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Minireview

The NADH: Ubiquinone Oxidoreductase (Complex I) of the Mammalian Respiratory Chain and the cAMP Cascade

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Recent work has revealed cAMP-dependent phosphorylation of the 18-kDa IP subunit of the mammalian complex I of the respiratory chain, encoded by the nuclear *NDUFS4* gene (chromosome 5). Phosphorylation of this protein has been shown to take place in fibroblast cultures in vivo, as well as in isolated mitochondria, which in addition to the cytosol also contain, in the inner-membrane matrix fraction, a cAMP-dependent protein kinase. Mitochondria appear to have a Ca^{2+}-inhibited phosphatase, which dephosphorylates the 18-kDa phosphoprotein. In fibroblast and myoblast cultures cAMP-dependent phosphorylation of the 18-kDa protein is associated with potent stimulation of complex I and overall respiratory activity with NAD-linked substrates. Mutations in the human *NDUFS4* gene have been found, which in the homozygous state are associated with deficiency of complex I and fatal neurological syndrome. In one case consisting of a 5 bp duplication, which destroyed the phosphorylation site, cAMP-dependent activation of complex I was abolished in the patient's fibroblast cultures. In another case consisting of a nonsense mutation, leading to termination of the protein after only 14 residues of the putative mitochondria targeting peptide, a defect in the assembly of complex I was found in fibroblast cultures.

KEY WORDS: Protein phosphorylation; mitochondria; complex I; cAMP-dependent protein kinase; *NDUFS4* gene.

INTRODUCTION

In mammalian mitochondria complex I (NADH: ubiquinone oxidoreductase EC 1.6.5.3) of the respiratory chain catalyzes the oxidation of NADH by ubiquinone, and conserves the free energy so made available as transmembrane proton-motive force (PMF). The reaction mechanism of this complex, the functional role of its numerous constituent subunits (Skehel *et al*., 1998; Walker, 1992), the biogenesis, and regulation are not yet fully understood. Complex I represents, at least under certain conditions, the rate-limiting step of the overall respiratory activity in mitochondria. Complex I deficiency represents one of the most severe and frequent of the disorders in the mitochondrial energy metabolism associated with human diseases (Robinson, 1998; Smeitink *et al.*, 2001; Smeitink and van den Heuvel, 1999).

Complex I consists of 43 subunits (Skehel *et al.*, 1998; Walker, 1992). Seven subunits (ND1–ND6 and ND4L) are encoded by the mitochondrial genome, the others are encoded by nuclear genes. The cDNA of the seven mitochondrial subunits (Chomyn *et al*., 1985, 1986) and of 35 nuclear subunits (Loeffen *et al.*, 1998; Smeitink *et al.*, 1998) have been sequenced. High resolution electron microscopy analysis of Neurospora crassa complex I shows this to have an L-shaped configuration with a peripheral arm protruding in the matrix and an hydrophobic mass inserted in the inner mitochondrial membrane (Guénebaut *et al.*, 1997). The bovine complex I can be fragmented in three moieties, the flavoprotein fraction (FP) consisting of three subunits, the iron–protein fraction (IP) with seven subunits, and the hydrophobic part

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Table I. Functions of Some Nuclear Encoded Subunits of Complex I

51 (NDUFV1)	Flavin, 4Fe-4S	NADH binding
24 (NDUFV2)	2Fe-2S	e^- transfer
75 (NDUFS1)	4Fe-4S	e^- transfer
23 (NDUFS8)	4Fe-4S	e^- transfer
20 (NDUFS7)	3Fe-3S	e^- transfer
14 (NDUFA7)	Q-binding	
10 (NDUFAB1)	acyl carrier protien	
18 (NDUFS4)	cAMP-dependent phosphorylation	

comprising the remaining subunits (Walker, 1992). The function of only a few of the subunits of complex I has so far been identified (Robinson, 1998; Walker, 1992) (see Table I). Besides those involved in the redox function, the SDAP 10 kDa subunit, sharing homology with acyl carrier proteins (Runswick *et al.*, 1991), appears to be involved in mitochondrial phospholipid metabolism (Robinson, 1998). Evidence that the 24 kDa subunit is a G protein has also been presented (Hegde, 1998).

Recentley Papa *et al.* discovered that the 18-kDa IP subunit of the mammalian complex I is phosphorylated by the cAMP-dependent protein kinase (Papa *et al.*, 1996; Sardanelli *et al.*, 1995). Following these findings investigations were carried out in fibroblast cultures which identified a role of this subunit, and its cAMP-dependent phosphorylation, in the physiopathology of mitochondrial energy metabolism (Papa *et al.*, 1999, 2001; Petruzzella *et al.*, 2001; Scacco *et al.*, 2000).

The 18-kDa IP subunit, (AQDQ N-terminus in bovine heart) (Walker *et al.*, 1992) is encoded by the nuclear *NDUFS4* gene (van den Heuvel *et al.*, 1998). It consists

