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cAMP-dependent protein kinase regulates post-translational processing and expression of complex I subunits in mammalian cells

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ABSTRACT

Work is presented on the role of cAMP-dependent protein phosphorylation in post-translational processing and biosynthesis of complex I subunits in mammalian cell cultures. PKA-mediated phosphorylation of the NDUFS4 subunit of complex I promotes in cell cultures in vivo import/maturation in mitochondria of the precursor of this protein. The import promotion appears to be associated with the observed cAMP-dependent stimulation of the catalytic activity of complex I. These effects of PKA are counteracted by activation of protein phosphatase(s). PKA and the transcription factor CREB play a critical role in the biosynthesis of complex I subunits. CREB phosphorylation, by PKA and/or CaMKs, activates at nuclear and mitochondrial level a transcriptional regulatory cascade which promotes the concerted expression of nuclear and mitochondrial encoded subunits of complex I and other respiratory chain proteins.

1. Introduction

Complex I (NADH–ubiquinone oxidoreductase E.C 1.6.5.3) is the largest enzyme complex of the respiratory chain of high eukaryotes. In mammals the complex has in addition to 14 essential subunits, conserved from prokaryotes to humans, 31 supernumerary subunits [1–5]. The functional role of the supernumerary subunits is not completely understood. Some of these participate in the assembly of the complex [6–9], and/or regulation of the functional activity by cellular transduction signals [5,9–13]. The complex which appears to function as a regulable pacemaker of mitochondrial oxidative phosphorylation [9–12], contributes by 40% to the energy linked proton translocation in the respiratory by a mechanism which is, however, not yet well defined [1–3,5].

In mammalian cells complex I is the major site of oxygen free radical production [10,14–21], it plays a critical role in cell growth, death, degeneration [22], and transformation [23,24]. Seven subunits of mammalian complex I are encoded by mitochondrial DNA, the other 38 by nuclear genes located in different chromosomes [1–3,5]. Complex I genes have been found to be hotspots for pathological mutations in humans and some of the subunits are particularly vulnerable to environmental stressors [22,25–31]. Much remains to be known on (i) how the expression of the nuclear and mitochondrial genes is regulated in a coordinated way [32–37]; (ii) the mechanism by which the subunits synthesized in the cytosol are imported in mitochondria and assembled in a 1 to 1 stoicheiometry ratio with those synthesized in the mitochondrial matrix [7,38]; (iii) how the biogenesis and functional capacity of the complex is regulated in different tissue-cells and adapted to the continuously changing energy demand of mammalian organs; (iv) how the activity of the complex is affected by subunit post-translational modifications like phosphorylation [9,39–43], acylation, oxidation-reduction [20,21] and proteolytic processing [44].

In this paper work on the role of the cAMP cascade and cAMP-dependent protein kinase in the regulation of the expression and functional capacity of complex I in human cell is presented.

Abbreviations: ATRA, all *trans* retinoic acid; CREB, cyclic-AMP response element binding protein; DMEM, high glucose Dulbecco's modified Eagle's medium; NDHFn, normal human dermal fibroblasts-neonatal; KB, human mouth epidermoid cells carcinoma; NHEK, normal human epidermal keratinocyte; PKA, cAMP-dependent protein kinase; PP2A, Protein phosphatase 2A

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Table 1A
cAMP-dependent protein kinase promotes the activity of complex I and lowers ROS level in mammalian cell cultures.

