Impaired complex III assembly associated with BCS1L gene mutations in isolated mitochondrial encephalopathy

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We investigated two unrelated children with an isolated defect of mitochondrial complex III activity. The clinical picture was characterized by a progressive encephalopathy featuring early-onset developmental delay, spasticity, seizures, lactic acidosis, brain atrophy and MRI signal changes in the basal ganglia. Both children were compound heterozygotes for novel mutations in the human bc1 synthesis like (BCS1L) gene, which encodes an AAA mitochondrial protein putatively involved in both iron homeostasis and complex III assembly. The pathogenic role of the mutations was confirmed by complementation assays, using a ΔBcs1 strain of Saccharomyces cerevisiae. By investigating complex III assembly and the structural features of the BCS1L gene product in skeletal muscle, cultured fibroblasts and lymphoblastoid cell lines from our patients, we have demonstrated, for the first time in a mammalian system, that a major function of BCS1L is to promote the maturation of complex III and, more specifically, the incorporation of the Rieske iron–sulfur protein into the nascent complex. Defective BCS1L leads to the formation of a catalytically inactive, structurally unstable complex III. We have also shown that BCS1L is contained within a high-molecular-weight supramolecular complex which is clearly distinct from complex III intermediates.

INTRODUCTION

Mitochondrial complex III (cytochrome bc complex, ubiquinol:cytochrome c oxidoreductase, CIII) catalyzes the electron transfer from succinate and nicotinamide adenine dinucleotide-linked dehydrogenases to cytochrome c in the respiratory chain. In mammals, the active enzyme is a dimer (CIII₂), where each monomer is composed of 11 different polypeptide subunits (1). Ten subunits are encoded by nuclear genes, whereas only one (cytochrome b) is encoded by the mitochondrial genome. In yeast, and possibly in mammals as well, the formation and function of the complex are accomplished through the action of several other nuclear gene products, which for instance provide the synthesis and incorporation of prosthetic groups and the assembly of the protein backbone.

Although rare, isolated CIII deficiency has been detected in a heterogeneous group of neuromuscular and non-neuromuscular disorders in children and adults, ranging from rapidly progressive encephalomyopathies with multi-organ failure to slowly progressive pure myopathies. Different pathogenic mutations in MT-CYTB, the mtDNA gene encoding cytochrome b, have been described (2), whereas nuclear mutations have so far been identified only in two genes: the bc1 synthesis like (BCS1L) gene, encoding the human homolog of the yeast protein bcs1p (7), a mitochondrial protein of the AAA (ATPases associated with a variety of cellular activities) family. BCS1L-deficient yeast strains display a selective loss of...
the Rieske iron–sulfur protein (RISP), a 22 kDa Fe–S protein that constitutes one of the catalytic centers of the complex. As a consequence, Δbcs1 strain has no detectable ubiquinol: cytochrome c reductase activity (8). Bcs1p was proposed to act as an ATP-dependant chaperone that physically interacts with the yeast pre-CIII, maintaining it in a competent state for the incorporation of RISP (9). The assembly of CIII is then completed by the addition of the smallest subunit Qcr10p (9).

BCS1L mutations have been documented in CIII-deficient infants exhibiting signs of both visceral and neurological involvement with lactic acidosis (3–5) and in an exclusively visceral congenital syndrome denominated GRACILE (fetal growth retardation, aminoaciduria, cholestasis, iron overload, lactic acidosis and early death, OMIM 603358) (4,10).

We found two unrelated CIII-deficient children with early-onset encephalopathy without any sign of visceral involvement, associated with novel BCS1L gene mutations. The effect of these mutations on CIII structure and function was investigated in both yeast and mammalian systems.

CASE REPORTS

Patient 1 is the only child of healthy, unrelated Italian parents. She was born at 30 weeks by cesarean section, small for gestational age (weight at birth, 800 gm). Birth was complicated by a severe respiratory distress requiring assisted ventilation. In the first months of life, failure to thrive was accompanied by muscle hypotonia and severe psychomotor delay, further aggravated by drug-resistant epileptic spasms. By 1 year of age, she presented with severe spastic quadriparesis with muscle wasting and no postural control. She was fed by nasogastric tube because of severe swallowing difficulties. Cognitive functions and interaction were relatively better preserved. Weight and height were normal for age; her face was moon-like, slightly dysmorphic, with a peculiar bristle and brittle hair. Lactate and pyruvate were increased in blood and CSF. EEG tracings displayed subcontinuous epileptic discharges during wakefulness and slow sleep. Brain MRI studies showed diffuse cerebral atrophy with slight signal change in the hemispheric white matter. Symmetrical signal abnormalities in the putamina and thalami were consistent with perinatal hypoxic-ischemic damage (Fig. 1). Further clinical course was characterized by the recurrence of high-frequency seizures, with parallel cognitive deterioration, and persistent lactic acidosis leading to vegetative state. The child died at 4 years of age.