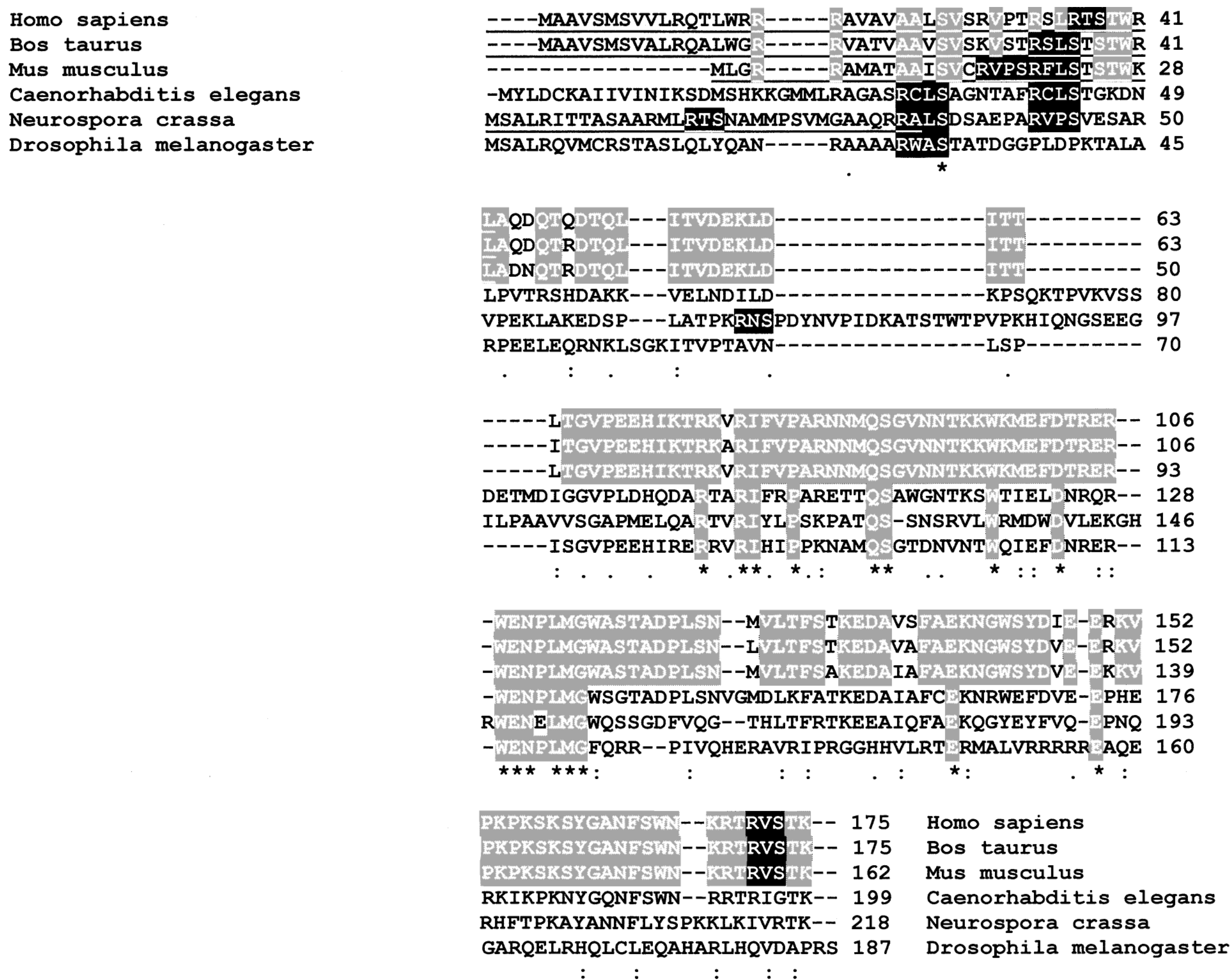

Fig. 1. Sequence alignment of the 18-kDa IP subunit of *H. sapiens*, *B. taurus*, *M. musculus*, *C. elegans*, *D. melanogaster*, complex I and their analogue 21-kDa subunit from *N. crassa*. Sequences were obtained from Swiss-Prot Database (see also Scacco *et al.*, 2000). Putative canonical phosphorylation consensus sites for PKA are shaded in black. Conserved residues are shaded in gray. Known mitochondrial import sequence are underlined.

of 175 aminoacids, which appear to be highly conserved in the known mammalian sequences, exhibiting around 90% interspecies homology (Fig. 1) (Bairoch *et al.*, 1997; Murdock *et al.*, 1999; van den Heuvel *et al.*, 1998; Walker *et al.*, 1992). The mature form of 133 aminoacids does not bind any prosthetic group. In mammals it has, at the carboxy-terminal position 129–131, a canonical (Pearson and Kemp, 1991) cAMP-dependent protein kinase phosphorylation consensus site (RVS), in which the serine residue is phosphorylated (Fig. 1) (Papa *et al.*, 1999). The protein has a leader sequence, removed after import into mitochondria (Walker *et al.*, 1992), that also contains a phosphorylation consensus site (RTS in the human protein, at position −7 to −5). The leader sequence has a number of positively charged and hydroxylated residues which are characteristic of import signals of nuclear encoded subunits of oxidative phosphorylation complexes (Hartl and Neupert, 1990).

The putative corresponding subunit in *Caenorhabditis elegans* (The C. elegans Genome Sequencing Consortium, 1998) shows a sequence homology with the mammalian 18-kDa IP subunit of around 45%, those of *Drosophila* (Adams *et al.*, 2000) and *Neurospora crassa* (Azevedo *et al.*, 1994) of around 20 and 25% respectively. It can be noted that in the proteins of these three species no consensus phosphorylation site is present in the C-terminus, but one in the *Drosophila* protein and two phosphorylation sites in the *C. elegans* and *N. crassa* are present in the N-terminal region. In the *N. crassa* protein one of these two phosphorylation sites encompasses the C-terminus of the leader sequence and the first two N-terminal residues of the mature protein (Fig. 1). It is not known whether the low homology and different location of the phosphorylation consensus sites, with respect to the mammalian protein, results in these organisms in a different functional response of complex I to cAMP.

