Differences in assembly or stability of complex I and other mitochondrial OXPHOS complexes in inherited complex I deficiency

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NADH–ubiquinone oxidoreductase (complex I) deficiency is amongst the most encountered defects of the mitochondrial oxidative phosphorylation (OXPHOS) system and is associated with a wide variety of clinical signs and symptoms. Mutations in complex I nuclear structural genes are the most common cause of isolated complex I enzyme deficiencies. The cell biological consequences of such mutations are poorly understood. In this paper we have used blue native electrophoresis in order to study how different nuclear mutations affect the integrity of mitochondrial OXPHOS complexes in fibroblasts from 15 complex I-deficient patients. Our results show an important decrease in the levels of intact complex I in patients harboring mutations in nuclear-encoded complex I subunits, indicating that complex I assembly and/or stability is compromised. Different patterns of low molecular weight subcomplexes are present in these patients, suggesting that the formation of the peripheral arm is affected at an early assembly stage. Mutations in complex I genes can also affect the stability of other mitochondrial complexes, with a specific decrease of fully-assembled complex III in patients with mutations in \textit{NDUFS2} and \textit{NDUFS4}. We have extended this analysis to patients with an isolated complex I deficiency in which no mutations in structural subunits have been found. In this group, we can discriminate between complex I assembly and catalytic defects attending to the fact whether there is a correlation between assembly/activity levels or not. This will help us to point more selectively to candidate genes for pathogenic mutations that could lead to an isolated complex I defect.

INTRODUCTION

Mitochondrial diseases are a heterogeneous group of multisystemic disorders mostly caused by the dysfunction of one or more enzyme complexes of the oxidative phosphorylation (OXPHOS) system. NADH–ubiquinone oxidoreductase (complex I) deficiency is a frequently diagnosed defect of the mitochondrial OXPHOS system generally associated with severe metabolic disorders of childhood, like progressive encephalomyopathy, leukodystrophy or Leigh syndrome. Patients show a wide spectrum of clinical presentations and many die very young because of a fatal dysfunction of tissues with high metabolic energy rates (1).

Mammalian complex I is an \(~980\,\text{kDa}\) holoenzyme that consists of two functional parts: a catalytic peripheral arm that protrudes into the matrix, and a membrane arm for proton translocation, which is embedded in the mitochondrial inner membrane (Fig. 1). Complex I is made up of at least 39 different structural proteins encoded by the nuclear genome and seven subunits encoded by the mitochondrial DNA (2). By using mild chaotropic detergents the complex has been resolved into various subcomplexes, each containing a different subunit composition (2). Although in some cases mitochondrial DNA mutations have been described as causing a complex I deficiency, they only account for a small percentage of the total number of complex I-deficient patients. In the majority of these patients mutations in nuclear genes are expected. The identification and genetic characterization of 45 human complex I nuclear structural genes has been completed (1,2). As a result of an extensive mutation detection program at our...
mutations within the same gene could lead to a similar assembly defect. Furthermore, a severe disturbance in complex I assembly was confirmed in one patient harboring an unknown nuclear mutation (19).

In order to study how these mutations affect the integrity of mitochondrial OXPHOS complexes, we have now extended the biochemical studies by using blue native gel electrophoresis (BN–PAGE) (20). Here we report our results in cultured skin fibroblasts from 15 complex I-deficient patients harboring mutations in complex I-related nuclear genes (Table 1). Six patients with known mutations in complex I subunits show low relative amounts of fully-assembled complex I that correlate with low levels of complex I activity detected in-gel, suggesting that complex I assembly or stability is compromised. By two-dimensional BN–PAGE, different patterns of complex I sub-products are detected in patients with different mutated genes. Two patients harboring unknown nuclear mutations present such low complex I activity/assembly levels, suggesting that the genetic defect should be found in a new complex I subunit or assembly factor. The remainder seven patients show almost normal levels of the fully-assembled holoenzyme with low complex I activities, compatible with a catalytic defect in which a mutation could be found in a factor participating in the activation of complex I. Finally, we have analyzed how the different mutations in complex I genes affect the stability of other OXPHOS complexes, demonstrating a specific decrease of intact mitochondrial complex III in patients with mutations in NDUF2 and NDUF4.

