Pathological Mutations of the Human *NDUFS4* Gene of the 18-kDa (AQDQ) Subunit of Complex I Affect the Expression of the Protein and the Assembly and Function of the Complex*

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Presented is a study of the impact on the structure and function of human complex I of three different homozygous mutations in the *NDUFS4* gene coding for the 18-kDa subunit of respiratory complex I, inherited by autosomal recessive mode in three children affected by a fatal neurological Leigh-like syndrome. The mutations consisted, respectively, of a AAAGTC duplication at position 466–470 of the coding sequence, a single base deletion at position 289/290, and a G44A nonsense mutation in the first exon of the gene. All three mutations were found to be associated with a defect of the assembly of a functional complex in the inner mitochondrial membrane. In all the mutations, in addition to destruction of the carboxyl-terminal segment of the 18-kDa subunit, the amino-terminal segment of the protein was also missing. In the mutation that was expected to produce a truncated subunit, the disappearance of the protein was associated with an almost complete disappearance of the *NDUFS4* transcript. These observations show the essential role of the *NDUFS4* gene in the structure and function of complex I and give insight into the pathogenic mechanism of *NDUFS4* gene mutations in a severe defect of complex I.

In mammals, the mitochondrial respiratory chain complexes and the F$_{1}$F$_{0}$ ATP synthase are constituted, in addition to the conserved mitochondrial encoded subunits, by a large number of nuclear encoded subunits. Evidence is now emerging that indicates that the structural subunits of respiratory chain complexes, although not necessary for the catalytic activity, can contribute to the assembly of the complexes and/or have a regulatory role.

NADH:ubiquinone oxidoreductase (E.C.1.6.5.3., complex I) of the respiratory chain in the inner mitochondrial membrane catalyzes the oxidation of NADH by ubiquinone and conserves the free energy thus made available as the proton motive force (1, 2). Bovine heart complex I is made up of 46 subunits amounting to a minimum molecular mass of $\approx$ 980 kDa (3, 4). Seven subunits are encoded by the mitochondrial genome (5), the others by nuclear genes (6). The protein, cDNA, gene sequence, and chromosome location of the human genes are now available (7, 8). Fourteen subunits of the mammalian enzyme have their counterparts in prokaryotic and *Neurospora crassa* complexes I. These subunits are likely to contribute the essential elements of the redox and proton translocating activities of complex I (1, 2). Complex I has an L shape, i.e. one arm is in the membrane plane, and the other peripheral arm protrudes into the matrix and contains all the redox cofactors of the complex (1, 4). As far as the mammalian enzyme is concerned, the following questions remain to be understood: (i) how the expression of the 39 nuclear encoded subunits is coordinated with that of the seven mitochondrial encoded subunits; (ii) how the 39 nuclear encoded subunits are imported into mitochondria and assembled together with the mitochondrial subunits in the inner membrane mature complex; and (iii) the role of the supernumerary 32 subunits. Evidence for functions of some of these subunits has been obtained (2, 4). Papa et al. have found that the 18-kDa subunit of mammalian complex I, encoded in humans by the nuclear *NDUFS4* gene and localized on chromosome 5 (6), is phosphorylated by the CAMP-dependent protein kinase (PKA) (9, 10). In murine and human cell cultures, phosphorylation of this subunit, which is dephosphorylated by a Ca$^{2+}$-inhibited phosphatase in mitochondria (11), has been found to be associated with stimulation of the activity of complex I and the overall respiratory activity (10, 12, 13).

Mutational analysis of complex I-deficient patients has revealed that the *NDUFS4* gene presents various mutations and might be considered a mutational hot spot (14). We have investigated the impact on the expression of the 18-kDa subunit and the assembly of the complex of three different homozygous *NDUFS4* gene mutations in three children affected by Leigh-like syndrome. The three mutations in the *NDUFS4* gene consisted, respectively, of a 5-bp duplication leading to a deduced mutated form of the protein with destruction of a phosphorylation consensus site and elongation of the carboxyl terminus by 14 residues (patient 1) (15), a point deletion in the middle part of the coding sequence leading to a truncated polypeptide (patient 2) (16), and a nonsense mutation causing premature termination after only 14 amino acids of the putative mitochondrial targeting sequence (patient 3) (17) (Fig. 1).