Cell lines	Depressed activity of complex I in serum-starved cell cultures	Promotion by cAMP of complex I activity	High level of ROS in cell cultures	cAMP induced decrease of ROS level	Ref
Balb/c 3T3 mouse fibroblasts	+	+ (cholera toxin activation of G-protein)	+ (serum-starved cells)	+	[45]
C2C12 mouse myoblasts	Not tested	+ (cholera toxin)	Not tested	Not tested	[47]
Skin human fibroblasts	+	+ (cholera toxin)	Not tested	Not tested	[46]
NHDF neonatal human dermal fibroblasts	+	+ (dibutyryl-cAMP, isoprotenerol)	+ (serum-starved cells)	+	[48]
Hela cells	+	+ (dibutyryl-cAMP)	+ (serum-starved cells)	+	[48]
Skin fibroblasts with NDUFS1 pathological mutations	Not tested	+ (dibutyryl-cAMP)	+	+	[29]
Retroviral-vector transfected human bone marrow mesenchimal cells	Not tested	+(dibutyryl-cAMP)	+	+	[49]

2. Post-translational regulation by PKA of the functional capacity of complex I

Various conditions have been found in which the functional capacity of complex I in human and mouse replicating cells is markedly depressed, as compared to other respiratory chain complexes, the decrease being counteracted by activation of the cAMP cascade or direct addition to cells of a permeant derivative of cAMP. These conditions, summarized in Table 1A, encompass withdrawal of cell growth factors introduced by serum starvation of human cell cultures [45–48], homozygous mutation in complex I NDUFS1 gene (75 kDa subunit) associated with neurological disease [29], retroviral-vector transfected bone-narrow-derived mesenchymal cells [49]. The depression of the NADH–ubiquinone oxidoreductase activity of complex I, was associated with remarkable enhancement of the cellular level of oxygen reactive species, which was also reversed by cAMP [29,45,48,49]. The rescue effects exerted by cAMP was found to be associated with PKA dependent phosphorylation of the nuclear encoded NDUFS4 (18 kd, AQDQ) subunit of complex I (Table 1B) [9,45,46]. The phosphorylation state of the NDUFS4 protein can be contributed, on its way from the cytosol to mitochondria, by the balance of the activities of PKA and protein phosphatase(s) (PP2A, PP2C, etc.), both present in the cytosol [50,51] and mitochondria [52,53].

The protein encoded by the human NDUFS4 gene and the corresponding genes in mammals and birds has a highly conserved C-terminus in which a canonical RVSTK phosphorylation site in the last residues, with the highest phosphorylation score for PKA, is present (http://www.cbs.dtu.dk/services/NetPhosK/). The impact on mitochondrial functions of pathological mutations in the coding sequence of the NDUFS4 gene has been characterized: a base duplication at position 466–470 in exon 5, which destroyed the RVSTK phosphorylation site in the carboxy terminus [54], a single base deletion at position 289/290 in exon 3, introducing a premature termination codon (PTC) [55] and a nonsense mutation in the first exon causing premature termination of the protein [56]. Not only in this last mutation, as expected, but also in the other two the entire 18 kDa (AQDAQ) subunit disappeared from the patient's fibroblasts [6]. All the three NDUFS4 mutations resulted in defective assembly of complex I, with the appearance of a non-functional lower molecular weight subcomplex [6] and complete suppression of the NADH ubiquinone oxidoreductase activity which did not respond any longer to cAMP activation [6]. In a mouse model NDUFS4 point mutation, resulting in a PTC truncating the last 10–15 aminoacids of the protein including the RVSTK phosphorylation site, was recently found to be lethal in the homozygous state and to result, in the heterozygous state, in a marked depression of Complex I activity in heart mitochondria [57].

The results presented in Fig. 1 show that serum starvation of fibroblast cultures, has no effect on the level of cAMP, but it results in marked promotion of protein phosphatase activity (PP2A type?) (see also [58,59]). The depression of the functional activity of complex I is rescued in 30 min by activation of cAMP production, elicited by addition of the β-adrenergic agonist isoprotenerol (Fig. 1), similarly to what was observed upon permanent activation of the G protein by cholera toxin or direct addition of dibutyryl-cAMP (see Table 1A). Inhibition of the phosphatase activity by okadaic acid is equally effective, in the same time span, in reversing the depression of the activity of complex I. The functional capacity of complex I thus depends on a dynamic balance between protein phosphorylation by PKA and dephosphorylation by protein phosphatase(s).

