Mutation in the NDUFS4 gene of complex I abolishes cAMP-dependent activation of the complex in a child with fatal neurological syndrome

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Abstract Evidence is presented showing that in a patient with fatal neurological syndrome, the homozygous 5 bp duplication in the cDNA of the NDUFS4 18 kDa subunit of complex I abolishes cAMP-dependent phosphorylation of this protein and activation of the complex. These findings show for the first time that human complex I is regulated via phosphorylation of the subunit encoded by the NDUFS4 gene. **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: NDUFS4 gene; Complex I; Human fibroblast; cAMP-dependent phosphoprotein

1. Introduction

In mammals complex I of the respiratory chain is made up of 43 subunits [1,2]. Seven subunits (ND1–ND6, ND4L) are encoded by the mitochondrial genome [3], the others by nuclear genes [1,4]. The cDNAs of 35 of the human nuclear genes coding for complex I subunits have been sequenced [5], but for most of them the function is unknown [5,6]. Papa et al. [7,8] have found that in bovine heart mitochondria the nuclear encoded 18 kDa subunit of complex I is phosphorylated by cAMP-dependent protein kinase. The mature 18 kDa protein, which does not bind prosthetic groups, has, in *Bos taurus* at position 129–131, a canonical cAMP-dependent protein kinase phosphorylation consensus site (RVS) in which S is the residue that is phosphorylated (Fig. 1) [9,10]. The protein has a leader sequence, removed after import in mitochondria [11], that also presents a phosphorylation consensus site (RSLS) at position −10–−7. The 18 kDa subunit of complex I appears to be highly conserved in the known sequences of mammals [11–13]. Further investigations [9,14] showed cAMP-dependent phosphorylation of the 18 kDa subunit and activation of complex I in mouse fibroblasts in vivo. Subsequently, van den Heuvel et al. [12] sequenced the cDNA of the NDUFS4 gene, encoding the 18 kDa subunit, in patients with complex I deficiency. In a child, with fatal neurological Leigh-like syndrome, they detected a homozygous 5 bp duplication in the cDNA of the 18 kDa subunit, resulting in destruction of the phosphorylation site. The mutation was transmitted by autosomal recessive mode of inheritance [12]. In skin fibroblasts from this patient we have now found that cAMP fails to promote phosphorylation of the 18 kDa subunit of complex I and to activate the complex. These findings show for the first time that human complex I is regulated via phosphorylation of the subunit encoded by the NDUFS4 gene.

2. Materials and methods

2.1. Materials

DMEM, PBS, trypsin (0.05%), EDTA (0.02%), penicillin, streptomycin, calf serum and fetal bovine serum were from EuroClone; decylubiquinone, IBMX, antimycin-A, rotenone, CCCP, protein-A Sepharose and mouse monoclonal antibody against phosphoserine from Sigma. Horseradish peroxidase conjugate goat anti-mouse IgG antibody, dodecylmaltoside, high-purity digitonin, okadaic acid and cholera toxin were from Calbiochem. ECL system from NEN Life Science. Hyperfilm-MP from Amersham International. Nitrocellulose membrane from Schleicher and Schuell.

2.2. Case report

Male patient (from a collection of complex I deficient patients from the Nijmegen Medical Center) born after normal pregnancy as the second child of healthy non-consaguineous parents. Hospitalized at the age of 8 months was diagnosed as a Leigh-like neurological syndrome [12]. The child died of cardiorespiratory failure at the age of 16 months. The patient exhibited normal values for routine clinical chemical blood and urine analyses. Lactic acid concentration in blood and cerebrospinal fluid were normal. Enzymatic analyses of a skeletal muscle biopsy and skin fibroblast cultures revealed a decreased activity of complex I and a slightly reduced complex III activity in both tissues [12].