Fibroblast cultures from the three patients exhibited severe depression of the normal activity of complex I, which also did not respond to activation by CAMP as observed in control cells from normal subjects (18). All mutations were found to be
associated with impairment of the assembly of a normal, functional complex in the inner mitochondrial membrane. In all three patients, in addition to destruction of the carboxyl-terminal phosphorylation site, no protein was detected with an antibody against the amino-terminal segment of the 18-kDa protein. Estimation of the steady-state level of the \textit{NDUFS4} transcript showed an almost complete disappearance of the transcript only in patient 2. These observations provide clues for an essential role of the \textit{NDUFS4} gene in respiratory metabolism and the pathogenic mechanism of its mutations in the human inborn neurological syndrome.

**EXPERIMENTAL PROCEDURES**

**Culture and Preparation of Fibroblasts—**Primary fibroblast lines established from skin biopsies of control subjects and three \textit{NDUFS4} mutated patients were grown in high glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 110 \textmu g/ml pyruvate, and 50 \textmu g/ml uridine (12). Fibroblasts from patient 3 were made available to us by Dr. Massimo Zeviani, National Institute "C. Besta," Milan, Italy. In the experiments depicted in Fig. 2a, once cells were at 75% confluence the medium was replaced by Dulbecco’s modified Eagle’s medium with 0.5% fetal bovine serum. Where indicated, after 3 days of serum starvation the cells were supplemented with 1 \mu g/ml cholera toxin (Sigma) and 100 \textmu M 3-isobutyl-1-methylxantine.
which results in a permanent increase of the intracellular cAMP concentration (12, 19). The fibroblasts were harvested and suspended in phosphate-buffered saline as described previously (12).

Enzymatic Assays—Freshly harvested fibroblasts were exposed to ultrasound energy at 0 °C and used immediately. All enzymatic assays were performed on the total cell lysate. Cytochrome c oxidase activity was determined as described (20). The \( V_{\text{max}} \) of rotenone-sensitive NADH:ubiquinone oxidoreductase activity, measured with decylubiquinone as substrate, was obtained from Lineweaver-Burk plots as described (12). The \( V_{\text{max}} \) of ubiquinol-cytochrome c oxidoreductase was obtained from Lineweaver-Burk plots using decylubiquinone as substrate as described (21).

Electrophoretic Procedures—Mitoplasts (inner membrane matrix fraction of mitochondria devoid of the outer membrane) were prepared from cells as described previously (12) in the presence of phosphatase inhibitors (5 mM NaF, 500 mM okadaic acid, and 1 mM sodium orthovanadate). Two-dimensional gel analysis (blue native PAGE/SDS-PAGE) of purified bovine heart mitochondria and mitoplasts from fibroblasts was carried out as described previously (12). After the second dimension (SDS-PAGE), gels were either silver-stained or transferred onto nitrocellulose for immunoblotting of subunits of complexes as described (12, 17). Rabbit antisera raised against either the 20 residues of amino terminus or the 20 residues of C terminus with phosphorylated Ser\(^{131}\) of the mature protein were produced to our specifications and used as immunodecoration with specific antibodies revealed that the subunits of mitochondria and mitoplasts from fibroblasts was carried out as described previously (12). After the second dimension (SDS-PAGE), gels were either silver-stained or transferred onto nitrocellulose for immunoblotting of subunits of complexes as described (12, 17). Rabbit antisera raised against either the 20 residues of amino terminus or the 20 residues of C terminus with phosphorylated Ser\(^{131}\) of the mature protein were produced to our specifications and purchased from NeoSystem, Strasbourg, France. Monoclonal antibodies against the NDUFA9, NDUFS3, NDUFS7, NDUFB6, NDUFS5, and NDUFA6 subunits of complex I, the core II subunit of complex III, and the \( \beta \) subunit of complex V were from Molecular Probes (Eugene, Oregon). The NDUFA5 antibody was kindly provided by Prof. J. E. Walker.

Extraction of RNA from Cultured Fibroblasts and Northern Blot Analysis—The steady-state level of the NDUFS4 transcript in the NDUFS4-mutated patients was assayed by filter hybridization on 10 µg of total RNA from 8–10 × 10⁶ cultured fibroblasts. RNA samples were loaded on a 6.6% formaldehyde-1.2% agarose horizontal gel, transferred onto a nylon membrane (Hybond-N+; Amersham Biosciences), and then hybridized using ExpressHyb Hybridization Solution (Clontech) with the NDUFS4 cDNA probe labeled by random primer labeling at a specific activity of 1.5–2 × 10⁶ cpm/µg. The same blot was used for successive hybridization with a β-actin cDNA control probe that revealed the presence of a single 2-kb band in all lanes. The transcript signals were analyzed by using a Bio-Rad Personal Molecular Imaging FX.