Patient 2 is the fourth-born from unrelated Moroccan parents; three older siblings are healthy. She was born at 36 weeks of gestational age and assisted for a mild respiratory distress. After a symptom-free period of 9 months, the girl presented with an acute psychomotor regression, muscle hypotonia and failure to thrive. Symptoms slowly progressed over the following months, leading to spastic quadriapresis with no postural control and severe mental impairment. Blood lactate was increased. Brain MRI was initially normal, but a second study, performed at age 2 years and 8 months, showed symmetric atrophy and abnormal signal intensity of the thalami, a small lesion in the left putamen and a slightly abnormal signal intensity of the posterior periventricular white matter.

The lateral ventricles were enlarged (Fig. 1). EEG tracings documented a diffuse, unusual fast activity (up to 30 Hz) during slow sleep, but no epileptiform discharges. The girl is now 4 years old, in stable, although severe, clinical condition. She has developed a sensory-neural hearing loss and, interestingly, she also displays brittle hair.

Apart from the CNS, both patients showed no abnormalities in any other tissue or organ, including the heart, liver, kidneys and hemopoietic system.

RESULTS

Biochemical analysis

A severe, isolated defect of complex III was documented in fibroblasts from patient 1 and in muscle from patient 2; a milder defect was also present in lymphoblastoid cells from patient 2, whereas no defect could be detected in fibroblasts of this patient (Table 1).

Sequence analysis of BCS1L

We first excluded mutations in the mtDNA MT-CYTB gene, which encodes apocytochrome b. We then analyzed the BCS1L gene and found that both patients were compound heterozygous for different missense mutations (Fig. 2A), which were absent in over 210 controls alleles and are not listed in any SNP database.

Considering the A of the first ATG in the BCS1L cDNA (NM_004328) as nucleotide position +1, patient 1 carried a 217C>T substitution in exon 1, which was inherited from the mother, and a 1102T>A transition in exon 7, which was inherited from the father. The 217C>T mutation predicts an arginine-to-cysteine change at position 73 (R73C) of the BCS1L protein (NP_004319). R73 is conserved in yeast, birds and mammals (Fig. 2B). The second mutation, 1102T>A, predicts a phenylalanine-to-isoleucine change at position 368 of the amino acid sequence (F368I). The F368 residue is highly conserved from yeast through mammals and is located in the AAA-sequence motif of the BCS1L poly-peptide (Fig. 2B).

Patient 2 harbored two different missense mutations in exon 3, a paternal 547C>T and a maternal 550C>T transitions. Both mutations predict an arginine to cysteine change in two adjacent amino acid residues (R183C and R184C), which correspond, respectively, to a lysine (K) and an alanine (A) in the yeast protein sequence.

Yeast complementation assays

In order to validate the pathogenic role of the missense mutations in the human BCS1L gene, we first performed complementation studies in BCS1L-defective strains of the yeast Saccharomyces cerevisiae.

The S. cerevisiae strain W303-1A, which carries the deletion abolishing the BCS1 gene, W303Δbcs1, displays an OXPHOS phenotype characterized by failure to grow in media containing 2% ethanol or other obligatory aerobic compounds, as the only carbon sources (8). Δbcs1 strain was transformed with wild-type and mutated human BCS1L cDNAs.

Table 1

<table>
<thead>
<tr>
<th>Patient</th>
<th>Mutation</th>
<th>Genomic position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>217C&gt;T</td>
<td>exon 1 +1</td>
</tr>
<tr>
<td>Patient 1</td>
<td>1102T&gt;A</td>
<td>exon 7</td>
</tr>
<tr>
<td>Patient 2</td>
<td>547C&gt;T</td>
<td>exon 3</td>
</tr>
<tr>
<td>Patient 2</td>
<td>550C&gt;T</td>
<td>exon 3</td>
</tr>
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</table>
cloned into the pYEX expression plasmid. The OXPHOS phenotype could be rescued by expressing the wild-type human (h) \( hBCS1L \) gene (Fig. 3A), as previously observed (3). The results obtained and shown in Figure 3A indicated that no correction was obtained by \( hbcs1l \) variants harboring any of the three mutated alleles R183C or R184C or F368I mutations (\( hbcs1l^{R183C}, hbcs1l^{R184C}, \) and \( hbcs1l^{F368I} \)). On the contrary, the transformants carrying the R73C mutation (\( hbcs1l^{R73C} \)) failed to show a significant growth defect in comparison with control strains. However, as patient 1 was a compound heterozygote for R73C and F368I, we analyzed the phenotype of the \( \Delta bcs1 \) strain carrying both mutations (\( hbcs1l^{R73C} \) and \( hbcs1l^{F368I} \)). The results obtained and shown in Figure 3 indicated that, in the presence of the two mutant alleles combined,