2.3. Fibroblast cell culture

Skin human fibroblasts, stored in liquid nitrogen, were grown in high glucose DMEM supplemented with 10% fetal bovine serum at 37°C till 75% confluence. This medium was then replaced with DMEM supplemented with 0.5% fetal bovine serum. After 3 days of serum starvation the cells were treated for 3 h with 1 μg/ml cholera toxin+100 μM IBMX, an inhibitor of cAMP phosphodiesterase. This treatment induces massive intracellular cAMP production [15]. The cells were harvested from Petri dishes with 0.05% trypsin, 0.02% EDTA, and phosphatase inhibitors (5 mM NaF, 500 nM okadaic acid, and 1 mM sodium orthovanadate). After addition of 50 ml of PBS, pH 7.4 with 5% calf serum, cells were collected by centrifugation at $500 \times g$.

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; IBMX, 3-isobutyl-1-methylxanthine; SDS–PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; CCCP, carbonyl-cyanide-*m*-chlorophenylhydrazone

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Fig. 1. Sequence alignment of the 18 kDa subunit of mouse, human and bovine complex I. Sequences were obtained from the Swiss protein database (see also [1,11–13]). The residue numbering refers to the mature mouse, human and bovine proteins. Invariant and homologous residues are shaded in gray. Canonical phosphorylation consensus sites for cAMP-dependent protein kinase are shaded in black.

2.4. Mitoplast preparation

For mitoplast preparation freshly harvested human fibroblasts, suspended in PBS, were exposed for 10 min on ice to 0.2 mg of digitonin/mg cellular protein. The mitoplast fraction was pelleted by centrifugation of the suspension at $14\,000 \times g$ for 10 min.

2.5. Electrophoresis and immunoblotting

Two-dimensional gel analysis (blue-native PAGE/SDS–PAGE) of the mitoplast fraction from human fibroblasts was performed as described in [14,16]. Mitoplast immunoprecipitation with a polyclonal antibody against the 75 kDa subunit of complex I, SDS–PAGE of the immunoprecipitate and immunoblotting with monoclonal anti-phosphoserine antibody and polyclonal antibody against the 24 kDa subunit of complex I were carried out as described previously [14]. Densitometric scanning of immunoblots was performed with a CAMAG TLC Scanner II and a D2500 Cromato intergrator, Merck Hitachi.

2.6. Cellular respiration

Human fibroblasts were suspended in PBS, and respiration was monitored by oxygen polarography.

2.7. Measurement of enzymatic activities

For measurements of the NADH:Q oxidoreductase activity, serum-starved fibroblasts were exposed to ultrasound energy for 15 s at 0°C to eliminate permeability barriers for exogenous NADH and decylubiquinone. The NADH:Q oxidoreductase activity of cells (5×10^5 cells) was determined at 25°C in 700 μl of 40 mM potassium phosphate buffer (pH 7.4), 5 mM $MgCl_2$ and 3 mM KCN with NADH as reductant and 200 μM decylubiquinone as oxidant following spectrophotometrically the oxidation of 1.25–25 μM NADH at 360–374 nm, $\Delta\varepsilon$ 2.01 mM^{-1} [14]. At each concentration of NADH the activity was measured in the absence (a) and presence of 1 μg/ml rotenone (b). The rotenone-sensitive NADH:Q oxidoreductase was the difference, a−b. Cytochrome *c* oxidase activity was determined as described in [17].

3. Results

Skin fibroblasts from the patient were cultivated under serum starvation for 3 days, which results in down-regulation of the cAMP-dependent phosphorylation and activity of complex I [14]. Fibroblasts were then treated for 3 h with cholera toxin, which induces massive intracellular cAMP production [15] and, under normal conditions, consequent activation of complex I [14]. Complex I, separated by blue-native PAGE of

Table 1
Effect of cholera toxin on enzymatic and respiratory activities of control and patient's serum-starved fibroblasts