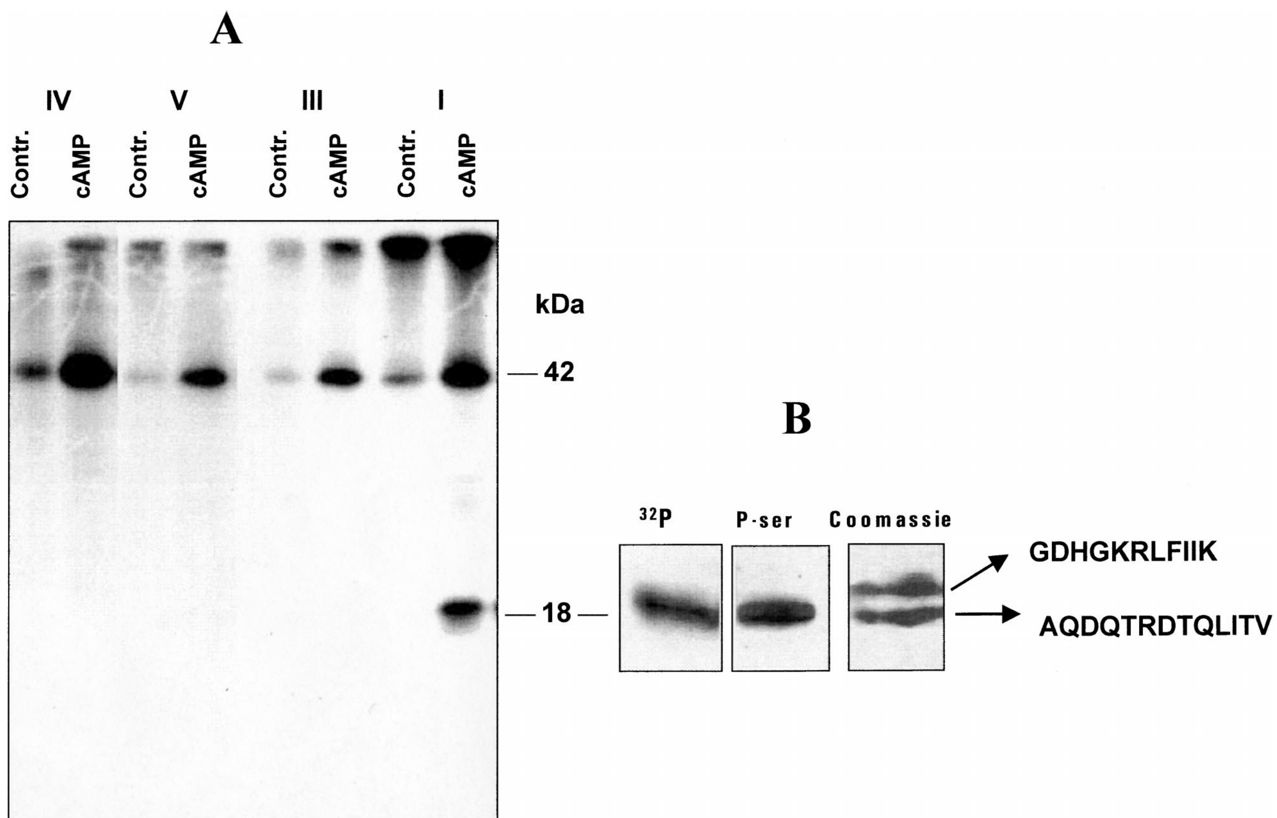

Fig. 2. (A) Two dimensional electrophoretic pattern of protein labelling by [γ-^{32}P]ATP in oxidative phosphorylation complexes from bovine heart mitochondria separated by blue native electrophoresis and resolved in their subunits by SDS-PAGE (reproduced from Sardanelli *et al.*, 1995). (B) Autoradiogram of ^{32}P labelling and immunodetection of phosphoserine in the 18-kDa (AQDQ) subunit of complex I. Purified complex I, 100 μg proteins, was incubated with 50 μg inner membrane proteins, 25 μM cAMP and 150 μM [γ-^{32}P]ATP. The 18-kDa AQDQ subunit was isolated from purified complex I by SDS-PAGE. After electrophoresis proteins were transferred from the gel to nitrocellulose membrane and exposed for autoradiography and phosphoserine immunodetection; the radioactive gel band was electrotransferred to immobilon membrane for aminoacid sequencing (Papa *et al.*, 1996).

cAMP-DEPENDENT PROTEIN KINASE AND PHOSPHATASE CATALYZE REVERSIBLE PHOSPHORYLATION OF THE 18-kDa SUBUNIT OF COMPLEX I IN MITOCHONDRIA

In the absence of added cAMP the only significant phosphorylation by [γ-^{32}P]ATP that can be detected in intact mitochondria is that of the protein band of 42 kDa, where the α subunit of pyruvate dehydrogenase migrates (Reed *et al.*, 1987). The addition of cAMP or of dibutyryl-cAMP results in the phosphorylation of three other protein bands of 29, 18, and 6.5 kDa respectively (Technikova-Dobrova *et al.*, 1994). In intact mitochondria the cAMP-dependent phosphorylation of these proteins by [γ-^{32}P]ATP is inhibited by carboxyactractyloside (Sardanelli *et al.*, 1996). Suppression of the cAMP-dependent phosphorylation of mitochondrial proteins by carboxyactractyloside, which inhibits transport of added ATP to the matrix side of the inner mitochondrial membrane (Brandolin *et al.*, 1993), in addition to providing unequivocal evidence for the existence of a functional PKA holoenzyme in the mitochondrial inner membrane matrix (mtPKA), shows that both the catalytic site of PKA and the mitochondrial proteins phosphorylated in the presence of cAMP are localized at the matrix side of the inner mitochondrial membrane. In mitoplasts, which consist of inner membrane vesicles and matrix with removal of the outer membrane, intermembrane space and cytosolic contaminants, the same pattern, as whole mitochondria, of cAMP-dependent serine phohosphorylation in protein bands of 29, 18, and 6.5 kDa is observed. cAMP-dependent phosphorylation of the protein bands of 18 and 6.5 kDa is associated with the inner membrane fraction (Sardanelli *et al.*, 1995).

The 2D electrophoretic pattern of inner membrane proteins of bovine heart mitochondria reveals, upon silver staining, the presence of numerous proteins of 42, 29, and 18 kDa (not shown). The overall pattern of phosphorylation of the inner membrane proteins, obtained in the presence of added cAMP, shows that few proteins of 42, 29, and 18 kDa are phosphorylated by [γ-^{32}P]ATP.