**Table 1. Biochemical activities**

<table>
<thead>
<tr>
<th>Patient (tissue)</th>
<th>CI/CS (nv)</th>
<th>CII/CS (nv)</th>
<th>CIII/CS (nv)</th>
<th>CIV/CS (nv)</th>
<th>CS (nv)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (fibroblasts)</td>
<td>12.8 (18.4 ± 7.6)</td>
<td>10.9 (13.5 ± 4.9)</td>
<td>34.7 (108.2 ± 21.7)</td>
<td>82.7 (124.6 ± 37.6)</td>
<td>198 (150 ± 50)</td>
</tr>
<tr>
<td>2 (fibroblasts)</td>
<td>24.6 (18.4 ± 7.6)</td>
<td>13.4 (13.5 ± 4.9)</td>
<td>99.9 (108.2 ± 21.7)</td>
<td>110.9 (124.6 ± 37.6)</td>
<td>98.5 (150 ± 50)</td>
</tr>
<tr>
<td>2 (lymphoblasts)</td>
<td>97% (100 ± 17)</td>
<td>–</td>
<td>66% (100 ± 3)</td>
<td>90% (100 ± 4)</td>
<td>92% (100 ± 2)</td>
</tr>
<tr>
<td>2 (muscle)</td>
<td>17 (20.65 ± 7.0)</td>
<td>16 (21.5 ± 6.5)</td>
<td>27.6 (108.2 ± 32.5)</td>
<td>109 (130 ± 5)</td>
<td>72.6 (145 ± 6)</td>
</tr>
</tbody>
</table>

nv, normal values; CS, citrate synthase.
\(^{\text{}\text{a}}\)nm/min/mg protein.
\(^{\text{}\text{b}}\)Expressed as % of control sample values.

**Figure 1.** Brain MRI. Patient 1: MR axial (A) and coronal (B) T2-weighted images show symmetric signal abnormalities in the putamina and thalami (arrows) and diffuse supratentorial atrophy. Patient 2: axial T2-w. images (C and D) show symmetric atrophy and abnormal signal intensity in the thalami and in the posterior periventricular white matter. The lateral ventricles are dilated mostly in their posterior parts.
oxidative growth was significantly reduced when compared with that of the wild-type allele. To evaluate whether this OXPHOS phenotype was due to dominance of the F368I mutation, we introduced it into the wt BCS1 parental strain.

The oxidative growth phenotype of this strain was indistinguishable from the wt strain (personal observation, Paola Goffrini), indicating that the F368I mutation behaved as a recessive trait in yeast, as it does in humans. Consequently,
**Table 2. Effect of mutations on respiration in haploid W303-Δ bcs1 strains**

<table>
<thead>
<tr>
<th>Allele&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Respiration&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>No allele</td>
<td>&lt;2</td>
</tr>
<tr>
<td>hBCS1L</td>
<td>54</td>
</tr>
<tr>
<td>hbcs1&lt;sup&gt;R&lt;/sup&gt;R73C</td>
<td>&lt;2</td>
</tr>
<tr>
<td>hbcs1&lt;sup&gt;R&lt;/sup&gt;F368I</td>
<td>&lt;2</td>
</tr>
<tr>
<td>hbcs1&lt;sup&gt;F&lt;/sup&gt;R73C</td>
<td>51</td>
</tr>
<tr>
<td>hbcs1&lt;sup&gt;F&lt;/sup&gt;F368I + hbcs1&lt;sup&gt;F&lt;/sup&gt;R73C</td>
<td>13</td>
</tr>
</tbody>
</table>

<sup>a</sup>Allele carried by the vector introduced into the strains.

<sup>b</sup>Expressed as μg O2/h/mg dry weight. All values are mean values of three independent experiments. In no case was the variation >10%.

The OXPHOS phenotype observed in the strain carrying both hbcs1<sup>R</sup>R73C and hbcs1<sup>F</sup>F368I alleles was due to the compound heteroallelic condition. Taken together, these results indicate that the BCSI mutations found in humans are deleterious in yeast.