	Control fibroblasts				Patient's fibroblasts			
	Serum-starved		+Cholera toxin		Serum-starved		+Cholera toxin	
	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m
	fmol/min/cell	μM NADH	fmol/min/cell	μM NADH	fmol/min/cell	μM NADH	fmol/min/cell	μM NADH
A. Enzymatic activities								
NADH:Q oxidoreductase	2.39 ± 0.15(8)	2.77 ± 0.40(8)	6.46 ± 0.37(8)	6.51 ± 1.13(8)	1.86 ± 0.35(5)	4.8 ± 3.08(5)	2.14 ± 0.47(5)	5.0 ± 2.4(5)
			$P < 0.001$	N.S.			N.S.	N.S.
	fmol/min/cell		fmol/min/cell		fmol/min/cell		fmol/min/cell	
Cytochrome *c* oxidase	9.87 ± 5.08(3)		13.53 ± 3.30(3)		19.76 ± 3.38(3)		19.91 ± 6.29(3)	
			N.S.				N.S.	
	femtoatoms O/min/cell		femtoatoms O/min/cell		femtoatoms O/min/cell		femtoatoms O/min/cell	
B. Substrates								
Glutamate+malate	3.38		12.23		4.29		5.49	
Ascorbate+TMPD	6.40		5.88		5.83		7.95	

Serum-starved human fibroblasts, from control and patient, and starved fibroblasts incubated, where indicated, 3 h with cholera toxin+IBMX, were suspended in phosphate-buffered saline with phosphatase inhibitors and immediately respiration and enzymatic activities were determined as described in Section 2 [14]. V_{max} and K_m were obtained from Lineweaver–Burk plots of the rotenone-sensitive NADH–ubiquinone oxidoreductase. Respiration was measured polarographically on fibroblast suspension ($1.5–2 \times 10^6$ cells) in 650 μl of 75 mM sucrose, 30 mM Tris–Cl, 50 mM KCl, 0.5 mM EDTA, 0.5 mM $MgCl_2$, 2 mM potassium phosphate buffer, pH 7.4, 1 μM CCCP (carbonyl-cyanide-*m*-chlorophenylhydrazone). Cells were permeabilized with 0.002% digitonin. Substrate concentrations were: 10 mM glutamate, 10 mM malate, 2 mM ascorbate and 0.2 mM TMPD (*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine). Complex I V_{max} and K_m and cytochrome oxidase activity values represent means ± S.E.M., with number of experiments in parentheses. The respiratory activities are the means of two measurements on different batches of fibroblasts.

the mitoplast fractions obtained from control and patient's fibroblasts, was resolved into constituent subunits by two-dimensional SDS–PAGE. The immunoblots with an antibody against phosphoserine, presented in Fig. 2A, show that the exposure of control fibroblasts to cholera toxin promoted serine phosphorylation in the 18 kDa subunit of complex I. In the patient's fibroblasts the same treatment did not result in phosphorylation of this subunit. In control fibroblasts promotion by cholera toxin treatment of serine phosphorylation in the 18 kDa subunit of complex I was also evident in the mitoplast fraction immunoprecipitated by a polyclonal antibody against the 75 kDa subunit of complex I (Fig. 2B). Failure of the cholera toxin treatment to promote phosphorylation of the 18 kDa subunit in the patient was confirmed in the immunoprecipitate of complex I.

It can be noted that the content of the 24 kDa subunit of complex I was in the patient's fibroblasts the same as in control fibroblasts (Fig. 2B,C). This indicates that the present mutation in the NDUFS4 did not impair assembly of complex I in mitochondria.

Table 1 summarizes data on the V_{max} and K_m values, obtained from typical Lineweaver–Burk plots, of the normal rotenone-sensitive NADH–ubiquinone oxidoreductase in control and patient's serum-starved fibroblasts, previously exposed to ultrasonic energy to eliminate permeability barriers for exogenous NADH and decylubiquinone. It can be seen that in serum-starved conditions, there was only small difference in the V_{max} of the rotenone-sensitive NADH–ubiquinone oxidoreductase activity in the patient's fibroblasts as compared to control fibroblasts. The cholera toxin treatment, which increased by more than two-fold the V_{max} of the rotenone-sensitive NADH–ubiquinone oxidoreductase activity in control fibroblasts, failed to produce activation of the activity of complex I in patient's fibroblasts. In both control and patient's fibroblasts cholera toxin treatment had no statistically significant effect on the K_m of NADH–ubiquinone oxidoreductase activity. It was also noted that the patient's fibroblasts showed enhancement of a rotenone-insensitive NADH–ubiquinone oxidoreductase activity, which was unaffected by cholera toxin treatment. The nature of this activity remains to be clarified. In control serum-starved fibroblasts the cholera toxin treatment enhanced by more than three-fold the overall respiration supported by NAD-linked substrate, but failed to exert significant effect on respiration in serum-starved patient's fibroblasts (Table 1). Cytochrome *c* oxidase (complex IV) was not depressed in the patient's fibroblasts as compared to control fibroblasts. Both in control and in patient's fibroblasts cholera toxin treatment had no significant effect on this activity (Table 1).