Addition of all *trans* retinoic acid (ATRA) to exponentially growing NHEK (normal human epidermal keratinocyte) culture arrests cell growth ([60], see also [61]). The arrest of cell growth is associated with enhanced phosphatase activity (Fig. 2 see [62,63]). The ATRA treatment results, like in the case of serum deprivation, in specific inhibition of complex I activity which is, also in this case, reversed by phosphatase inhibition with okadaic acid or activation of PKA by dibutyryl-cAMP (Fig. 2). It can be mentioned here that among many proteins whose expression is enhanced by ATRA, there is the GRIM-

Table 1B
Experimental evidence showing PKA-mediated serine phosphorylation in the RVS site of the conserved C-terminus of the NDUFS4, 18 kDa subunit of complex I.

Biological material	Experimental observations	Ref
Balb/c 3T3 mouse fibroblasts, C2C12 mouse myoblasts	Permanent activation of G protein by cholera toxin promotes immunodetected serine phosphorylation of 18 kDa subunit(s) of complex I	[45,47]
Blue native human isolated complex I from normal and patient's fibroblasts with NDUFS4 mutations	Cholera thoxin promotes immunodetected serine phosphorylation of 18 kDa subunit. Phosphorylation of this protein band is missing in patient's fibroblasts with pathological mutations of the NDUFS4 gene resulting in disappereance of the protein product detected by specific antibodies raised against the N-terminus and phosphorylated C-terminus of the protein	[6,22,46]
Purified bovine heart complex I	Two dimensional IEF/SDS-PAGE separation, ^{32}P-labelling by [γ-^{32}P]ATP and immunodetection with the specific antibody show PKA-mediated phosphorylation of the NDUFS4 isoform	[9]

Note: Unpublished observations from the author's laboratory based on ^{32}P labelling and TLC phosphoamino acid mapping show that “in vitro” PKA phosphorylates serine 173 in the carboxy terminus of purified human NDUFS4 protein obtained by expression in *E. coli* of a recombinant construct of the entire human NDUFS4 cDNA.