Separation of the five complexes of oxidative phosphorylation by blue-native electrophoresis of the inner membrane fraction of bovine heart mitochondria, incubated with [γ-^{32}P]ATP, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the individual complexes has shown that cAMP promotes ^{32}P-labelling of the 18-kDa protein band of complex I (Fig. 2) (Sardanelli *et al.*, 1995). Furthermore it has been found that the purified catalytic subunit of PKA catalyzed, in the presence of [γ-^{32}P]ATP, ^{32}P-labelling of the 18-kDa AQDQ subunit of the purified complex I from bovine heart mitochondria (Papa *et al.*, 1996). Besides the purified catalytic subunit of PKA, the inner membrane fraction of bovine heart mitochondria also catalyzed cAMP-dependent ^{32}P-labelling of the 18-kDa subunit in a sample of purified complex I. Immunoblotting analysis with a specific monoclonal antibody showed that the residue phosphorylated in the 18-kDa subunit was a serine (Fig. 2).

Immunoblot analysis with specific antibodies revealed the presence of both the regulatory (RII–PKA) and the catalytic subunit (C-PKA) in bovine heart mitochondria. RII–PKA is present both the cytosol and mitochondria, where it is distributed between the inner membrane and matrix fraction. As expected a large amount of C-PKA is present in the cytosol. A significant level of C-PKA is also detected in mitochondria, where it is particularly enriched in the matrix fraction (Technikova-Dobrova *et al.*, 2001).

An important breakthrough in understanding the intracellular distribution of PKA, was provided by the discovery of a new family of proteins (protein kinase A anchor proteins, AKAP), which specifically anchor

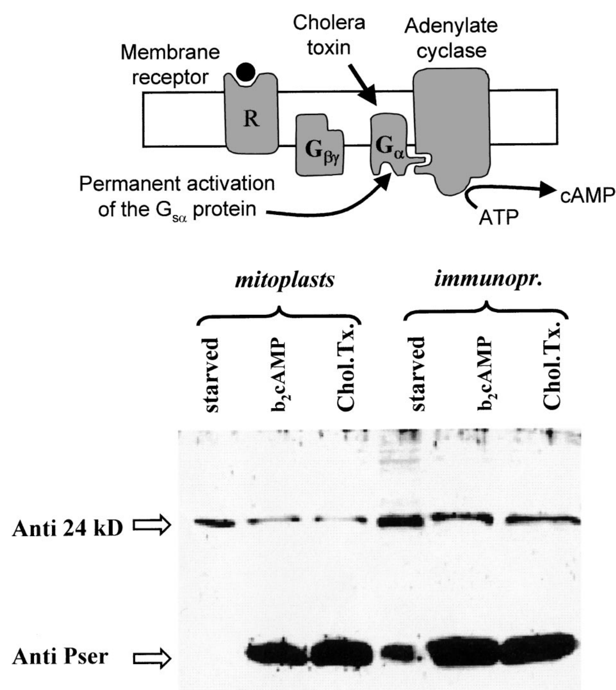

Fig. 3. cAMP production in 3T3 fibroblasts cultures induced by cholera toxin. Cholera toxin A-subunit induces ADP-ribosylation and activation of the α-subunit of the stimulatory G protein. The activation of Gs α increases the activity of adenylate cyclase resulting in enhanced level of cAMP. Immunodetection of phosphoserine-containing proteins and of the 24-kD subunit of complex I in mitoplasts from mouse fibroblasts and in solubilized proteins immunoprecipitated from mitoplasts with an antibody against the 75-kD subunit of complex I (reproduced from Scacco *et al.*, 2000).

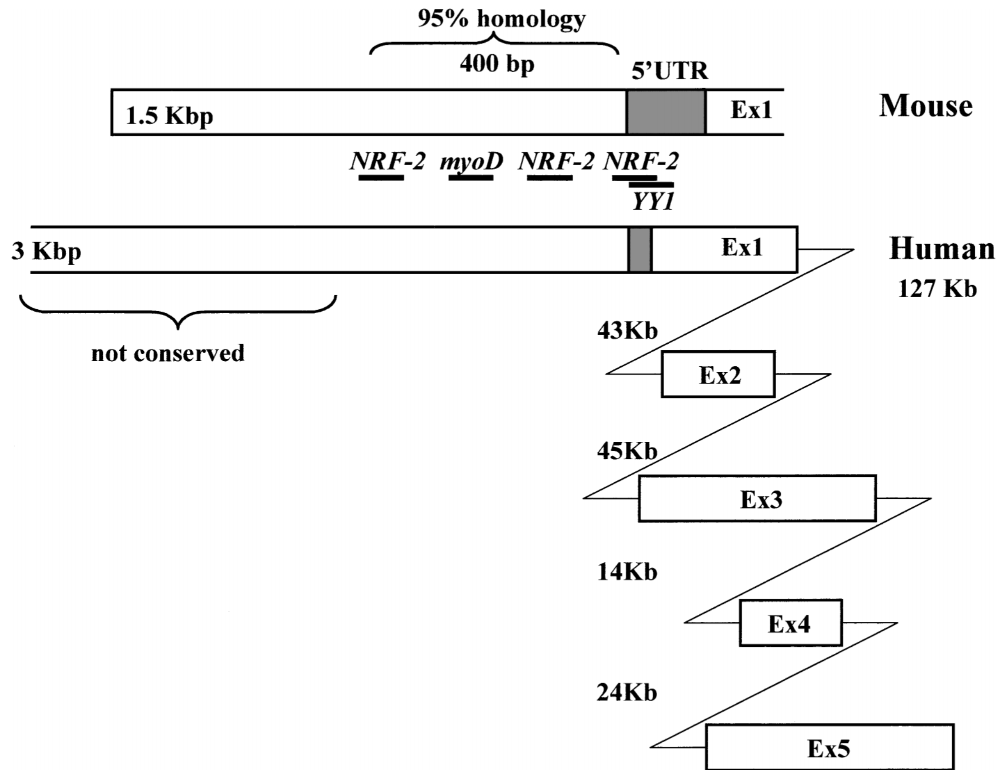

Fig. 4. Structure of the human and mouse *NDUFS4* genes with promoter elements.