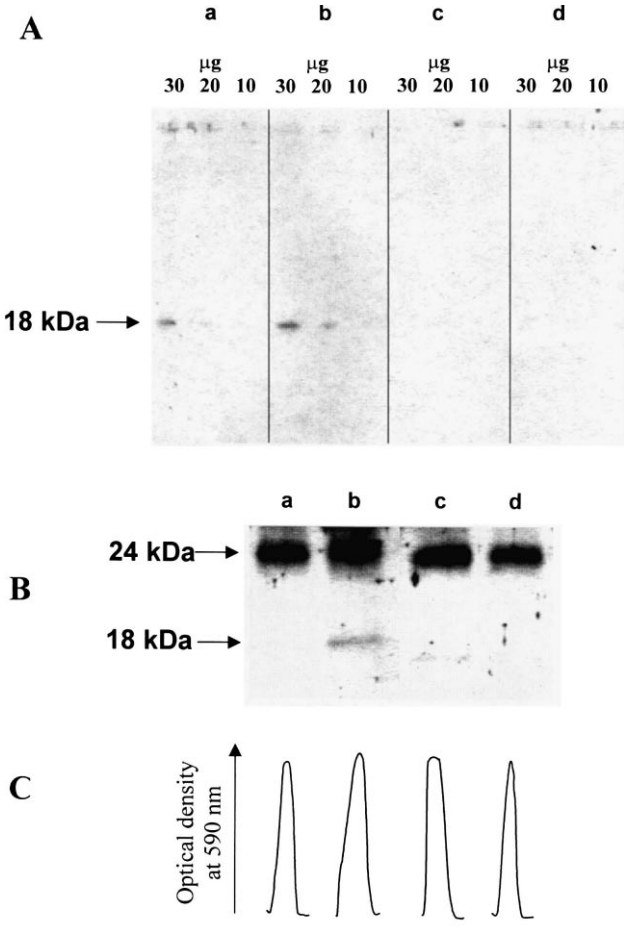

Fig. 2. Immunodetection of phosphoserine-containing proteins in human fibroblasts. A: Western blot of SDS–PAGE of complex I, separated by blue-native PAGE of mitoplasts [16] from serum-starved human fibroblasts, immunodecorated with anti-phosphoserine antibody. Mitoplasts (10, 20, 30 μg proteins) from control fibroblasts (a), control fibroblasts treated with cholera toxin (b), patient's fibroblasts (c), patient's fibroblasts treated with cholera toxin (d), were applied to a 5–13% blue-native gradient gel. Complex I band was cut from the gel and runned in a two-dimensional SDS–PAGE, blotted onto nitrocellulose, incubated with mouse anti-phosphoserine antibody and developed using ECL system. B: Western blot of mitoplasts fraction immunoprecipitated with a polyclonal antibody against the 75 kDa subunit of complex I. Mitoplasts from fibroblasts were solubilized in 1% laurylmaltoside and incubated overnight with the antibody against the 75 kDa subunit of complex I, preadsorbed on protein-A activated Sepharose. The immunoprecipitate was dissolved in lysis buffer, resolved by SDS–PAGE, blotted onto nitrocellulose, immunodecorated with mouse anti-phosphoserine and rabbit anti-24 kDa subunit of complex I antibodies and developed using ECL system. C: Densitograms of the 24 kDa subunit immunoblots.