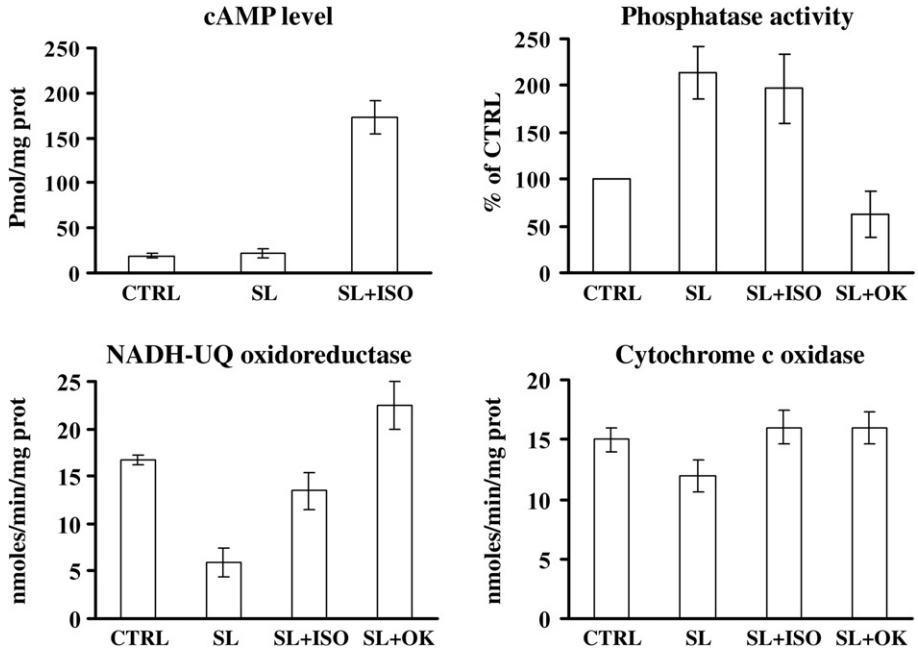

Fig. 1. Level of cAMP and activities of phosphatase (PP2A type), NADH–ubiquinone oxidoreductase and cytochrome *c* oxidase in serum-starved fibroblasts. Effect of the β-receptor agonist isoprotenerol. NHDFn (normal human dermal fibroblast-neonatal) cells grown in the exponential phase in DMEM at 37 °C, 5% CO_2 air mixture in the presence of 10% fetal bovine serum (CTRL) were subjected to 48 h serum limitation (0.5% FBS) (SL). Serum-starved cells were then treated for 35 min with 1 μM isoprotenerol (SL + ISO) or for 2 h with 0.1 μM Okadaic acid (SL + OK). For cAMP assay the culture medium was aspirated and 1 ml 0.1 M HCl was added to the cell layer followed by 10 min incubation at 37 °C. The lysed cells were scraped into an Eppendorf tube. The samples were centrifuged and the supernatants used to determine cAMP concentration by a direct immunoassay kit (Assay Designs). Total protein concentration was determined by a Bio Rad protein assay. Protein phosphatase (PP2A type?) activity was assayed photometrically using a Ser/Thr phosphatase assay kit (Upstate Biotechnology). NADH–ubiquinone oxidoreductase and cytochrome *c* oxidase activities were determined as described in [6] in the mitoplast fraction of the cells. The histogram shows the means (expressed as percentage of control) of three different experiments ± S.E.M. For other experimental details see [6].

19, retinoid-interferon induced mortality factor [64,65]. GRIM-19 was initially detected in the nucleus [66–68], but subsequently found to represent a bona fide constituent of complex I (subunit B16.6) [69]. GRIM-19 is essential for the assembly of the complex [70] and is found to be phosphorylated in vivo [43].

The finding that pathological mutations in the NDUFS4 coding sequence result in defective assembly of complex I [22] as well as the observation that cAMP fails to reproduce in isolated mitochondria [71] the effect exerted on complex I activity and ROS balance in cell cultures "in vivo" [45–48] led to the discovery that cAMP-dependent phosphorylation of the NDUFS4 subunit of the complex promotes the import in mitochondria of this nuclear encoded protein [72]. It can be recalled that work from other laboratories has provided evidence showing that PKA promotes the import in mitochondria of proteins like cytochrome P4502B1 [73], CY2E1 [74] and glutathione S-transferase (GSTA4-4) [75].

The NDUFS4 subunit of complex I, like the NDUFB11 (ESSS) subunit, has a canonical positively charged leader sequence (Fig. 3) characteristic