RESULTS

The integrity of human mitochondrial complex I is altered by mutations in nuclear-encoded subunits

Mitochondria isolated from cultured skin fibroblasts of complex I-deficient patients (Table 1) in which mutations have been described in subunits NDUF2 (3), NDUF4 (5,21), NDUF7 (6) and NDUF8 (7) were run under native conditions in a BN-PAGE system and subjected to a complex I in-gel activity assay (Fig. 2A). As expected, low complex I activities were confirmed in all the patient samples when compared with control levels. A second activity below 440 kDa is normally observed in all samples tested, which include rho zero cells (unpublished data), and it could correspond to other NADH dehydrogenases present in mitochondria. A duplicate gel was blotted and incubated with antibodies raised against the complex I subunits NDUF9 and NDUF3 (Fig. 2B and C, respectively). Decreased levels of fully-assembled complex I were observed in all cases, which correlate with the low complex I activities found in the patients. These results suggest that disturbances in the assembly pathway or in the stability of mitochondrial complex I are the direct cause of the decreased activity levels. This effect seemed to be more pronounced in two patients with mutations in NDUF8 (patient 1) and NDUF2 (patient 3). Moreover, patients 5 and 6 carrying mutations in the NDUF4 gene accumulated high levels of a lower molecular weight subcomplex (~800 kDa, gray arrow) that showed no complex I activity in Figure 2A, as has been recently described (18). The same product was observed in
three additional patients with different mutations in NDUFS4 (data not shown). Other subcomplexes containing subunit NDUFS3 were commonly present in patients and control samples (Fig. 2C, small black arrows). Surprisingly, patient 3 (P229Q mutation in NDUFS2) repeatedly showed a band at a position higher than complex I (Fig. 2C, dashed arrow). It also appeared in the NDUFS4 patients 5 and 6 under different electrophoretic conditions (data not shown). In patient 3, this band was more intense than the one corresponding to the fully assembled complex (estimated ratio 2 : 1) and it could correspond to the presence of mitochondrial supercomplexes I,III₂ or I,III₂IV₁ (22). To check this possibility and, at the same time, analyze the expression levels of native complexes IV and III in the different patients, the same blot was incubated with antibodies against core protein 2 (complex III) and COX I (complex IV) subunits (Fig. 2E and D, respectively). Presence of complexes III or IV was not detected at the high molecular weight range, suggesting that the upper band would contain only complex I, maybe representing an inactive conformation of this complex (it does not show NADH dehydrogenase activity in the in-gel activity assay). Further studies are necessary to fully confirm this hypothesis.

Mutations in NDUFS2 and NDUFS4 subunits affect the stability of mitochondrial complex III

When we compared the ratios between native mitochondrial complexes, it appeared that the levels of the fully assembled complex III dimer (~600 kDa) were clearly decreased in the patients harboring different mutations in NDUFS2 (patients 3 and 4) and NDUFS4 (patients 5 and 6), were slightly decreased by the mutation in NDUFS7 (patient 2), but not affected by the mutation in NDUFS8 (Fig. 2E). This result supports the idea of a physical interaction between both complexes. In agreement with this, a combined complex I + III deficiency has been described for three patients carrying mutations in the NDUFS4 gene (5,21). A similar reduction in the levels of complex III was observed in two out of three additional patients harboring different mutations in NDUFS4, making it a total of four patients. All these patients harbor missense mutations leading to premature stop codons in the central part of the protein. In the remainder patient with normal assembled complex III (4), the mutation is located very near to the carboxy terminus of the protein, suggesting that this region is important for the activity of complex I but not relevant for the stability of mitochondrial complex III (Ugalde et al., manuscript in preparation). Because all the patients showed normal cytochrome c oxidase (COX) enzymatic activities and the enzymatic values for complex I were calculated relative to COX (Table 1), the antibody against COX I subunit was also used to normalize for the expression levels of mitochondrial complex IV (Fig. 2D). Fully assembled COX levels were slightly increased in patients 4, 5 and 6 when compared with the control, but this is probably due to differences in sample loading in this particular experiment.