**Cytochrome content and respiratory activity in hBCS1l yeast mutants**

Measurement of the mitochondrial cytochrome content is an index of the structural integrity of the respiratory chain complexes. The strains carrying allegedly pathogenic mutations (hbcs1<sup>R</sup>R183C, hbcs1<sup>R</sup>R184C and hbcs1<sup>F</sup>F368I) displayed a significant reduction of cytochromes, whereas the transformants carrying the hbcs1<sup>R</sup>R73C mutant allele exhibited a cytochrome profile similar to that of the strain transformed with the wild-type hBCS1L gene (Fig. 3B). However, the Δbcs1 strain carrying both hbcs1<sup>R</sup>R73C and hbcs1<sup>F</sup>F368I alleles again displayed a significant cytochrome reduction. In the same transformants, the respiration activity paralleled the cytochrome profile, indicating that the presence of the pathological alleles hbcs1<sup>R</sup>R183C, hbcs1<sup>R</sup>R184C and hbcs1<sup>F</sup>F368I, as well as the combination of the hbcs1<sup>R</sup>R73C and hbcs1<sup>F</sup>F368I alleles, severely impaired the respiratory capacity of the cells (Table 2).

**Western-blot analysis sodium dodecyl-sulfate–polyacrylamide gel electrophoresis**

To establish the presence and steady-state levels of the BCS1L protein (BCS1Lp) and CIII subunits in our mutant patients, we performed western-blot (WB) analysis on sodium dodecyl-sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) in different samples. Using a specific anti-BCS1Lp antibody, we showed that the amount of BCS1Lp cross-reacting material (CRM) in fibroblasts of patient 1 and in skeletal muscle of patient 2 was similar to, or slightly lower than, that found in corresponding controls. However, the CRM of the RISP was ~30–50%, depending on the tissue, to that of control samples. This reduction was specific, as the CRM amount for three other CIII proteins (Core1, Core2 and subunit VII) was similar in patients and controls (Fig. 4).

**WB analysis on one-dimension blue-native gel electrophoresis**

To evaluate the subunit composition and physical state of CIII in BCSI mutants, we first carried out one-dimension blue-native gel electrophoresis (1D-BNGE) on dodecylmaltoside-treated samples from fibroblasts of patients 1 and 2 (and controls) and from lymphoblastoid cells of patient 2 (and controls). 1D-BNGE WB analysis using an antibody against the complex III Core2 subunit showed the presence of two bands, one corresponding to the 480 kDa bona fide CIII dimer (CIII<sub>2</sub>) and the second to a higher molecular weight (MW) protein species. Unlike the lower band, the higher band reacted also with an anti-COX1 antibody and was therefore considered a CIII<sub>2</sub> + CIV supercomplex. This is the typical pattern obtained from cell samples prepared using dodecylmaltoside as the solubilizing detergent (11). The intensity of the two bands was comparable in the samples from the patients and the controls. However, an intermediate size band was present in the fibroblast samples of both patients, which was consistently absent in the controls. This band migrated between the bands corresponding to CIII<sub>2</sub> and the CIII<sub>2</sub> + CIV supercomplex, contained Core1, Core2, and also COX1 CRM and was therefore considered to correspond to an aberrant supercomplex composed of CIV plus an intermediate assembly complex of CIII<sub>2</sub> (indicated as subCIII<sub>2</sub> + CIV thereafter and in Figs 5 and 6). In fibroblasts of patients 1 and 2 and in lymphoblasts of patient 2, the RISP CRM was markedly reduced in CIII<sub>2</sub> and in the CIII<sub>2</sub> + CIV supercomplex and was undetectable in subCIII<sub>2</sub> + CIV. This result indicates that the levels of fully assembled CIII were very low and that most of the Core1 and Core2 CRMs were part of a RISP-less pre-CIII<sub>2</sub> complex. The latter appears to be capable of dimerizing and forming a supercomplex with CIV. As shown in Figure 5A, the assembly levels of CI, CII and CIV were very similar in patients 1 and 2 and in controls, again indicating a specific CIII abnormality.

We next analyzed the muscle sample from patient 2. As shown in Figure 5A, 1D-BNGE WB analysis using anti-Core2 revealed lower amount of fully assembled CIII<sub>2</sub> in the patient versus age-matched control samples. No CIII<sub>2</sub> + CIV supercomplex was detected in any of the muscle samples used in this analysis.

**WB analysis on 2D-BNGE**

The assembly state of CIII was further investigated by WB analysis on 2D-BNGE (see Materials and Methods). In dodecylmaltoside-treated fibroblast and lymphoblast lysates from patients 1 and 2, Core1 and Core2 CRMs were present at positions corresponding to CIII<sub>2</sub> and CIII<sub>2</sub> + CIV. However, Core1 and Core2 subunits were also found in low-MW complexes (Fig. 6), conceivably representing partially assembled CIII species. Neither subCIII<sub>2</sub> + CIV nor the low-MW assembly intermediates were immunovisualized in the control samples, where Core1 and Core2 subunits were exclusively detected in CIII<sub>2</sub> and the CIII<sub>2</sub> + CIV supercomplex. RISP was not detected in patient 1 and was considerably reduced in patient 2, whereas it was present in CIII<sub>2</sub> and CIII<sub>2</sub> + CIV of the control samples.