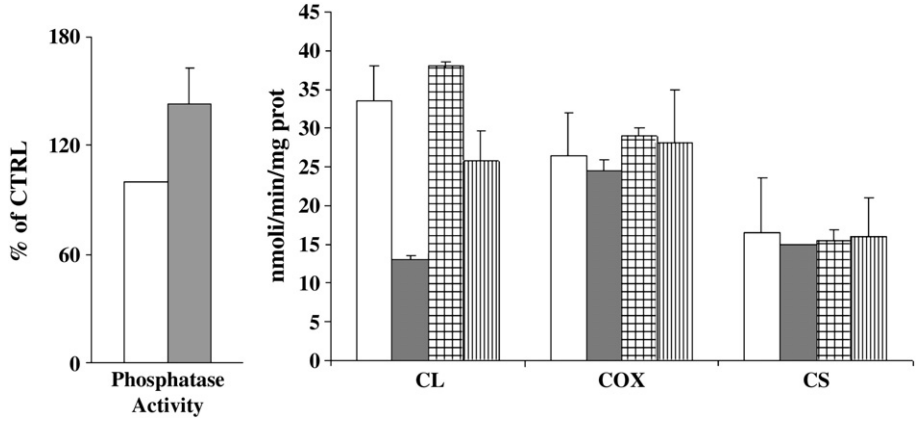

Fig. 2. Activation by ATRA of phosphatase activity and inhibition of complex I activity in NHEK cell cultures. Recovery of complex I inhibition by okadaic acid or dibutyryl-cyclic-AMP. Where indicated NHEK cultures were grown 72 h in the presence of 20 μM ATRA at the conditions described in the legend to Fig. 10. The Phosphatase activity was measured as described in the legend to Fig. 1. The activity of complex I, complex IV and citrate synthase was determined as in [6]. Open bars, controls; gray bars, cells treated with ATRA; squared bars, cells after ATRA treatment were exposed for 1 h to 100 nM okadaic acid; dashed bars, cells after ATRA treatment were exposed for 1 h to 100 μM dibutyryl-cyclic-AMP. For experimental details see [6].

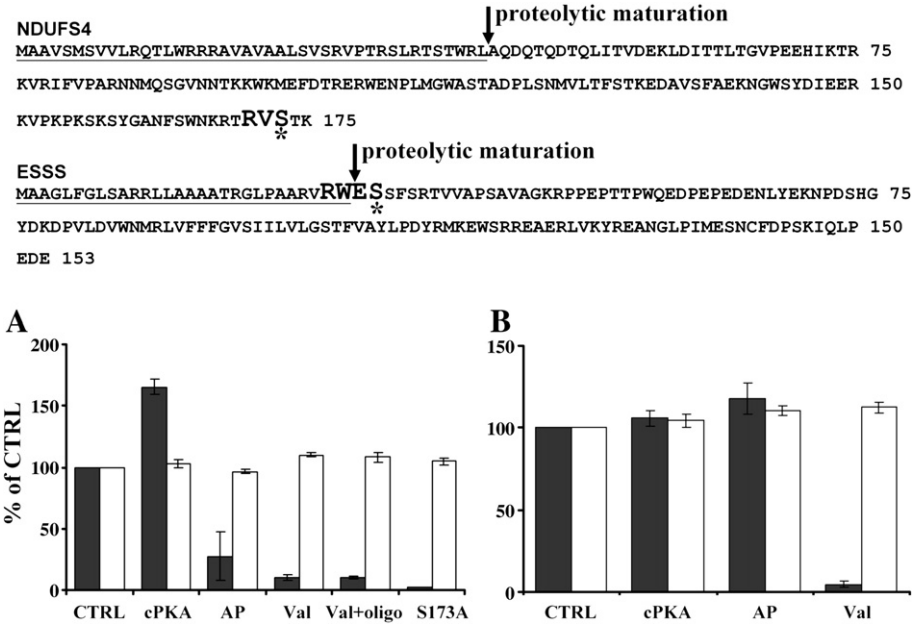

Fig. 3. Binding to the surface of rat liver mitochondria of the full-length precursor of the NDUFS4 subunit of complex I and its import/maturation in the inner mitochondrial compartment. At the top the human NDUFS4 and ESSS sequences are presented. The presequences are underlined and consensus phosphorylation sequences for cAMP-dependent protein kinase are shown in bold characters with asterisks under the phosphorylable serines (EMBL Data Bank). Full-length human NDUFS4 wild type or mutated Ser173Ala NDUFS4 (panel A) or full-length human ESSS (panel B) were synthesized in the RRL system in the presence of [^{35}S] methionine and then added to a mitochondrial import mixture containing rat liver mitochondria and an ATP energy supplying system as described in [72]. After 35 min incubation at 30 °C mitochondria were spun down from the import mixture before or after trypsin treatment (1 μg per 50 μg mitochondrial proteins, 35 min on ice) and analyzed by SDS-PAGE and autoradiography. Panel A, histograms of the densitometric analysis of radioactive spots expressed as % of the control (CTRL), of the trypsin sensitive bound precursor (empty squares) and the trypsin resistant mature form (filled squares) of the NDUFS4 wild type (CTRL) and serine/alanine 173 mutated form (S173A) after in vitro mitochondrial import in the absence and in the presence of cPKA, alkaline phosphatase (AP), cPKA plus valinomycin, cPKA plus valinomycin and oligomycin. Panel B, histograms of the densitometric analysis, of radioactive spots expressed as % of the control (CTRL), of the trypsin sensitive bound precursor (empty squares) and the trypsin resistant mature form (filled squares) of the ESSS protein (CTRL) after in vitro mitochondrial import in the absence and in the presence of cPKA, AP and valinomycin. Data reproduced with permission from [72]. For experimental details see [72] and text.