The signals from the blots were quantified and the complex I/complex IV and complex III/complex IV ratios were used as numerical values for the expression levels of fully assembled complex I and III, respectively (Table 2). An apparent lack of

### Table 1. Clinical, biochemical and genetic characteristics of the investigated complex I deficient patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Gene</th>
<th>CI fraction</th>
<th>Mutation</th>
<th>Biochemistry</th>
<th>CI activity (%)</th>
<th>Other activities (%)</th>
<th>Phenotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>NDUFS8</td>
<td>P1</td>
<td>P79L/R102H</td>
<td>2x4Fe-4S clusters</td>
<td>69 a</td>
<td>All normal</td>
<td>Leih Syndrome</td>
<td>Loeffen (7)</td>
</tr>
<tr>
<td>P2</td>
<td>NDUFS7</td>
<td>P2</td>
<td>V122M</td>
<td>4Fe-4S cluster (N2)</td>
<td>68 a</td>
<td>All normal</td>
<td>Leih Syndrome</td>
<td>Triepels (6)</td>
</tr>
<tr>
<td>P3</td>
<td>NDUFS2</td>
<td>P3</td>
<td>P229Q</td>
<td>H transact./Ubiqu Binding</td>
<td>39 a</td>
<td>All normal</td>
<td>HCEM</td>
<td>Loeffen (3)</td>
</tr>
<tr>
<td>P4</td>
<td>NDUFS4</td>
<td>P4</td>
<td>W97X</td>
<td>cAMP dep. phosphorylation</td>
<td>59 a/47b</td>
<td>CIII 98a/87b</td>
<td>Leigh-like Syndrome</td>
<td>Budde (5)</td>
</tr>
<tr>
<td>P5</td>
<td>NDUFS4</td>
<td>P5</td>
<td>R228Q</td>
<td>cAMP dep. phosphorylation</td>
<td>36 a/30b</td>
<td>CIII 100a/88b</td>
<td>Leigh Syndrome</td>
<td>Budde (5)</td>
</tr>
<tr>
<td>P6</td>
<td>NDUFS4</td>
<td>P6</td>
<td>R106X</td>
<td>cAMP dep. phosphorylation</td>
<td>60 a/59b</td>
<td>CIII 100a/88b</td>
<td>Leigh-like Syndrome</td>
<td>Budde (5)</td>
</tr>
<tr>
<td>P7</td>
<td>Unknown</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>45 a</td>
<td>All normal</td>
<td>FILA</td>
<td>—</td>
</tr>
<tr>
<td>P8</td>
<td>Unknown</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>39 a</td>
<td>All normal</td>
<td>UEM</td>
<td>—</td>
</tr>
<tr>
<td>P9</td>
<td>Unknown</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>60 a</td>
<td>All normal</td>
<td>FILA</td>
<td>—</td>
</tr>
<tr>
<td>P10</td>
<td>Unknown</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>85 a</td>
<td>All normal</td>
<td>PCA</td>
<td>—</td>
</tr>
<tr>
<td>P11</td>
<td>Unknown</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>27 a</td>
<td>All normal</td>
<td>Leigh Syndrome</td>
<td>—</td>
</tr>
<tr>
<td>P12</td>
<td>Unknown</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>36 a</td>
<td>All normal</td>
<td>Leigh-like Syndrome</td>
<td>—</td>
</tr>
<tr>
<td>P13</td>
<td>Unknown</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>73 a</td>
<td>All normal</td>
<td>FILA</td>
<td>—</td>
</tr>
<tr>
<td>P14</td>
<td>Unknown</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>42 a</td>
<td>All normal</td>
<td>PCA</td>
<td>—</td>
</tr>
<tr>
<td>P15</td>
<td>Unknown</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>27 a</td>
<td>All normal</td>
<td>Leigh Syndrome</td>
<td>—</td>
</tr>
</tbody>
</table>

