs initiating from each of the first three ATG, but not with ATG number 4 possibly because the sequence between the third to fourth ATGs contains a critical signal for mitochondrial importation in yeast (18).

The highest complementation efficiency was achieved with constructs initiating at the third ATG, this sequence is the most similar to the yeast mitochondrial importation pre-sequence. However, analysis of COQ2 genes in other mammalian species such as dog and rat revealed that they contain only one possible ATG initiation codon, which corresponds to the human fourth ATG. We are currently investigating the functional significance of the four ATG codons in human cells.

Both the mutated human COQ2 gene and its yeast homolog engineered to harbor the corresponding mutation, failed to fully complement COQ2 deficient yeast strain. Nevertheless, complementation studies clearly indicate that mutated proteins still retain some enzymatic activity because yeast can grow on non-fermentable substrates and accumulate some amount of CoQ6, although at a significantly lower rate than the wild-type strains. Direct biochemical assays on patients P1 and P2 fibroblasts confirm the presence of residual enzymatic activity, which correlates with the amount of Coq2p detected by western blot. The residual Coq2 function is probably essential for embryogenesis, and may explain why the patients did not present symptoms of the disease until 1 year of age. In both patients P1 and P2, COQ2 mRNA and Coq2 peptides are also hyper-expressed, suggesting that there is a feedback control mechanism regulating COQ2 expression in cells.

Interestingly, yeast cells transformed with the mutated genes show accumulation of DMQ, an intermediate of coenzyme Q biosynthesis that is synthesized by reactions downstream of Coq2p. This apparent paradox can be explained by the fact that enzymes that catalyze coenzyme Q biosynthesis in yeast are thought to function in a multienzyme complex (20). In this case, the mutation in Coq2 proteins not only impairs enzymatic function of Coq2p, but probably affects the whole complex, interfering also with some of the downstream enzymatic steps.

In a second set of experiments, we analyzed the effect of COQ2 deficiency in fibroblasts. We have previously shown that a CoQ10 analogue, decyl-ubiquinone, can rescue the complex II + III enzymatic defect in vitro (12). We have now checked the effect of ubiquinone supplementation in vivo on cell proliferation and mitochondrial complexes activities. Supplementation of human cells with different isoforms of CoQ shows that a small amount can reach inner mitochondrial membrane (23). Using this approach, we have shown that long-term incubation of deficient fibroblasts with CoQ10 increases significantly the activity of both complexes I + III and II + III. These results support the cause of mitochondrial dysfunction on the deficiency, and would explain the positive results observed after the treatment of deficient patients with CoQ10 (10,12).

We then analyzed if we could modulate the growth deficient phenotype in these cells. Surprisingly, the effect of uridine addition was even more pronounced than that of CoQ10.
suggesting that this phenotype is largely due to an insufficient supply of nucleotides rather than to an impairment of ATP production. We believe that the relative lower efficiency of CoQ10 in rescuing the defect in proliferation is probably due to a low efficiency of its incorporation in cells (1,23).

Taken together, our results demonstrate the pathogenicity of the COQ2 mutation and that yeast is an optimal model to study the effects of mutations in COQ genes, because it is sensitive enough to unveil defects due to missense mutations. The high homology of yeast and human COQ genes may allow complementation studies to identify other human genes involved in CoQ10 deficiency. Moreover, we demonstrate that the pathogenesis of CoQ10 deficiency is related not only to a defect in bioenergetics, but also to an impairment of pyrimidine metabolism.

**Materials and Methods**

**Fibroblast cultures**

Fibroblasts from CoQ10 deficient patients (P1 and P2) (12,16), from patients with cerebellar ataxia and CoQ10 deficiency (10) and controls were plated in separate six-well plates (40 000 cells/well) and cultured using DMEM with 20% fetal calf serum (FCS). Also, HeLa cells were grown in a similar procedure. Supplemental CoQ10 pre-diluted in FCS was added to the plates at a final concentration of 10 μM.

**Cloning of human and yeast COQ2**

yCOQ2 was cloned into pEGFP-N1 (Promega) and in pYES2.1V5HisTOPO (Invitrogen) vectors using primers listed in Table 3. Initially, hCOQ2 was cloned in a two-step protocol similarly to what has been described (17). pEGFPN1 was used as intermediate vector because of convenient cloning sites. The 5’ portion corresponding to each 0 COQ portion was directly cloned from reverse transcriptase (RT)-PCR amplified from total fibroblast RNA in

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence 5' → 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCOQ2-Ext(ATG1)</td>
<td>5'-ATGACCGCAATTTCAACA-3'</td>
</tr>
<tr>
<td>hCOQ2-Ext(ATG2)</td>
<td>5'-GGAGGATGAGGAAAGTCT-3'</td>
</tr>
<tr>
<td>hCOQ2-Fint &amp; Rint</td>
<td>5'-TGTACCAATGATATATACATGTC-3'</td>
</tr>
<tr>
<td>yCOQ2-Ext</td>
<td>5'-TGGACCGAAGAAGTTATATCAACTA-3'</td>
</tr>
<tr>
<td>yCOQ2-Ext</td>
<td>5'-TTCAGAAGCCACAGCCTGTC-3'</td>
</tr>
<tr>
<td>yMUTAG-F &amp; R</td>
<td>5'-GATATGTTGCGATATATCCGGTG-3'</td>
</tr>
<tr>
<td>yMUTAG-F &amp; R</td>
<td>5'-GCATATGTTGCGATATATCCGGTG-3'</td>
</tr>
</tbody>
</table>

Bold-face character is the mismatched nucleotide used in site-directed mutagenesis.