of the $\Delta\psi$ and ATP dependent TOM/TIM mitochondrial import system [76]. The results presented in Fig. 3A show that the $\Delta\psi$ dependent import and maturation in isolated mitochondria of [^{35}S] methionine-labelled precursor protein, produced "in vitro" by expression in the reticulocyte lysate system of the full-length NDUFS4 cDNA, is promoted when phosphorylated by added PKA and inhibited by phosphatase. The import/maturation in isolated mitochondria of the NDUFS4 protein, which is completed within 60 min, is completely suppressed by site specific substitution of serine 173 with alanine in the C-terminal RVS phosphorylation site. The $\Delta\psi$ dependent mitochondrial import/maturation of the NDUFB11 protein is, on the contrary, unaffected by PKA and phosphatase (Fig. 3).

In similar experiments Lazarou et al. [38,77] showed that in vitro-synthesized NDUFS4 protein is within 60 min incorporated in the mature complex I in human fibroblast mitochondria. This is practically the same time span in which in cell cultures cAMP stimulates the activity of complex I and lowers ROS level (Table 1A) [6,45–48].

In "vivo" observations in Hela cells transfected with a construct of the wild NDUFS4 cDNA showed that the precursor form of the over-expressed protein is detectable in the cytosolic fraction, whilst in the mitochondrial fraction only the mature NDUFS4 protein is present [72]. Treatment of transfected HeLa cells with H89, prevents the accumulation in mitochondria of the mature NDUFS4 protein. When HeLa cells are transfected with a construct of the NDUFS4 cDNA with S173A substitution, the precursor form of the NDUFS4 protein accumulates in the cytosol, but no mature NDUFS4 protein, in addition to the endogenous content, appears in mitochondria [72].

The results presented in Fig. 4 show that the stimulation by isoprotenerol of the activity of complex I in serum-starved human fibroblast cultures is completely abolished by CCCP or by valinomycin, which a collapsing mitochondrial $\Delta\psi$, suppresses import/maturation in mitochondria of newly synthesized NDUFS4 protein (see Fig. 3). These observations, taken together, indicate that the activity of complex I depends on a continuous exchange of pre-existing, possibly damaged, NDUFS4 subunit in the complex with the newly synthesized protein [see also 77]. The stimulation of NADH–ubiquinone oxidoreductase activity, effected in a time span of 60 min by cAMP, is likely to be associated with stimulation of import/maturation of the precursor of the NDUFS4 protein, phosphorylated by PKA, and its maturation and functional association in complex I. The present observations are consistent with the mechanism of complex I biogenesis, pointed out by studies of Lazarou et al. [77], involving two complementary processes: synthesis of mtDNA-encoded subunits to seed de novo assembly and exchange of pre-existing subunits with newly imported ones to maintain complex I homeostasis. These authors propose that subunit exchange may act as an efficient mechanism to prevent the accumulation of oxidatively damaged subunits that would otherwise be detrimental to mitochondrial oxidative phosphorylation.