Enzymatic activities were measured in triplicate in at least two independent patient-derived samples. The values for the residual complex I and complex III activities are given as a percentage of the lowest control values (set as 100%) after anormalization by COX or by the matrix enzyme citrate synthase. HCEM, hypertrophic cardiomyopathy and encephalomyopathy; FILA, fatal infantile lactic acidosis; PCA, progressive cerebellar ataxia.
correlation between the residual complex I enzymatic activities shown in Table 1 and the amount of fully assembled complex I quantified in Table 2 occurred in some patients. For instance, patients 1 and 2 presented a similar complex I activity but the differences in the amount of complex I calculated in Table 2 were bigger (7 and 50%, respectively). These differences might come from the mitochondrial extraction method used in each assay. For the biochemical activity measurements, differential centrifugation in glucose gradient was used, whereas for the BNE assay, digitonin extraction was used. Although digitonin is considered a mild detergent, it could enhance the disruption of the complex and the loss of complex I activity under circumstances that would promote the loss of stability of the complex (such as certain mutations in complex I subunits). If this is the case, this treatment does not seem to affect the levels of fully assembled complex I in control fibroblasts.

Reversibly, patients with mutations in \textit{NDUFS2} and \textit{NDUFS4} showed a relatively important decrease of assembled complex III levels with very limited or no complex III enzymatic defect. Possibly the reduction in the levels of fully assembled complex III is not sufficient to reach the threshold necessary to provoke a marked decrease on complex III activity, as it has been shown in a parkinsonism-related complex III deficiency (23).

**Table 2.** Numerical values for the expression levels of mitochondrial complexes I, III and IV in patients with known mutations in nuclear-encoded subunits

<table>
<thead>
<tr>
<th>Patient</th>
<th>CI</th>
<th>CIII</th>
<th>CIV</th>
<th>CI/CIV</th>
<th>CIII/CIV</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>P1</td>
<td>7 ± 1</td>
<td>100 ± 4</td>
<td>95 ± 4</td>
<td>0.07</td>
<td>1.05</td>
</tr>
<tr>
<td>P2</td>
<td>50 ± 2</td>
<td>87 ± 3</td>
<td>105 ± 7</td>
<td>0.48</td>
<td>0.83</td>
</tr>
<tr>
<td>P3</td>
<td>10 ± 0</td>
<td>59 ± 3</td>
<td>103 ± 5</td>
<td>0.10</td>
<td>0.57</td>
</tr>
<tr>
<td>P4</td>
<td>24 ± 2</td>
<td>55 ± 6</td>
<td>136 ± 8</td>
<td>0.18</td>
<td>0.40</td>
</tr>
<tr>
<td>P5</td>
<td>24 ± 4</td>
<td>64 ± 5</td>
<td>125 ± 7</td>
<td>0.19</td>
<td>0.51</td>
</tr>
<tr>
<td>P6</td>
<td>38 ± 4</td>
<td>73 ± 6</td>
<td>121 ± 5</td>
<td>0.31</td>
<td>0.60</td>
</tr>
</tbody>
</table>

To calculate the expression levels of complexes I and III, three independent blue native blots were quantified and the values normalized to those obtained from complex IV. Values below the normal expression range are highlighted in bold.

To calculate the expression levels of complexes I and III, three independent blue native blots were quantified and the values normalized to those obtained from complex IV. Values below the normal expression range are highlighted in bold.