Synthesis of cDNA and Quantitative Real Time PCR of NDUFS4 Transcript from the Three NDUFS4-mutated Patients—Aliquots of total RNA from cultured fibroblasts extracted by TRIzol® (Invitrogen) were reverse-transcribed by using the oligo-dT primer with avian myeloblastosis virus (AMV) reverse transcriptase KnaseH minus (Finnzymes, Espoo, Finland). The determination of the expression profile of the NDUFS4 transcript was performed by two-step reverse transcription PCR and online measurement of PCR products based on the use of QuantiTect SYBR Green PCR kit (Qiagen, Cologne, Germany) with BIORAD instrument iCycler iQ, version 2.033 (Bio-Rad). The PCR reactions were performed in a total volume of 25 µl containing an aliquot of 2.5 µl of each cDNA, 7.5 pmol of primers, and 12.5 µl of QuantiTect SYBR Green PCR kit in a iCycler. The NDUFS4 amplification was generated by use of the forward primer Real-F-30 (5′-GGTG-TACTGAGGCAGACGTTGT-3′) and the reverse primer R-143 (5′-GAAGGTGAAGGTCGGAGT-3′). The PCR conditions were 20 s at 94°, 30 s at 59 °C, and 45 s at 72 °C for 45 cycles. For the amplification of GAPDH, the primers F-5′-TGTGCTGGCCAAATCCATGG-3′ and R-5′-CATGGTTGGAATCATATTGGAA-3′ were used. The PCR program was followed by a melting curve analysis tools (data not shown). In contrast, the position of complex III activity, as evidence exists supporting the possibility that alterations in the NDUFS4 gene product may affect complex III (16).

Assembly of the Respiratory Complexes—Two-dimensional blue native/SDS-PAGE was used to examine the assembly of the respiratory complexes and the \( \text{F}_0\text{F}_1 \) ATP synthase (complex V) of mitoplasts from fibroblast cultures of control subjects and patients. Coomassie staining of bovine heart mitochondria was carried out to reveal the separation of oxidative phosphorylation complexes and their relative position in the native blue-PAGE (Fig. 3a). A functional complex I, which was directly detected on the first dimension native gel by the specific reagent nitro-blue tetrazolium (22) in the case of the control mitoplasts, did not reach a level of activity detectable in the gels of the mitoplasts from the three patients (Fig. 3b). The constituent subunits of the complexes were resolved in the second dimension (SDS-PAGE) in all three patients, no normally assembled subunits of complex I could be detected by silver-staining in the position in which they appeared in the control fibroblasts (Fig. 4). Weak silver-stained subunit-bands of the complex were, however, seen lined up in a position closer to complex V. The result was verified by subunit immunostaining of the complexes. As expected, an antibody against the phosphorylated carboxyl terminus of the 18-kDa subunit showed that this segment of the protein was absent in all the patients, both in the normal and lower molecular weight position of complex I (Fig. 5a). In addition, an antibody against the amino-terminal segment of the 18-kDa subunit also showed that this segment of the protein was absent in all the patients either at the normal or the lower molecular weight position of complex I (Fig. 5b). Immunodetection of the NDUFA5 subunit of complex I showed that this also moved in all three patients from its normal position, in the control fibroblasts, to the lower molecular weight position of the complex closer to complex V, whose \( \beta \)-subunit was detected by its specific antibody. Immunodecoration with specific antibodies revealed that the subunits of complex I encoded by the nuclear genes NDUFA9, NDUFS3, NDUFS7, NDUFB6, NDUFS5, and NDUFA6 also moved to the same lower molecular weight sub-complex position (data not shown). In contrast, the position of complex III relative to complex V did not change in the three patients as shown by both the silver-staining and the immunodetection of the core II subunit of this complex (Figs. 4 and 5).

\(^1\) The abbreviations used are: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NMD, nonsense-mediated decay; nt, nucleotide(s); contig, group of overlapping clones.
By double immunoblotting the 18-kDa subunit with specific antibodies against the phosphorylated form of the carboxyl terminus and the amino terminus of the subunit, we found that, not only in the case of nonsense mutation (patient 3) as expected but also in the case of the other mutations (patients 1 and 2), the amino terminus segment of the protein could not be detected in the total fibroblast extracts, in addition to the absence of the phosphorylated carboxyl terminus (Fig. 6).