PKA to different cellular structures (Pawson and Scott, 1997; Rubin, 1994). More recently two mammalian mitochondrial AKAPs which bind both RI and RII isoforms have been identified, D-AKAP1 (Huang *et al.*, 1997) and D-AKAP2 (Wang *et al.*, 2001). Alternative N-terminus splice variants seem to control the direction of D-AKAP1 either to mitochondria or endoplasmic reticulum (Huang *et al.*, 1999). D-AKAP2 has a positively charged N-terminus which might serve for its preferential localization in mitochondria (Wang *et al.*, 2001).

The 18-kDa subunit of complex I after its phosphorylation by PKA can be dephosphorylated by a phosphatase associated with the inner mitochondrial membrane, which in contrast to the Ca^{2+}-dependent phosphatase of the pyruvate–dehydrogenase complex (Reed *et al.*, 1987), is inhibited by Ca^{2+} (Signorile *et al.*, 2001).

cAMP-DEPENDENT PHOSPHORYLATION OF THE 18-kDa SUBUNIT OF COMPLEX I AND ACTIVATION OF THE COMPLEX IN CELL CULTURES IN VIVO

Whilst mitochondrially encoded proteins can only be phosphorylated by mtPKA, nuclear encoded proteins can be first phosphorylated in the cytosol, where the major pool of PKA is localized, and then imported in mitochondria where they can also be phosphorylated. Thus, a complex pattern of phosphorylation–dephosphorylation in the two cell compatments can take place, the physiological significance of which remains to be understood. Clues for the elucidation of these aspects are emerging from studies on the occurrence and role of cAMP-dependent phosphorylation of mitochondrial proteins in cell cultures.

Investigations along these lines have shown that an intracellular increase in the level of cAMP, induced by activation of adenylate cyclase with cholera toxin in 3T3 BALB/c mouse fibroblasts in the serum starved resting state, promoted phosphorylation of the 18-kDa subunit of complex I associated to the inner mitochondrial membrane (Fig. 3) (Scacco *et al.*, 2000). This phosphorylation was accompanied by marked enhancement of the activity of complex I as well as by stimulation of mitochondrial respiration supported by NAD-linked substrates. A short term stimulatory effect on complex I activity was also produced by incubation with dibutyryl-cAMP of mitoplasts isolated from starved fibroblasts (Scacco *et al.*, 2000). Thus phosphorylation of the 18-kDa subunit by PKA regulates the activity of complex I; through this effect a rise in the cellular level of cAMP can result in activation of mitochondrial respiration and ATP synthesis.

THE *NDUFS4* GENE

The human *NDUFS4* gene, coding for the 18-kDa IP subunit of complex I, is localized by BLAST search at position 5q11.1 of chromosome 5. The BLAST search reveals 5 exons with 100% identity with the cDNA of the gene, which are intercalated by four large introns (PUBMED GENBANK) (Fig. 4). The first exon is preceeded by a short 5′ UTR. The promoter region presents a 400-bp segment which shares 95% homology with the promoter of the mouse gene. The overall size of the human *NDUFS4* gene amounts to 127 Kb, showing this to be a relatively large gene. In the conserved segment of the promoter nucleotide sites for the transcription factors NRF2, myoD, and YYI are localized. Investigations are being carried in collaboration with Richard Scarpulla of the Northwestern University in Chicago in order to better characterize the promoter region of the gene and its regulation.

PATHOLOGICAL MUTATIONS IN THE HUMAN *NDUFS4* GENE

Loeffen *et al.* (2000), examining numerous tissue specimen from children affected by encefalomyocardiopathies with suspected oxidative phosphorylation disorders, found complex I deficiency to represent the most frequent of OXPHOS defects (23% isolated deficiency, 32% deficiency combined with defects in other respiratory complexes).

After the finding of Papa *et al.* (Papa *et al.*, 1996; Sardanelli *et al.*, 1995) that the 18-kDa subunit of bovine