**Figure 2.** BN–PAGE analysis of mitochondrial complexes I, III and IV in fibroblasts from patients with characterized mutations in nuclear-encoded complex I subunits. (A) In-gel activity assay of mitochondrial complex I confirming a decrease in all the activities of patients compared to control samples. A non-specific NADH dehydrogenase activity below 440 kDa is indicated on the left with an arrowhead. (B) A second gel was run in duplicate and western blot analysis was performed using antibodies against complex I subunits NDUFA9 and (C) NDUFS3, or (D) against complex IV subunit COX I plus NDUFA9 or (E) complex III core protein 2. A high molecular weight band containing NDUFS3 on patient P3 is indicated by a dashed arrow. The accumulation of the typical 800 kDa subcomplex observed in NDUFS4 patients (patients 5 and 6) is indicated by a gray arrow. Lower subcomplexes that are common to control and patient samples are indicated by small black arrows. CI, fully assembled complex I (~980 kDa); CIII, mitochondrial complex III dimer (~600 kDa); CIV, mitochondrial complex IV (~200 kDa); CIII + CIV indicates the presence of a supercomplex containing both complexes that is frequently observed on BN–PAGE. The positions in the gel of the ferritin monomer (440 kDa) and dimer (880 kDa) used as molecular mass standards are indicated on the left.
Distinct patterns of low molecular weight subcomplexes in patients with mutations in nuclear structural genes

To study in more detail how mutations in nuclear genes affected complex I assembly or stability, mitochondrial native (sub)complexes were separated in a second dimension SDS–PAGE system, blotted and incubated with antibodies against three complex I subunits: NDUFS3, localized in the peripheral arm and NDUFS5 and NDUFA9, localized in the peripheral and membrane arm boundary (Fig. 3). Three different effects were observed: (i) the repeated presence of lower amounts of fully-assembled complex I in patients’ fibroblasts relative to controls; (ii) different patterns of low molecular weight subcomplexes that contain the NDUFS3 subunit; and (iii) similar accumulation of an ~800 kDa subcomplex in patients with different mutated genes. These results confirm that the assembly and/or stability of mitochondrial complex I is severely affected by these mutations. The most dramatic effects were observed in the two patients with a heterozygous P79L/R102H mutation in the NDUFS8 subunit and with a homozygous P229Q mutation in NDUFS2 (data not shown), where the signals for complex I subunits were almost undetectable. In all the patients NDUFS3-containing subcomplexes below 450 kDa accumulated, probably indicating that the formation of the peripheral arm is interrupted at very early stages of the assembly process. Subunits NDUFS5 and NDUFA9 were mostly present in the ~800 kDa subcomplex, suggesting their incorporation at a later stage of complex I assembly. According to the results obtained in the first dimension, the accumulation of this late subcomplex is especially abundant in patients harboring mutations in the NDUFS4 gene (Fig. 3, lower panel).

Differences in catalytic versus assembly defects in patients harboring unknown mutations in nuclear genes

The same analysis was extended to nine fibroblast cell lines from patients in which mutations have been discarded in nuclear and mitochondrial complex I structural genes (24). Again, a complex I deficiency was confirmed in all the patients by a complex I in-gel activity assay (Fig. 4A). This decrease was more clearly noticeable when the band corresponding to complex I was normalized by the unspecific NADH dehydrogenase activity (Fig. 4A). However, a complex I assembly/activity correlation was only found in patients 11 and 15 (Fig. 4B). In these cases the levels of fully-assembled complex I were very low and the levels of complex I activity were almost undetectable, suggesting an assembly defect as the primary cause of the decreased complex I activity. A lower molecular weight subcomplex also accumulated in patient 15 (Fig. 4B, gray arrow). Remarkably, patient 11 is the same in which an assembly defect was previously described by other means (19). These data indicate that these two patients are good candidates for carrying mutations in a yet unknown complex I subunit or assembly factor. In the rest of this second group of patients complex I activities were low but the levels of fully-assembled complex I were normal, so the impairment of complex I activity is most probably due to a catalytic defect. The pattern and expression levels of native complexes III (Fig. 4C) and IV (data not shown) were also analyzed, but no significant differences were detected between patients and controls.