3. Expression of complex I subunits. The role of phosphoCREB

Lack of growth factors, effected by serum deprivation of replicating mammalian cells in the exponential growth phase, results in arrest of cell growth, with cells going to rest in the G0 phase. G0 cells re-enter, upon serum addition, in the G1 protein synthetic phase of the cycle [78,79]. The arrest of cell growth effected by serum starvation and its restoration by serum is a complex phenomenon involving down and up regulation, respectively, of complex events in the down-stream signal transduction networks recruited in the cellular response to growth factors [79,80].

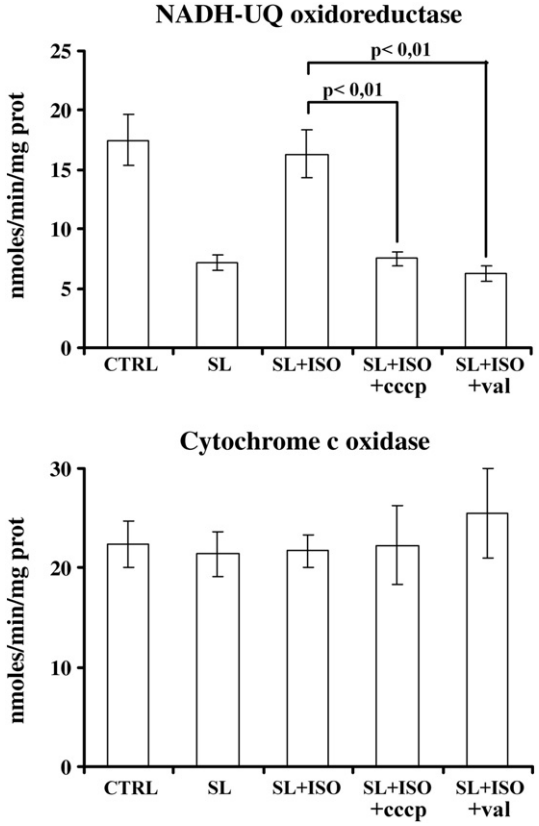

Fig. 4. CCCP and valinomycin prevent the stimulatory effect exerted by the β-receptor agonist isoprotenerol on complex I activity in serum-starved fibroblast culture. NHDFn cells grown in the exponential phase in DMEM in the presence of 10% fetal bovine serum, FBS, (CTRL) were subjected to 48 h serum limitation (0.5% FBS) (SL). Serum-starved cells were then treated for 35 min with 1 μM isoprotenerol in the absence (SL + ISO) or in the presence of the 2 μM CCCP (SL + ISO + CCCP), or of 5 μM valinomycin (SL + ISO + val). After treatment cells were harvested and the mitoplast fraction prepared as described in [6]. Complex I and complex IV activities represent the means ± S.E.M. of three or more determinations in the mitoplast fraction of the cells. Experimental details as in Fig. 1 and [6].

Fig. 5 shows the growth arrest of exponentially growing NHDFn human fibroblasts caused by serum starvation and their subsequent re-entry in the cell cycle upon serum addition. The cell-growth promoting effect of the serum is completely prevented if added together with H89, a permeant inhibitor of PKA.

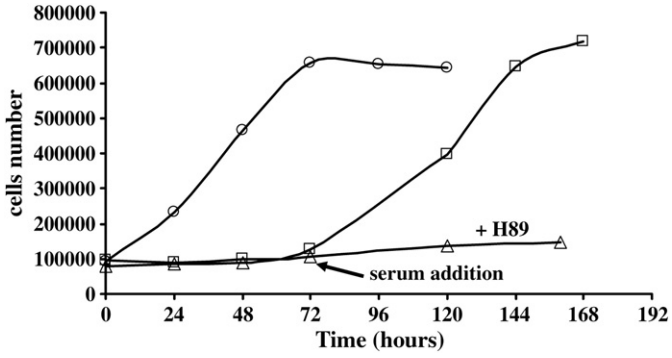

Fig. 5. Arrest of human fibroblast growth by serum starvation, serum induced fibroblast growth and prevention of the effect by H89. Growth curves of NHDFn fibroblasts was followed by inoculating 100,000 cells/well in 100 mm petri dishes and daily cell counting for 7 days. Cell growth in standard culture conditions supplemented with 10% FBS (circles), under 72 h serum limitation with 0.5% FBS (squares), followed, where indicated, by the addition of 10% FBS in the absence (squares) or in the presence of 5 μM H89 (triangles).