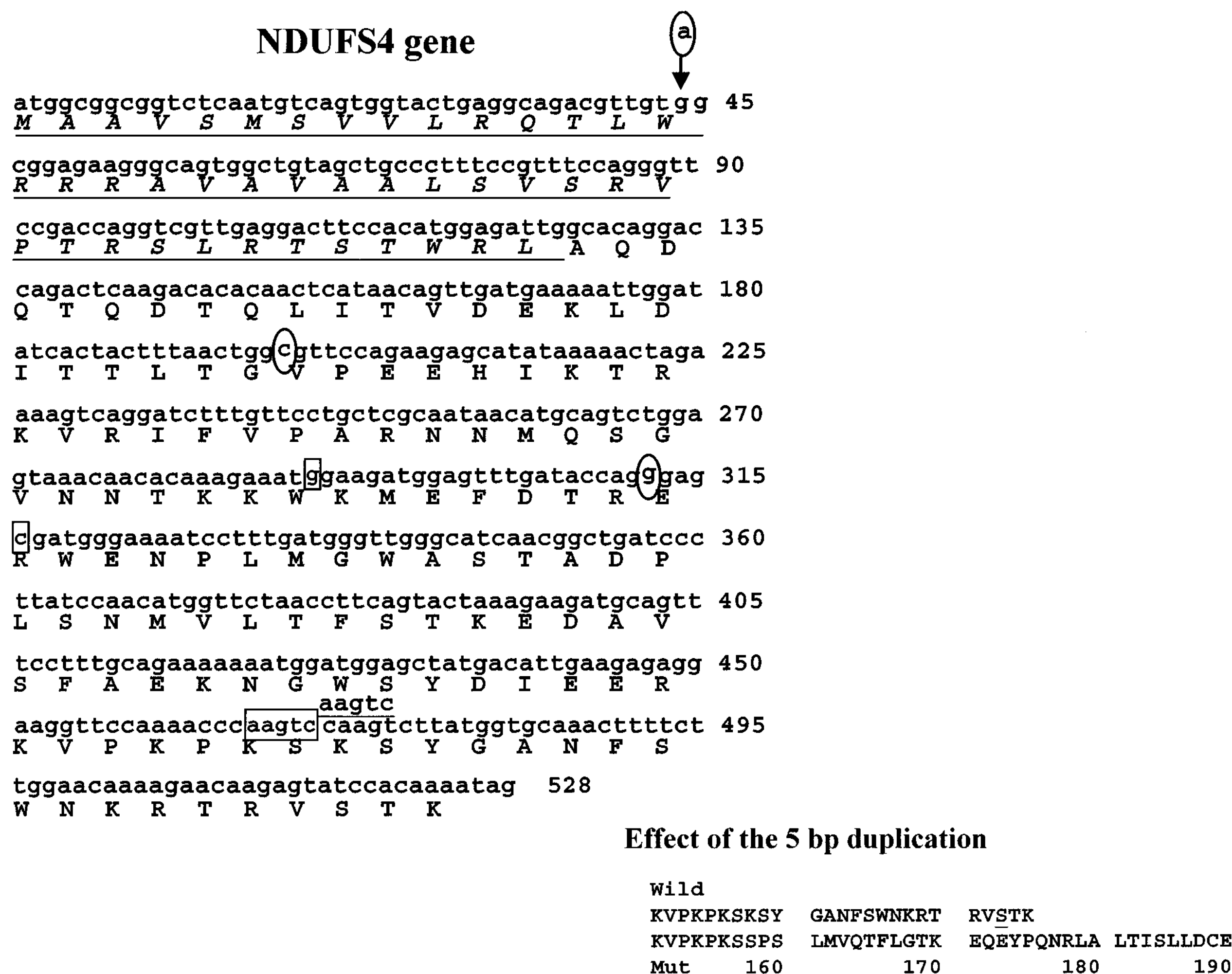

Fig. 5. Sequence of human *NDUFS4* cDNA. Pathogenic mutation and polymorphisms found in our laboratory are circled. Mutations reported by the Nijmegen group are boxed. The effect of the 5-bp duplication on the sequence of the carboxy-terminus of the protein is also shown (reproduced from Petruzzella *et al.*, 2001).

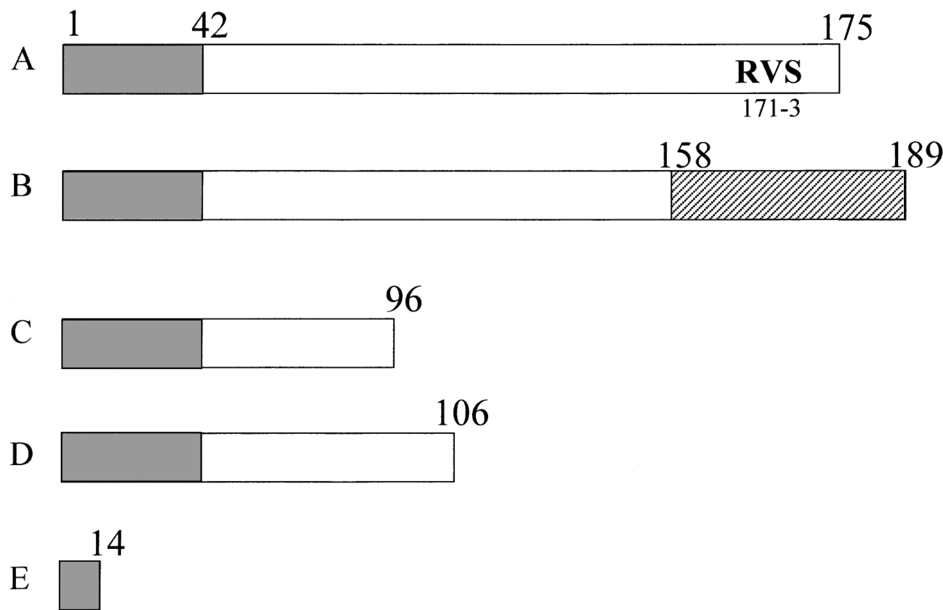

Fig. 6. Wild type (A) and mutated forms (B, from van den Heuvel *et al.*, 1998, C and D, from Budde *et al.*, 2000, E, from Petruzzella *et al.*, 2001) of the protein encoded by the *NDUFS4* gene. The import presequence is in gray. The frameshift of 5-bp duplication is shaded.

complex I can be phosphorylated by PKA, van den Heuvel *et al.* (van den Heuvel *et al.*, 1998) started their mutation analysis in complex I-deficient patients with examination of the 18-kDa *NDUFS4* gene. Sequence analysis of this gene, cloned by RT-PCR, revealed in children who died at the age of 16 months with a fatal neurological syndrome, a homozygous 5-bp duplication of the cDNA sequence at position 466–470 (AAGTC) (Fig. 5), resulting in a shift of the translational reading frame with destruction of the phoshorylation consensus site and elongation of the C-terminus of the protein by 14 residues (van den Heuvel *et al.*, 1998) (Figs. 5 and 6). Both parents were heterozygous for this mutation, thus showing an autosomal recessive mode of inheritance. Investigations on skin fibroblast cultures from this patient showed that the 5-bp duplication abolished cAMP-dependent phosphorylation of the 18-kDa subunit of complex I and cAMP activation of the normal rotenone-sensitive NADH: ubiquinone oxidoreductase activity of the complex and of the NAD-linked respiratory activity (Papa *et al.*, 2001). These findings showed, for the first time, that human complex I is regulated via phosphorylation of the subunit encoded by the *NDUFS4* gene. In the last months three other patients with different mutations in the *NDUFS4* gene have been identified. Two children with complex I deficiency from different families, died of fatal neurological syndrome, presented respectively a deletion of G at position 289 or 290, with change of the tryptophan-96 codon to a stop codon, and a C to T transition at position 316, with change of the GCA arginine codon to a stop codon (Budde *et al.*, 2000). Both mutations which segregated in the families with an autosomal recessive mode of inheritance, caused in the homozygous patient's protein premature stop with destruction of the phosphorylation consensus site in the carboxy terminal region (Budde *et al.*, 2000). Sequence analysis of mitochondrial and nuclear candidate genes of complex I in three other children with complex I deficiency, died of fatal neurological syndrome, revealed in one of them another different mutation in the *NDUSF4* gene (Petruzzella *et al.*, 2001). This mutation, which also segregated in the patient's family with an autosomal recessive mode of inheritance, consisted in the patient of a homozygous G to A transition at nucleotide position +44 of the coding sequence of the gene, changing a tryptophan codon (TGG) to a stop codon (TAG) with premature termination of the protein after only 14 amino acids of the putative mitochondrial targeting peptide.