DISCUSSION

Complex I deficiency comprises a number of clinically heterogeneous disorders of energy metabolism of which Leigh (or Leigh-like) syndrome is the most commonly diagnosed. Unlike complex IV-deficient Leigh syndrome patients, in whom mutations have been mostly found in complex IV assembly factors such as SURF1 or SCO2, all mutations responsible for complex I deficiencies detected so far are located in complex I structural genes (25,26). Patients with mutations in the complex I nuclear-encoded subunits NDUFS4,
NDUFS7 and NDUFS8 plus, in our hands, ~50% of the patients in whom no mutations have been found yet, present with this devastating disorder (Table 1). Mutations found in the NDUFS2 gene have been specifically associated with hypertrophic cardiomyopathy and encephalomyopathy (3). Despite genetic and clinical differences, in these patients there seems to be a common pathogenic mechanism leading to complex I or IV deficiencies that would involve a problem in the proper assembly or stabilization of the mitochondrial OXPHOS complexes. This in turn would lead to a decrease of OXPHOS activities and to structural changes in the mitochondrial inner membrane that subsequently could induce proton leakage, accumulation of toxic reactive oxygen species and release of apoptotic-inducing factors.

According to this hypothesis, it has been recently demonstrated that complex I-deficient Leigh syndrome patients with mutations in NDUFS4 or in the mitochondrial ND6 gene show low levels of native mitochondrial complex I together with an accumulation of lower intermediate assemblies (18,27). In rodent cells, complex I assembly and activity are affected by the lack of the NDUFA1-homolog subunit, MWFE (28). In Neurospora crassa, complex I nuclear-encoded subunits play a central role in the assembly and function of the holoenzyme (29). In this paper we show that, in six complex I-deficient patients where mutations in nuclear subunits have been found, complex I assembly and/or stability are severely compromised. Decreased levels of fully-assembled complex I correlate with the low complex I activities found in the patients, suggesting an assembly/stability defect as the primary pathogenic mechanism. To explain the causes we must take into account different factors, like the position and conservation of the mutations, the localization of the mutated subunits within the complex and the individual role of each subunit within the overall function of complex I. All the mutations are found in highly conserved amino acid positions during evolution, but maybe some positions are functionally