Similar results were obtained in the muscle sample from patient 2, where the Core1 and Core2 subunits were detected in CIII<sub>2</sub> and also in complexes of lower MW (Fig. 6). RISP CRM was present in CIII<sub>2</sub> in amount comparable to that of Core1 and Core2 CRMs, but was absent in the Core1-
Core2-containing low-MW species. These species were absent in the control sample.

**WB analysis of BCS1Lp**

By 1D- and 2D-BNGE WB analyses using a specific anti-BCS1L antibody, the BCS1L CRM was found in fibroblasts and lymphoblasts of both patients and controls as part of two high-MW complexes, a prominent 400 kDa and a less abundant 350 kDa species. These BCS1Lp-containing complexes were considerably smaller than pre-CIII2 and did not include either Core1 or Core2 nor RISP (Figs 5 and 6). In addition, the same 400 kDa BCS1Lp complexes were detected in 143B0 cells, where the assembly of CIII cannot take place due to the absence of cytochrome b (Fig. 5B). When the same experiments were carried out after boiling the samples for 5 min, no high-MW BCS1Lp CRM was detected by both BNGE systems, indicating that the complex was composed of protein species non-covalently linked to each other (data not shown).

By WB of SDS–PAGE, a single band corresponding to a protein of ~50 kDa could be detected in muscle samples (Fig. 4). However, virtually no BCS1L-CRM could be immunovisualized by 1D- or 2D-BNGE WB. These results may reflect the existence of functional differences between different cell types, for instance, in the turnover of CIII or in the expression and supramolecular state of BCS1L itself.

**DISCUSSION**

We report on two unrelated children with isolated CIII deficiency due to novel mutations in the BCS1L gene. The children shared a very similar clinical picture, characterized by exclusively neurological symptoms, MRI-documented involvement of thalami and supra-tentorial white matter and lactic acidosis. The overall course was progressive, although both patients have survived beyond 3 years of age. A peculiar kinky-like, brittle hair was present in both and could be a relevant clinical clue, possibly addressing the diagnosis in future cases.

BCS1L-related CIII deficiency was first described by de Lonlay et al. (3) in six patients from four unrelated families suffering from connatal metabolic acidosis, neonatal proximal tubulopathy, liver failure and encephalopathy, leading to death in the first months of life. Several BCS1L-mutated patients have been described thereafter, including three British infants and two Spanish siblings whose clinical picture was, again, characterized by signs of both neurological and visceral involvement (4,5) (Table 3). Besides CIII defect, a specific S78G homozygous point mutation in BCS1L is responsible for the GRACILE syndrome (4,10), a fatal multisystem disorder belonging to the Finnish disease heritage. The clinical picture of GRACILE children somehow resembles that of some CIII-deficient patients (i.e. lactic acidosis, tubulopathy and liver disease). However, neither neurological signs nor CIII deficiency has been documented in the GRACILE syndrome.
Taken together, the clinical findings in previously reported patients, as well as in our patients, suggest that \textit{BCS1L} defects underlie a phenotypic continuum, ranging from the GRACILE syndrome to pure early-onset encephalopathy (Table 3). In order to explain this clinical and biochemical heterogeneity, the \textit{BCS1L} protein has been proposed to play at least two different cellular functions in humans, that is, iron homeostasis, related to the GRACILE syndrome, and CIII assembly, related to the OXPHOS deficiency phenotypes (4). Moreover, the recent observation that the embryonic expression pattern of \textit{BCS1L} in the mouse neural tube is different from that of other mitochondrial proteins suggests a possible morphogenetic role for \textit{BCS1L} in the development of neural structures deriving from the floor plate (12).