4. Discussion

The observations presented allow to draw a number of conclusions. In the patient the 5 bp duplication in the NDUFS4 gene, which destroys the phosphorylation site in the 18 kDa subunit of complex I [12], abolishes cAMP-dependent phosphorylation of the 18 kDa mitoplast protein band attributed to complex I and activation of the normal rotenone-sensitive NADH–ubiquinone oxidoreductase activity of the complex in fibroblast cultures. Independent controls showed that the NDUFS4 protein was present in the fibroblasts of the patient. Whilst the present work was being completed three new mutations in the NDUFS4 gene were found in patients with complex I deficiencies. All these mutations resulted in destruction of the consensus phosphorylation site in the C-terminus of the NDUFS4 protein ([18] and V. Petruzzella, R. Vergari, I. Puzziferri, D. Boffoli, E. Lamantea, M. Zeviani and S. Papa, manuscript in preparation). These findings provide substantial evidence showing that cAMP-mediated intracellular signal transduction, through serine phosphorylation of the NDUFS4 18 kDa subunit of complex I regulates the activity of the complex in cAMP-responsive mammalian tissues. Since complex I represents, at least under

certain conditions, the rate-limiting step of the mitochondrial respiratory chain, up-regulation of complex I by cAMP-induced phosphorylation of the NDUFS4 18 kDa subunit can activate the overall cellular respiratory activity and aerobic ATP production.

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References

[1] Walker, J.E. (1992) Q. Rev. Biophys. 25, 253–324.
[2] Skehel, J.M., Fearnly, I.M. and Walker, J.E. (1998) FEBS Lett. 438, 301–305.
[3] Chomyn, A., Cleeter, M.W., Ragan, C.I., Riley, M., Doolittle, R.F. and Attardi, G. (1986) Science 234, 614–618.
[4] Smeitink, J. and van den Heuvel, L. (1999) Am. J. Hum. Genet. 64, 1505–1510.
[5] Smeitink, J.A., Loeffen, J.L., Triepels, R.H., Smeets, R.J., Trijbels, J.M. and van den Heuvel, L.P. (1998) Hum. Mol. Genet. 7, 1573–1579.
[6] Robinson, B.H. (1998) Biochim. Biophys. Acta 1364, 271–286.
[7] Sardanelli, A.M., Technikova-Dobrova, Z., Scacco, S.C., Speranza, F. and Papa, S. (1995) FEBS Lett. 377, 470–474.
[8] Papa, S., Sardanelli, A.M., Cocco, T., Speranza, F., Scacco, S.C. and Technikova-Dobrova, Z. (1996) FEBS Lett. 379, 299–301.
[9] Papa, S., Sardanelli, A.M., Scacco, S. and Technikova-Dobrova, Z. (1999) FEBS Lett. 444, 245–249.
[10] Pearson, R.B. and Kemp, B.E. (1991) Methods Enzymol. 200, 62–81.
[11] Walker, J.E., Arizmendi, J.M., Dupuis, A., Fearnley, I.M., Finel, M., Medd, S.M., Pilkington, S.J., Runswick, M.J. and Skehel, J.M. (1992) J. Mol. Biol. 226, 1051–1072.
[12] Van den Heuvel, L., Ruitenbeek, W., Smeets, R., Gelman-Kohan, Z., Elpeleg, O., Loeffen, J., Trijbels, F., Mariman, E., de Bruijn, D. and Smeitink, J. (1998) Am. J. Hum. Genet. 62, 262–268.
[13] Bairoch, A., Bucher, P. and Hofmann, K. (1997) Nucleic Acids Res. 25, 217–221.
[14] Scacco, S., Vergari, R., Scarpulla, R.C., Technikova-Dobrova, Z., Sardanelli, A., Lambo, R., Lorusso, V. and Papa, S. (2000) J. Biol. Chem. 275, 17578–17582.
[15] Gopalakrishnan, L. and Scarpulla, R.C. (1994) J. Biol. Chem. 269, 105–113.
[16] Klement, P., Nijtmans, L.G., Van den Bogert, C. and Houstek, J. (1995) Anal. Biochem. 231, 218–224.
[17] Cooperstein, S.J. and Lazarow, A. (1951) J. Biol. Chem. 189, 665–670.
[18] Budde, S.M.S., Van den Heuvel, L.P.W.J., Janssen, A.J., Smeets, R.J.P., Buskens, C.A.F., DeMeirleir, L., Van Coster, R., Baethmann, M., Voit, T., Trijbels, J.M.F. and Smeitink, J.A.M. (2000) Biochem. Biophys. Res. Commun. 275, 63–68.