Steady-state Level of the NDUFS4 Gene Transcript—To assess the steady-state amount of NDUFS4 mRNA in the three patients, a Northern blot was performed on total RNA samples extracted from the fibroblasts of a normal child and the fibroblasts of the three NDUFS4 mutant patients. The Northern blot was hybridized with a labeled PCR fragment corresponding to the entire NDUFS4 cDNA and then probed for the nuclear encoded cytoplasmic β-actin gene, which estimates the amount of total RNA loaded. Although in the fibroblasts of patients 1 and 3 the expected NDUFS4 transcript (~650 nt) showed a level practically equal to that of control fibroblasts, it was not detectable in the fibroblasts of patient 2 (Fig. 7A). This finding was confirmed by quantification of the NDUFS4 transcript by real time PCR, which enabled determination of the NDUFS4 transcript with respect to the GAPDH transcript used as an internal reference. The PCR results showed that, although the relative amount of the NDUFS4 transcript in the fibroblasts of patients 1 and 3 was the same as that in the control fibroblasts, it was reduced to ~30% of the control in patient 2 (Fig. 7B).

To determine whether nonsense-mediated decay (NMD) was...
induced in the \textit{NDUFS4} transcript that harbored the W97X nonsense mutation, primary fibroblasts from patient 2 carrying the premature termination codon were exposed to puromycin. This protein synthesis inhibitor has been shown to inhibit NMD (23). Puromycin fully restored the \textit{NDUFS4} mRNA level in the cells of patient 2, whereas the removal of the protein inhibitor gradually restored the amount of the transcript to a level comparable with that of the untreated cells (0 h) (Fig. 7C).

**DISCUSSION**

In patients with complex I deficiency, mutations in the nuclear encoded subunits of the complex have been found in six genes. The \textit{NDUFS4} gene is one of the most “hit” (with mutations) among these genes (14). Searching with the \textit{NDUFS4} cDNA sequence by Basic Local Alignment Search Tool Nucleotide (BLASTN) in the Human Genome Resources web site, two contigs derived from assembled genomic sequence data, including both draft and finished sequences, have been identified. The contigs NT006693 (147208 nt) and 12731439 (122694 nt), not full-length and found at NCBI and Celera, respectively, are split into five exons following the AG/GT rule. Each of the first two introns spans for $\sim 42$ kb, thus accounting for the large size of the overall gene. A BLAST search in the EST data base (dbEST) has disclosed several cDNA clones extending the

**FIG. 4.** Two-dimensional gel electrophoresis of bovine heart mitochondria (BHM) and mitoplasts from control child and patient fibroblasts. Respiratory complexes (I, III, and IV) and ATP synthase (V) were separated by blue native electrophoresis, and their constituent subunits were resolved in a second dimension by SDS-PAGE. Gels were silver-stained to detect polypeptide patterns of oxidative phosphorylation complexes. For further details, see “Experimental Procedures.”

**FIG. 5.** Immunoblot of two-dimensional gel electrophoresis of bovine heart mitochondria (BHM) and mitoplasts from control child and patients’ fibroblasts. Respiratory complexes (I, III, and IV) and ATP synthase (V) separated by blue native electrophoresis and resolved in their subunits in a second dimension by SDS-PAGE were blotted onto nitrocellulose and tested with different antibodies. The positions of subunits of complexes I, III, and V were detected by specific antibodies against the phosphorylated carboxyl terminus (a) and the amino terminus (b) of the NDUFS4 and NDUFA5 subunits (a and b), the core II subunit of complex III (a and b), and the \( \beta \)-subunit of complex V (a and b). According to its relative position to complex V, complex I exhibited in the three patients a molecular mass $\sim 200$ kDa lower as compared with those in bovine heart mitochondria and control fibroblasts. For further details see “Experimental Procedures.”

**FIG. 6.** Western blot analysis of the phospho-carboxyl terminus and the amino terminus of the NDUFS4 subunit of complex I in whole protein extracts from control (Wt) and patient fibroblasts. SDS-PAGE slabs were tested with specific antibodies against the phospho-carboxyl terminus (Anti PC-terminus) and the amino terminus (Anti N-terminus) of the NDUFS4 subunit. For further details see “Experimental Procedures.”
length of the published 5’-UTR to a size of at least 24 nt.