Our two patients were compound heterozygotes for novel missense mutations in the \textit{BCS1L} gene, which impaired the correct assembly of the respiratory chain CIII. The defect of CIII activity varied in different cell types of our patients and was notably absent in fibroblasts of patient 2. Such variability, and particularly milder or no defect in fibroblast cell culture compared with solid tissues, e.g. muscle or liver, is frequently observed in many mitochondrial disorders and has been specifically reported in association with \textit{BCS1L} mutations (3). Nevertheless, several lines of evidence support the deleterious effects of these mutant variants. Complementation studies on a \textit{bcs1}-deficient yeast strain showed that human cDNAs harboring three of the mutations (F368I, R183C and R184C) failed to restore the yeast respiratory competence. In contrast, as already reported for other \textit{BCS1L} missense changes (3), the R73C mutation found in patient 1 was able to complement the OXPHOS deficiency of the \textit{Δbcs1} yeast strain. Although not conserved during evolution, the R73 residue is conserved between mammals and yeast. In yeast, the corresponding amino acid (R109) is located within an N-terminal region containing the information required for \textit{bcs1p} targeting and sorting into the mitochondrial inner membrane. In \textit{S. cerevisiae}, three different sequence elements have been identified in this region (13): (i) a transmembrane domain (amino acid residues 51–68), anchoring the protein to the inner membrane; (ii) an amphipathic α-helical structure containing the internal targeting sequence (positions 69–83); (iii) an import-auxiliary region (residues 84–126) strongly binding to the TOM complex, which is crucial for the proper translocation of \textit{bcs1p} across the mitochondrial outer membrane and for its insertion into the inner membrane (14). This import mechanism is likely to be conserved between yeast and mammals because the human \textit{BCS1L} protein shows the same sequence of elements and is internalized into mitochondria without cleavage of a targeting peptide (7). Our complementation results suggest that the R73C mutation alone is not sufficient to impair the
correct sorting and targeting of the BCS1L protein. However, when the Δbscl yeast cells were transformed with alleles carrying both mutations found in patient 1 (R73C and F368I), no OXPHOS complementation was observed. These results clearly indicate that the combination of the two mutant proteins is responsible for the biochemical defect leading to disease. In addition, this finding strongly supports the idea that BCS1L polypeptides must interact with each other in order to be functionally active (see subsequently).

A role for bcs1p in the late steps of CIII assembly was first suggested by the observation of a selective loss of RISP in the OXPHOS-deficient Δbscl yeast strain (8). According to the current yeast model (9,15), the assembly of CIII is a dynamic, stepwise process, which starts from the formation of different subcomplexes: the cytochrome b subcomplex, the cytochrome c1 subcomplex and the core proteins subcomplex, composed of the Core1 and Core2 proteins. These subcomplexes are believed to assemble to form a cytochrome bc1 pre-complex embedded in the inner mitochondrial membrane, prior to the insertion of RISP and Qcr10p. Yeast bcs1p is engaged in this latter step (9). Our results are in agreement with the yeast model, suggesting that the same sequence of events is conserved in humans. Both our BCS1L-mutant patients showed a selective reduction in the steady-state levels of RISP, as detected by WB analysis on SDS-PAGE. In addition, Core1 and Core2 CRMs were detected in considerable amount as part of low-MW species in 2D-BNGE, whereas RISP was consistently absent. Taken together, these observations suggest that, in humans as in yeast, BCS1Lp facilitates the incorporation of RISP in the pre-CIII complex. The low-MW CIII species detected in different mutant cell types could then be either assembly intermediates, due to a block in the assembly process, or, more likely, CIII degradation products. As RISP is incorporated late in CIII, failure for this subunit to be inserted is expected to determine the instability of the nascent complex, rather than impair the early steps of its assembly.

The identification of CIII2 + CIV and CI + CIII2 + CIV supercomplexes in yeast (16) and mammalian systems (17–19) supports the idea of a structural and possibly functional interdependence of these respiratory chain complexes to form what is known as the mitochondrial ‘respirasome’ (20). In particular, both CII2 and CIV are needed in order to maintain the stability of CI (21,22), possibly via the formation of a CIII2 + CIV supercomplex. Although functionally defective in ubiquinol-cytochrome c oxido-reductase activity, patient-1 fibroblasts had virtually normal levels of assembled CIII2, most of which, however, appeared to lack RISP. This RISP-less CIII2 species was nevertheless capable of forming a CIII2 + CIV supercomplex. Interestingly, mutant cells showed normal levels of CI, CIV and CII, all of which were functionally active. Likewise, the activity of CI was normal in muscle of patient 2. Taken together, these findings indicate that the assembly of CI depends on the physical presence of assembled CIII species and not on their catalytic activity, as also suggested by studies based on the pharmacological inhibition of CIII (21).

The effects of defective BCS1L on CIII assembly were different in fibroblasts and skeletal muscle. In the first, we found that the levels of assembled CIII2 and CIII2 + CIV supercomplex were comparable to those found in control cells, but contained much lower amounts of RISP CRM. In mutant muscle, assembled CIII2 was drastically reduced compared with controls, but RISP was present, and its amount was similar to that of other CIII subunits such as Core1 and Core2 proteins. These findings could reflect the existence of tissue-specific differences in the relative abundance and/or assembly of CIII subunits, mainly involving the incorporation of RISP into pre-CIII.