**Table 2.** Effect of supplementation of CoQ10 on the CoQ-dependent mitochondrial complexes activities in human fibroblasts

<table>
<thead>
<tr>
<th>Samples</th>
<th>Complex I + III (nmol/min/mg protein)</th>
<th>10 μM CoQ10</th>
<th>Complex II + III (nmol/min/mg protein)</th>
<th>10 μM CoQ10</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>Control</td>
<td>410 ± 36</td>
<td>445 ± 27</td>
<td>No addition</td>
</tr>
<tr>
<td>P1</td>
<td>248 ± 25</td>
<td>386 ± 44**</td>
<td>470 ± 37</td>
<td>3.6 ± 0.12**</td>
</tr>
<tr>
<td>P2</td>
<td>301 ± 37</td>
<td>480 ± 56**</td>
<td>3.1 ± 0.15**</td>
<td></td>
</tr>
</tbody>
</table>

*Significant versus no addition.

**Table 3.** Oligonucleotides used in PCR amplifications

<table>
<thead>
<tr>
<th>Primer name</th>
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<tbody>
<tr>
<td>hCOQ2-F</td>
<td>5'-ATGACCGCAATTTCAACA-3'</td>
</tr>
<tr>
<td>hCOQ2-R</td>
<td>5'-GGAGGATGAGGAAAGTCT-3'</td>
</tr>
</tbody>
</table>

**Figure 4.** Growth of CoQ10 deficient fibroblasts can be restored by exogenous CoQ10 or uridine. (A) Addition of CoQ10 to culture media containing 20% FCS induced significant increases of growth of fibroblasts of patients harboring the COQ2 mutation compared with control fibroblasts. Black bars: no CoQ10. Grey bars: plus 10 μM CoQ10. a: Significant versus no addition of uridine. (B) Growth of fibroblasts in media containing 10% FCS supplemented with 10 μM uridine. Black bars: control fibroblasts; grey bars: fibroblasts harboring the COQ2 mutation (patient 2). a: Significant versus no addition of uridine (P < 0.05).
a single reaction in order to analyze patient samples. Site-directed mutagenesis of wild-type yCOQ2 was carried out by PCR using primers yMUTAG-F and yMUTAG-R. All inserts were directly sequenced using standard protocols and the ABI-Prism 310 automated sequencer (Perkin Elmer).

Yeast complementation analysis
Yeast coq2 mutants were transformed with the pYES2 vector containing the different versions of hCOQ2, and both wild-type and site-directed mutant yeast COQ2 and the empty vector for complementation experiments. Growth rate on non-fermentable carbon source (YPG medium) was determined as a marker of functional complementation.

CoQ determination
CoQ10 was extracted after addition of 4 ml hexane-ethanol (5/2 v/v) and vortexed for 2 min. After centrifugation at 1000 g at room temperature for 5 min, the upper phase was carefully transferred into a 20-ml glass scintillation vial (performed twice for each sample). The combined extract was evaporated to 4 l of the extract was added to 4 ml hexane-ethanol (5/2 v/v) and vortexed for 2 min. After centrifugation at 1000 g at room temperature for 5 min, the upper phase was carefully transferred into a 20-ml glass scintillation vial (performed twice for each sample). The combined extract was evaporated under a gentle stream of N2 gas and the residue was dissolved to 4 l of the extract was added to 4 ml hexane-ethanol (5/2 v/v) and vortexed for 2 min. After centrifugation at 1000 g at room temperature for 5 min, the upper phase was carefully transferred into a 20-ml glass scintillation vial (performed twice for each sample). 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Mitochondrial enzyme activities
Mitochondria-enriched fractions were obtained according to Fernández-Ayala et al. (23). and used to determine complex I + III and II + III activities. In both cases, mitochondria were incubated in 40 mM sodium phosphate buffer pH 7.5 plus 0.25 mM KCN and containing either 0.2 mM NADH or 5 mM succinate, and reduction of beef-heart cytochrome c (0.5 mM) was monitored spectrophotometrically at 550 nm for 5 min. To discriminate the rotenone-sensitive NADH-cytochrome c reductase activity (Complex I + III) from the rotenone-insensitive NADH-cytochrome c reductase activity due to the NADH-cytochrome b1 reductase located in the outer mitochondrial membrane, 5 lM rotenone was added for the last 2 min of incubation.

Statistical analysis
All results are expressed as mean ± SEM. Serial measurements were analyzed by using two-way ANOVA with Tukey’s post-hoc test using SigmaStat software from SPSS Science (Chicago, IL). The level of significance was set at P < 0.05.

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Conflict of Interest statement. None declared.

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