This nonsense mutation completely suppressed the normal rotenone-sensitive NADH:UQ oxidoreductase activity of the complex in the patient's fibroblast cultures, both in the absence and presence of cholera toxin-induced cAMP production, and prevented its normal assembly in the inner mitochondrial membrane. The 18-kDa subunit is apparently located in a strategic position within the complex, at the junction between the peripheral mass protruding in the matrix and the membrane moiety of complex I

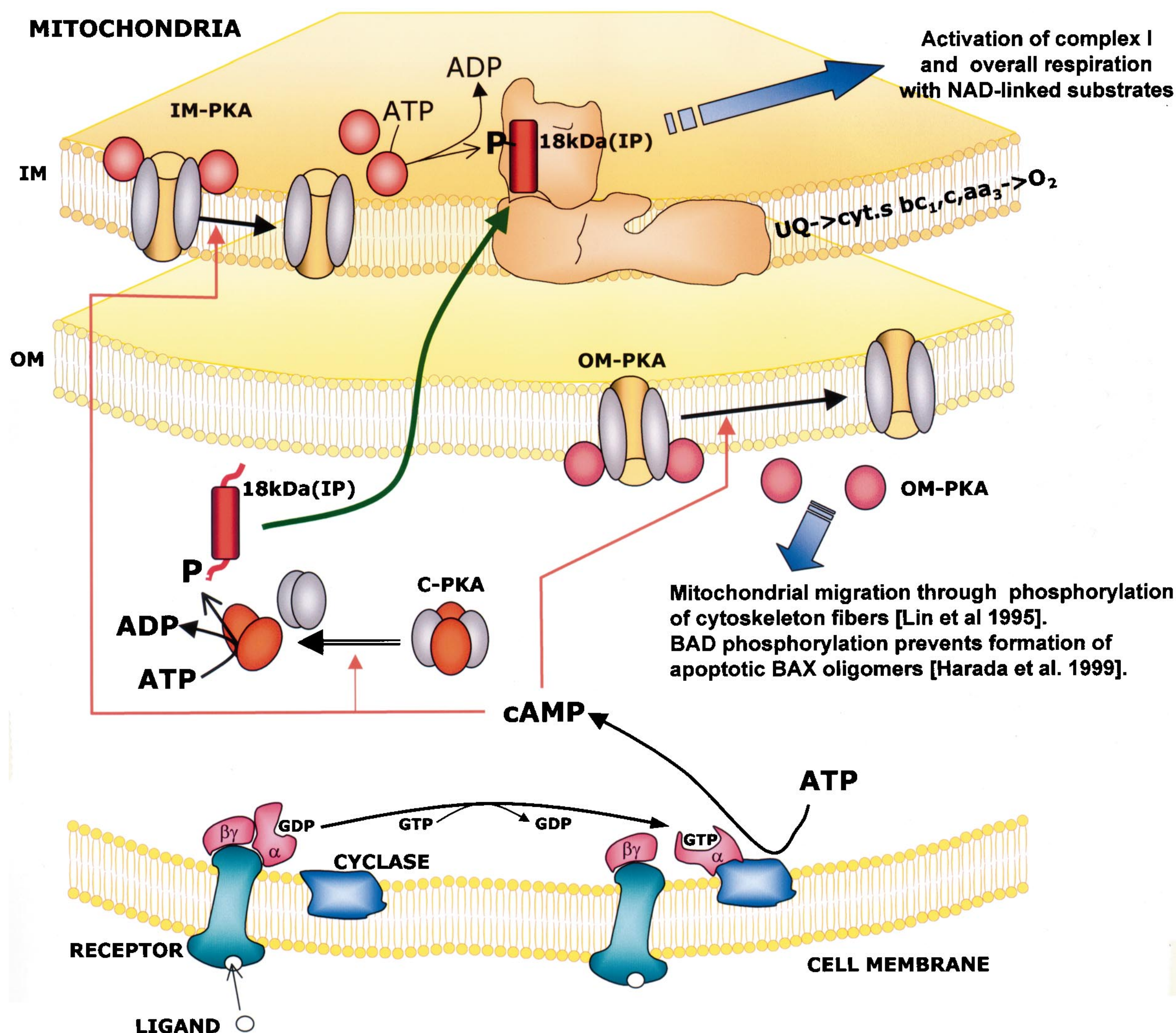

Fig. 7. Scheme showing the phosphorylation of the 18-kDa IP AQDQ subunit of complex I induced by activation of the cAMP cascade in response to binding of a neurohormonal ligand at the plasma membrane specific receptor. Activation of PKA in the cytosol and mitochondrial membranes. PKA (regulatory subunit in gray) is shown to be anchored to the mitochondrial membrane by AKAP proteins shown in yellow. The processes activated by the outer mitochondrial membrane PKA are also indicated.