By 1D- and 2D-BNGE, we immunodetected the BCS1L protein within two supramolecular complexes in proliferating...
Table 3. Clinical features of BCS1L-related disorders

| Descent | GRACILE | Complex-III-deficient patients | Finnish | British | British | British | Turkish | Turkish | Turkish | Turkish | Turkish | Turkish | Spanish | Spanish | Italian | Moroccan |
|---------|---------|--------------------------------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| Sex     | 8 M/17 F | M | F | F | F | F | M | F | M | F | M | F | F | F | F |
| Gestational age (week) | 37.9 | 38 | 39 | 38 | 38 | 39 | Term | 41 | 39 | Term | 38 | 30 | 36 |
| IUGR | + | + | + | + | + | − | + | + | − | + | + | + | 3 months | 9 months |
| Age at onset | Birth | Birth | Birth | Birth | Birth | Birth | Birth | Birth | Birth | Birth | Birth | Birth | |
| Lactic acidosis | + | + | + | + | + | + | + | + | + | + | + | + | |
| Age at death | <4 months | 2 days | 42 days | 105 days | 3 months | 6 months | Alive (9 years) | 2 years | Alive (5 months) | 3 weeks | 3 months | Deceased (4 years) | Alive (4 years) |
| Tubulopathy | + | + | + | + | + | + | + | + | − | + | − | − | − |
| Hepatopathy | + | − | + | + | + | + | + | + | + | + | + | + | − | |
| Hypoglycemia | − | − | − | − | − | − | − | − | − | − | − | − | |
| Failure to thrive | + | + | + | + | + | + | + | + | + | + | + | + | |
| Encephalopathy | − | − | − | − | − | − | − | − | − | − | − | − | |
| Hypotonia | − | + | + | + | + | + | + | + | − | − | − | − | |
| Seizures | − | − | − | − | − | − | − | − | − | − | − | − | |
| Microcephaly | + | + | + | + | + | + | + | + | + | + | + | + | |
| Other symptoms | Blindness, deafness | Bradykinesia, ataxia, hypotonia, poor feeding | Brisk tendon reflexes | Ventilation disturbances | Acute myoglobinuria | ND | | | |
| Brain MRI | Normal (US) | ND | ND | ND | ND | ND | Atrophy | Leigh syndrome | Leech syndrome | ND | Normal | Delayed myelination |
| Iron overload | + | ND | ND | ND | ND | ND | ND | ND | ND | ND | + | + | − | − |
| CIII defect | − | + | + | + | + | + | + | + | + | + | + | + | |
| Mutation | S78G | R56X | R327A | S78G | S277N | P99L | P99L | R155P | R355M | R45C | R45C | R73C | R183C | |
|            | V327A | R144Q | S277N | P99L | R45C | R45C | F368I | R184C | | | | | | |
cultured cell lines. These complexes were clearly distinct from CIII₂ in both size and composition because Core1-, Core2-, and in control cell lines, RISP-CRMs, were consistently absent in the former but present, as expected, in the latter. In addition, the same complex was detected in ρ₀ cells, i.e. cells that are completely devoid of assembled CIII₂. Similar results have previously been shown in yeast (9). These concordant findings suggest that, in both organisms, the interaction between the BCS₁L complex and CIII₂ is either labile, non- stoichiometric or both (9).

BCS₁L is a member of the AAA family of proteins, which plays different roles in many cellular processes, but shares the common property of inducing conformational changes in target proteins or nucleic acids (23). AAA proteins may associate to form ring-shaped oligomeric (usually hexameric) complexes. A mechanism common to these proteins is to transfer the mechanical tension developed by the conformational changes of the ring induced by the ATPase cycle to the substrate, thus allowing protein unfolding. DNA-unwinding, etc. (24,25). As an AAA domain-containing protein, it is likely for BCS₁Lp to interact with itself, eventually forming a homo-oligomer. As previously mentioned, this hypothesis is indeed supported by the observation that bcs₁l¹⁶⁷⁺ and bcs₁l¹⁶₇⁻ homodimers but not heterodimers were formed and interacted in vitro (26).

DNA-fragments were assessed on agarose gel, purified and sequenced. Molecular analysis

MATERIALS AND METHODS

Biochemical analysis

Biochemical assays of the individual respiratory chain complexes were carried out according to Bugiani et al. (26) on the following material: cultured skin fibroblasts (patient 1 and patient 2), lymphoblasts (patient 2) and muscle homogenate (patient 2). Enzymatic activity of each complex was normalized to the parasite cytochromes. Overlapping fragments of the BCS₁L coding region were PCR-amplified, and the products were assessed on agarose gel, purified and sequenced.

Molecular analysis

Molecular analysis was performed on genomic DNA extracted from either muscle or fibroblasts. Overlapping fragments of the BCS₁L coding region were PCR-amplified, and the products were assessed on agarose gel, purified and sequenced.

Yeast strains and culture media

The genotypes of the yeast strains were as follows: W303-1A: MATa ade2–1 leu2–3,112 ura3–1 his3–1 trp1–1 BCS₁; W303Δbcs1 MATa ade2–1 leu2–3,112 ura3–1 his3–1 trp1–1Δbcs1::HIS3 (8).