Our results show that the three different mutations detected in the NDUFS4 gene in the three patients in the homozygous state (Fig. 1) were all associated with the following: (i) depression of the V_max of the normal rotenone-sensitive NADH:ubiquinone oxidoreductase, which was insensitive to cAMP stimulation; (ii) defective assembly of the complex, with the appearance of a non-functional lower molecular weight subcomplex; and (iii) undetectability of both the phosphorylated carboxyl terminus (as expected from the mutations) and the amino terminus. Disappearance of the amino-terminal seg-

ment was expected in the case of nonsense mutation (patient 3) but not in the other two cases, i.e. in the 5-bp duplication of the cDNA at position 466–470 (AAGTC) (patient 1) and the point deletion at position 289/290, introducing a stop codon corresponding to position 97 of the protein (patient 2) (Fig. 1).

Quantitative analysis of the NDUFS4 transcript showed that, although in the fibroblasts of patients 1 and 3 its level was practically equal to that of control fibroblasts, it was much lower in the fibroblasts of patient 2. These findings show that the deletion at position 289/290, leading to introduction of a stop codon, can result in a transcript that is rapidly degraded with suppression of the synthesis of the truncated 18-kDa subunit. The low level of NDUFS4 mRNA appears to be due to the elicitation of a mechanism of NMD (24), a mechanism of quality control that ensures fidelity of gene expression. This is an RNA surveillance pathway that detects and destroys aberrant mRNAs containing premature termination codons (see also Refs. 25 and 26). The 289/290 mutation, which introduces a stop codon in the open reading frame of patient 2, is located 60/61 nt upstream of the junction between exons 3 and 4 (Fig. 1) and is therefore a candidate for NMD (25, 26).

The NDUFS4 mRNA steady-state level was, on the other hand, not affected in the other two mutations in the gene. In these cases the transcripts, even those with an altered sequence, maintained a level corresponding to the wild-type transcript. In the case of patient 3, the mutation makes possible the synthesis of only the first 14 amino acids of the mitochondrial targeting pre-sequence so that a functional or even a partially functional mitochondrial protein cannot be generated. This nonsense mutation falls within the first half of exon 1, and an in-frame AUG resides 213 nt after the premature termination codon (Fig. 1) which could protect the transcript from NMD (27).

Translation of this aberrant mRNA could lead to the production of an amino-terminal truncated NDUFS4 altered protein that, however, would be unable to localize into mitochondria because of the lack of the entire mitochondrial targeting sequence encoded by exons 1 and 2. The absence of the putative protein product of the mutated gene of patient 1 harboring the 5-bp duplication (Fig. 1) could be due to a defective translation of the mutated transcript and/or proteolytic degradation of this long translated product. Proteolytic systems regulate the stability of mitochondrial inner membrane proteins (28). One role of these systems is the removal of non-assembled polypeptides from mitochondrial membranes (28, 29).

Our results are consistent with the occurrence of a multi-step quality control process at the transcriptional and/or the post-transcriptional level that avoids the presence in the cell of abnormal polypeptides resulting from mutations in the NDUFS4 gene. The lack of the expression of a wild-type 18-kDa subunit of complex I prevents the assembly of a normal functional complex of ~1000 kDa. A defective sub-complex of an apparent molecular mass of ~800 kDa is apparently formed in all the three NDUFS4 mutant fibroblasts. This complex, which was still associated with the mitochondrial inner membrane, showed a low, if any, level of NADH:ubiquinone oxidoreductase activity. Evidence has been presented for the stepwise assembly in the N. crassa complex I (30). Impact of mutations in mammalian structural genes on the assembly of complex I has been described for the nuclear NDUFA1 gene (31) and for the mitochondrial ND6 (32) and ND4 (33).

Our finding of an apparently normal assembly and redox activity of complex III in patients harboring three different NDUFS4 mutations in the homozygous state seems to exclude an impact of this complex I gene on the structure/function of complex III. This conclusion is supported by a mutational analysis of the NDUFS4 gene that we carried out on a different
cohort of 20 patients with a combined complex I and complex III deficiency detected in muscle samples. No mutations were in fact found in the NDUFS4 gene of this group of patients.

A defect in the activity of the complex can result in a deficiency of the overall process of oxidative phosphorylation (10, 13) This will be extremely deleterious in cells with high and variable energy demand, like those of brain regions, heart, and skeletal muscle. This and the large number of genes coding for constituent subunits, as well the possible existence of assembly genes (34), seems to explain why deficiency of complex I represents the most frequent of the human mitochondrial hereditary disorders of oxidative phosphorylation (~ 40% of disorders with an estimated occurrence of 1:10,000 live births). This could also be related to the finding of complex I deficiency in aging and neurodegenerative age-associated disorders (35, 36).

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