(Walker, 1992). These observations thus show that the 18-kDa subunit, in addition to its role in regulation of the activity of complex I by the cAMP cascade, also plays a critical role in its assembly. In *N. crassa*, inactivation of the *nuo21* gene coding for the 21-kDa subunit of complex I, considered to be orthologous to the mammalian *NDUFS4* 18-kDa subunit (Videira, 1998), produced alterations in the catalytic activity and subunit assembly of complex I, which need to be further clarified (Ferreirinha *et al.*, 1999). In an animal model of mitochondrial myopathy and cardiomyopathy, recently created by inactivation of the heart/muscle-specific isoform of the adenine nucleotide translocator (Graham *et al.*, 1997), upregulation of the expression of the 18-kDa subunit has been reported, suggesting that *NDUSF4* is an important gene involved in mitochondrial biogenesis and function (Murdock *et al.*, 1999). It is remarkable that, although the nonsense mutation in the *NDUFS4* gene resulted in suppression of the normal assembly of a functional complex I, the patient with such a defect survived until the age of 7 months. Also, the other patients, with different mutations in the same gene, survived a few months after birth (Budde *et al.*, 2000; van den Heuvel *et al.*, 1998). It is conceivable that, in these patients, a metabolic condition is set up in which the glycerol–phosphate shuttle, which mediates mitochondrial oxidation of glycolytic NADH bypassing complex I,

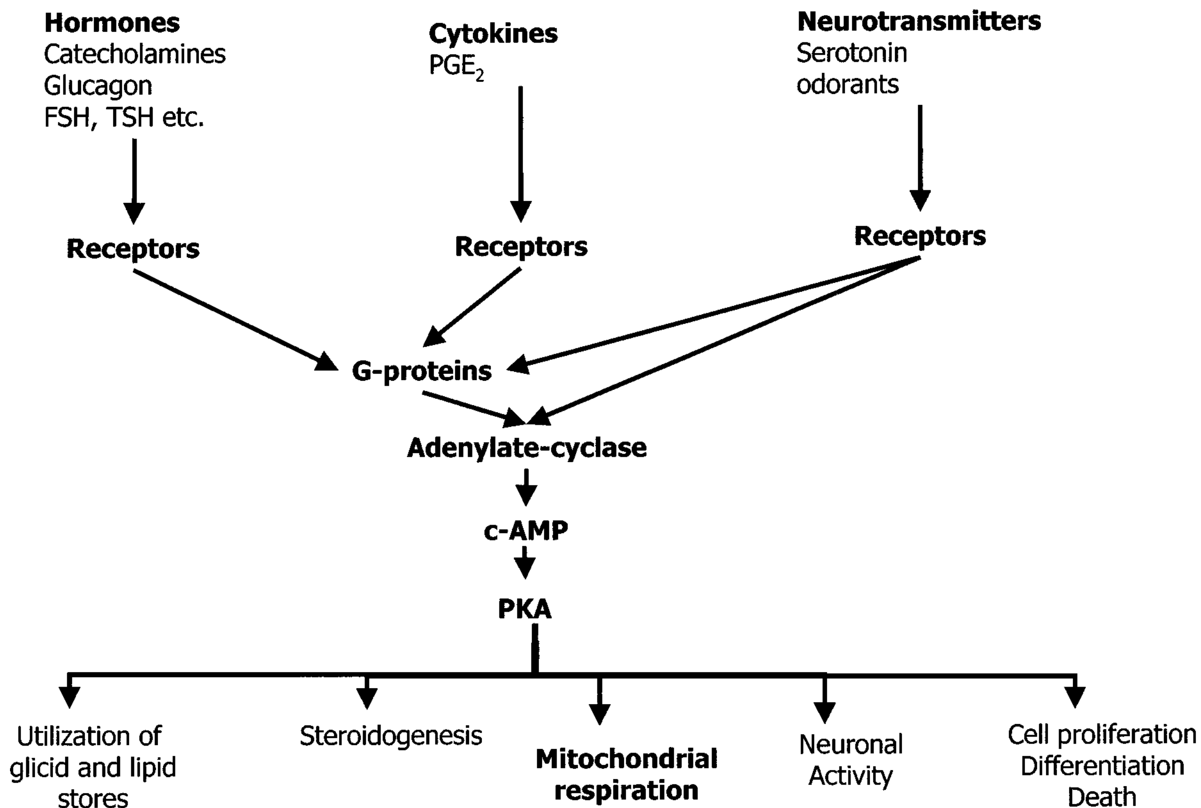

Fig. 8. Signal transduction by the cAMP cascade.

is able to substitute for complex I in supporting mitochondrial energy metabolism, at least in part and under the limited functional activities in the first months of life. Evidence for a significant contribution of NADH shuttles in sustaining mitochondrial energy metabolism and glucose-induced insulin secretion in pancreatic islets has been obtained in transgenic mice (Eto *et al.*, 1999).

CONCLUSIONS

The work reviewed in this paper provides evidence showing that the 18-kDa IP subunit of complex I, encoded by *NDUFS4* gene localized in chromosome 5, is involved in the normal assembly of the complex and mediates, through its phosphorylation by PKA, regulation of the activity of the complex by the cAMP cascade (Fig. 7). Through this process cAMP can regulate the overall mitochondrial respiratory activity, under those conditions in which complex I is the rate limiting step. As the cAMP cascade mediates numerous hormone and neurotrasmitters signals (Fig. 8), PKA regulation of complex I represents an important extension of the cAMP signal function in mammalian cells (Fig. 8). Further work should enable us to elucidate the regulation of the expression of the *NDUFS4* gene in mammalian cells, the mechansim by which the 18-kDa subunit is involved in the assembly of the complex and the possible role and mechanism of regulation of complex I activity by cAMP-dependent phosphorylation of the 18-kDa subunit in different cell types.

The finding in 3 years of four families bearing the mutations in the coding region of the *NDUFS4* gene shows this to represent a hotspot of mutations in the genetic apparatus of oxidative phosphorylation. This as well as the finding of pathological mutations of other nuclear genes coding for subunits of complex I (Benit *et al.*, 2001) might be related to the finding of complex I deficiency in ageing (Lenaz *et al.*, 1998) and in certain human diseases (Schapira, 1998), as well as to its apparent involvement in apoptosis (Fontaine and Bernardi, 1999).

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