Cells were cultured in the YNB medium [0.67% yeast nitrogen base without amino acids (Difco), supplemented with appropriate amino acids and bases to a final concentration of 40 μg/ml. Various carbon sources were added at 2% (w/v). Media were solidified with 20 g/l agar. For the cytochromes, absorption spectra were grown to late-log phase in the YNB medium supplemented with 0.5% glucose.

Site-directed mutagenesis and yeast complementation studies

The S. cerevisiae expression vector pYEX-BX (Clontech) was employed. The wild-type human BCS₁L cDNA (accession no. NM_004328) was cloned in BamHI-digested pYEX plasmid under the control of CUP₁ promoter. The point mutations corresponding to the four mutant variants found in the patients were generated using the QuickChange Site Directed Mutagenesis kit from Stratagene, and the oligonucleotide pairs (the mutated base is in bold and underlined) are as follows:

Pt 1.1 F: 5'–CACGCGCCACAGTACCTGTACTCAGCA CTCAG-3';
Pt 1.1 R: 5'–CTGAGGTGCTGACGTTTACGTTGACTGCG GGGT-3';
Pt 1.2 F: 5'–CCCATGTCTCCAGAGATCTATCCAGGG CAGGC-3';
Pt 1.2 R: 5'–GGCTGCCCTGAGATCTCCTGAGA CATCTGG-3';
Pt 2.1 F: 5'–CCCTTTTGCTATCCATGCGCCCGCGGCG ACCACTG-3';
Pt 2.1 R: 5'–CAGTGGTGCGGCCGACGATGATAG CCAAGGGG-3';
Pt 2.2 F: 5'–CTTTTTCATATCCACGCGTGGCGGCCG ACCATG-3';
Pt 2.2 R: 5'–GAATCTAGTGCCCGCGCCGACGCGG ATAGCCAAAGGG-3'.

After mutagenesis, sequences of inserts were verified on both strands. Wild-type and mutated human BCS₁L cDNAs in pYEX-BX vectors were used to transform a yeast strain, using the lithium acetate method (27).

In order to obtain strains co-transformed with both BCS₁ and BCS₁L alleles, we constructed the pYEX1-D1B1 vector by inserting the selectable marker TRP₁ and removing the URA3 marker. The TRP₁ open-reading frame was PCR-amplified from plasmid pFL35 using the following primers: 5'–CCGGTGATCCCGGGGGAAAGA TACGCA-3' and 5'–GGCGGGTGACGCTGGGGTAGCGAGT CCTCAG-3'. These primers resulted in the formation of a 4.8-kb insert, which was cloned into the pYEX1-C1 vector by digesting with NcoI and XhoI.

Yeast strains were grown on YNB medium supplemented with 0.5% glucose and 2% galactose, or YNB medium supplemented with 0.5% glucose and 2% arabinose. Anti-BCS₁L antibodies were used for Western blot analysis; polyclonal anti-CIII₂ antibodies were used for immunoprecipitation. The following antibodies were used for WB analysis: polyclonal anti-BCS₁L (ProteinTech Group), monoclonal anti-Complex III 10 kDa subunit, anti-Porin (VDAC)
(MitoSciences), anti-Complex III (Core1 subunit, Core2 subunit and RISP), anti-complex I (39 kDa subunit) and anti-complex II (30 kDa subunit), all purchased from Molecular Probes, Invitrogen. The signal obtained with each antibody was detected with the ‘ECL western blotting detection analysis system’ from Amersham and quantified using the Quantity One software (Bio-Rad).

Protein quantification in human tissues

WB of total cell protein lysates was used to estimate the steady-state levels of the BCS1L protein, the complex III subunits Core1, Core2, RISP and VII and the VDAC protein. Samples were run through a 12% acrylamide/bisacrylamide SDS-PAGE and electroblotted onto nitrocellulose filters. The blots were probed with specific antibodies as shown in Figure 4.

Assembly state analysis

The detection of the assembled respiratory complexes in control and patient skin fibroblast and lymphoblast cell lines, as well as in muscle biopsies, was performed by using BNGE. Samples were obtained from 2 × 10^6 cultured fibroblasts, 6 × 10^6 lymphoblasts or 25 mg of muscle biopsy, as described by Nijtmans et al. (30), with slight modifications. Fifteen to twenty microliters of the sample was loaded and electrophoresed in a 13% gradient non-denaturating 1D-BNGE. The lane was excised, treated for 1 h at room temperature with 2% mercaptoethanol and then run through a 16.5% tricine-SDS–polyacrylamide gel, using a 10% spacer lane was used. For WB analysis, the gels were electroblotted onto nitrocellulose filters and sequentially immunostained with the specific antibodies. The blots were probed with specific antibodies as shown in Figure 4.

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Conflict of Interest statement. None declared.

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