Figure 4. Mitochondrial complex I catalytic versus assembly defects can be distinguished in fibroblasts from isolated complex I-deficient patients by BN–PAGE analysis. Mitochondria were isolated and analyzed as described in Figure 1. (A) Complex I in-gel activity assay of mitochondrial complex I. The unspecific NADH dehydrogenase activity below 440 kDa is indicated with arrowheads. The bands corresponding to complex I activity were quantified and normalized by the levels of the non-specific NADH dehydrogenase. The ratios between both activities are shown below each lane. (B) Western blotting analysis of native complex I with antibodies against complex I subunit NDUFA9. The presence of a lower molecular weight subcomplex is indicated with a gray arrow. (C) Same analysis with an antibody against mitochondrial complex III core protein 2. The top band indicated as CI is a residual band from incomplete stripping of the membrane after probing for complex I. CI, CIII and CIV are mitochondrial complexes I, III and IV, respectively. The positions of the ferritin monomer (440 kDa) and dimer (880 kDa) used as molecular mass standards are indicated on the left.
more relevant than others and provoke a more dramatic complex I defect. All the mutations analyzed are located in subunits forming the peripheral arm of the complex, therefore these mutations are expected to affect the formation and catalytic activity of this specific fragment. Little is known about the function of the so-called complex I accessory subunits, but studies mainly performed in *N. crassa* and *Y. lipolytica* have shed light on the precise role of the core subunits. The most evident complex I defect was found in patients with P79L/R102H amino acid changes in NDUFS8 and a P229Q substitution in NDUFS2, in whom native complex I was almost undetectable. The homologs of NDUFS7 and NDUFS2 have been described in *Y. lipolytica* to form the catalytic core for ubiquinone binding, a process in which NDUFS8 is also involved (16). Reconstruction in *Y. lipolytica* of the human pathogenic mutations found in the NDUFS7 and NDUFS8 genes did not lead to an impaired complex I assembly but only to a complex I catalytic defect (15). The same occurred with mutations in highly conserved residues of the NDUFS7 and NDUFS2 homologs (30,31). However, disruption of any of these three subunits in *N. crassa* showed impaired assembly of the peripheral arm, accumulation of the membrane arm and a consequent catalytic defect (32–34). The differences found in complex I defects between our complex I-deficient patients and the yeast model give evidence of the necessary precaution that must be taken when mimicking human physiological conditions in lower species. All our patients with mutations in NDUFS4 also showed a marked decrease in the levels of fully-assembled complex I together with high levels of a defective subcomplex of ∼800 kDa not present in controls, as already described (18). Additionally, the presence of lower bands between ∼100–450 kDa containing complex I NDUFS3 subunit was detected both in control and all patient samples. These subcomplexes possibly correspond to assembly intermediates from the pathway recently proposed for complex I assembly (35). In a second dimension, distinct patterns of lower molecular weight subcomplexes were clearly observed in patients carrying mutations in different complex I subunits. The low amounts of fully-assembled complex I and the relative accumulation of NDUFS3-containing subcomplexes below 450 kDa suggest that the formation of the peripheral arm is interrupted at very early stages of the assembly process. This result is in agreement with the complex I assembly model in which the NDUFS3 subunit would form part of assembly intermediates since the beginning of the process (35). Because of the malfunction of the peripheral arm, the membrane arm might have problems getting incorporated and form the final assembled holoenzyme. Then subunits NDUFS5 and NDUFA9, present in the peripheral and membrane arm boundary region, would get accumulated at a later stage of assembly forming, together with the peripheral arm, an incomplete intermediate of ∼800 kDa. This is especially noticeable in patients with mutations in the NDUFS4 gene. The two mutations analyzed here lead to a truncated version of NDUFS4. It has been previously reported in other NDUFS4 patients that this intermediate contains a number of complex I subunits including NDUFS3, NDUFS5 and NDUFA9, but not NDUFS4 itself (18). These results suggest that NDUFS4 would be incorporated at this late stage in the assembly process, as has been reported (35), and that the lack of NDUFS4 would hamper the next step in the formation of fully-assembled complex I. The possibility also exists that the accumulation of lower molecular weight subcomplexes in these patients could not be only due to an assembly defect or to a lower rate of incorporation of the different subunits into the complex, but also to a lower complex I stability and its consequent disruption. Additional experiments must be performed in order to reach definitive conclusions.

In the remainder complex I-deficient patients harboring unknown mutations, catalytic versus assembly (or stability) defects have been easily distinguished by BN–PAGE. Two patients show a similar complex I assembly defect as established in the patients carrying mutations in nuclear-encoded subunits. Mutations in complex I subunits have been discarded in 45 out of the 46 complex I subunits, but the last of these subunits remains to be identified (2). These two patients are candidates to carry mutations either in this complex I subunit or in a complex I assembly factor. Our identification of the human homologue of a *N. crassa* complex I assembly protein, CIA30, proves the existence of such factors (36). Unfortunately, no disease-causing mutations have been found in any of these genes yet. In the rest of this second group of patients the levels of fully-assembled complex I are normal. The impairment in complex I activity cannot be explained by a defect in complex I assembly but to a pure catalytic defect, suggesting a different pathogenic mechanism leading to an isolated complex I deficiency. In these cases, mutations should be found in nuclear genes that regulate crucial steps in the activation of mitochondrial complex I. Examples of such candidate genes could be found in proteins involved in the phosphorylation of complex I subunits that could participate in the complex I activation/inactivation transition process, like mitochondrial-specific isoforms of cAMP dependent kinases or phosphatases (37,38). In support of this, it has been shown in different species that mitochondrial complex I reversibly alternates between an active and a de-activated conformation that probably depends on the cell respiratory status (39–41).

Finally, how these mutations affect the stability of native mitochondrial complex I and IV in our patient group has been analyzed. In different experiments, the steady-state levels of fully-assembled complex IV did not vary substantially in these patients when compared with controls. However, the levels of the native complex III dimer were clearly decreased in the patients harboring mutations in NDUFS2 and NDUFS4, but not significantly altered by the mutations in NDUFS7 or NDUFS8 genes, or in any patient with unknown mutations in nuclear genes. This finding is in agreement with the combined complex I + III deficiency described for three patients carrying mutations in the NDUFS4 gene (5,21). Reversibly, a specific mutation in cytochrome *b* has been found to lead to a combined complex III + I deficiency in a patient with progressive exercise intolerance (42). These results suggest a physical interaction between complexes I and III that could be mediated through these subunits. Experimental evidence supports this idea. The association of the mitochondrial respiratory chain complexes into higher supramolecular structures called supercomplexes seems to be well established in different organisms, including mammals (43). In *Y. lipolytica*, the ubiquinone-binding 49 kDa subunit (homolog of NDUFS2) seems to be located in an external position near the tip of the peripheral arm.
By cross-linking studies, a direct interaction between the homologs of NDUF52 and NDUF54 (19 and 18 kDa subunits, respectively) has been observed in bovine (45). It would explain the similar phenotypes found in patients with mutations in these two genes. In these patients carrying mutations in NDUF52 or NDUF54, the decreased stability of mitochondrial complex III is accompanied by a slight or no reduction of complex III activity. This could be explained by internal compensatory activation mechanisms or by the fact that a loss of bigger amounts of assembled complex III could be necessary before getting a considerable decrease of complex III activity, as has been suggested in cybrid clones harboring increasing amounts of mutant cytochrome b (23). Finally, the first report demonstrating that complex I mutations interfere with the function or assembly of other OXPHOS complexes has been recently described in a Caenorhabditis elegans model (electronic manuscript) (46). Summarizing, by BNE we have been able to differentiate catalytic versus assembly/stability defects in complex I-deficient patients and to study the effects of these mutations on the stability of other mitochondrial OXPHOS complexes. Using this technique will not only expand the cell biological knowledge of the mitochondrial energy metabolism, but it will also be useful as an additional tool to improve the pre- and postnatal diagnostic possibilities in patients with a mitochondrial complex I deficiency.

MATERIALS AND METHODS

Patients

Table 1 summarizes the genetic, clinical and biochemical features of the patients’ fibroblasts analyzed in this study. Patients and mutations were previously reported (3,5,6,7,14,21,24,47).

Cell cultures

Fibroblasts were obtained from skin biopsies and cultured in M199 medium (Life Technologies) supplemented with 10% fetal calf serum (FCS) and 100 IU/ml penicillin and 100 IU/ml streptomycin.

Complex I assembly and in-gel activity studies

One- and two-dimensional BN–PAGE and mitochondrial complex I in-gel activity assays were performed with digitonin-isolated mitochondria from patients’ fibroblasts as previously described (48). Proteins were transferred to a PROTRAN® nitrocellulose membrane (Schleicher and Schuell). Western blotting was performed using primary antibodies against complex I subunits NDUFA9, NDUSF3 and NDUSF5, against the core protein 2 and COX I subunits from mitochondrial complexes III and IV, respectively, and against subunit α from H+/F1-ATP synthase (Molecular Probes). Peroxidase-conjugated anti-mouse immunoglobulins (Molecular Probes) were used as secondary antibodies. The signal was detected by using ECL® plus reagents (Amersham Biosciences) and the quantification of the blots was performed using ImagePro-Plus 4.1 image analysis software (Media Cybernetics, Silver Spring, MD, USA).

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