Fig. 6 shows that serum addition to serum-starved fibroblasts, after a lag some hours, starts to promote the NADH–ubiquinone oxidoreductase activity of complex I, markedly depressed in the serum-starved cell, reaching a maximum effect at 24 h treatment, after which a decline ensues. Serum starvation/supplementation has practically no effect on complex IV activity. Serum promotion of the functional capacity of complex I, which is much delayed with respect to the post-translational cAMP/PKA stimulation of the catalytic activity of complex I, is evidently associated with stimulation of protein biosynthesis, which characterizes the G1 phase of the cell cycle, in which fibroblasts are re-introduced from the G0 phase by serum supplementation. Still the serum promoting effect on the functional capacity of complex I is completely suppressed, like the promotion of cell growth, by the PKA inhibitor H89 (Fig. 6). Protein phosphorylation by PKA, in addition to direct post-translational effect on the activity of complex I, appears thus to be involved also in its biosynthetic expression. Evidence of this is provided by the results presented in Fig. 7 which show that serum addition to serum-starved fibroblasts induces an increase in the cellular level of the nuclear encoded GRIM 19 and 39 kDa (NDUFA9) subunits of complex I, reaching a maximum at 24 h. This effect also is prevented by H89.

A role of PKA-mediated protein phosphorylation in the expression of subunits of complex I might involve a cascade of transcription (co) activators [81–83]. This starts with the cAMP-response element binding protein CREB (cyclic-AMP response element binding protein) whose activity depends, in fact, on phosphorylation by PKA. CREB belongs to the CREB/ATF1 family of cAMP/Ca^{2+} responsive transcription factors [84,85]. The transcriptional activity of CREB is induced through serine phosphorylation in its conserved kinase inducible domain (KID) by PKA [86], Ca^{2+}/calmodulin protein kinases (CaMKs) [83] and other kinases [85,87,88]. The phosphorylation dependent activation of CREB involves its interaction with basal transcription factors, adaptor(s), constitutive and inducible coactivators, the TATA box and POL II in gene promoters [85,88,89]. The CREB transcriptional complex controls the expression of the transcriptional coactivator PPARγ coactivator 1α (PGC-1α) [90] (Fig. 10). Induced expression of PGC-1α, a member of the PGC-1 family of coactivators, activates in turn a transcriptional regulatory cascade which amplifies the impact of CREB mediated signal transduction on mitochondrial biogenesis [91]. This involves in a down-stream sequence NRF1, NRF2 and the mitochondrial transcriptor factor TFAM (see Fig. 10). TFAM controls the transcription of both the heavy and light mtDNA strands interacting on the D-loop with additional transcription factors and the mitochondrial RNA polymerase [82,92,93].

A second process has more recently been discovered which contributes to coordinate the expression of nuclear and mitochondrial genes. This consists in the translocation of CREB from the cytoplasm into mitochondria where it binds to the mtDNA D-loop and activates the biosynthesis of mitochondrial encoded subunits [94–97]. Findings have been presented [94] showing that in addition to the nucleus, CREB is also localized in the inner mitochondrial compartment of rat brain. The CREB protein, extracted from synaptic mitochondria, was shown to be phosphorylated by PKA [94]. Lee et al. [95] showed that, in neuronal cell cultures CREB protein binds to the mtDNA D-loop. In these cells the stable overexpression of mito-tagged CREB increased the transcript level of ND2, ND4, and ND5 mitochondrial genes of complex I. Disruption of CREB activity in mitochondria, by overexpression of dominant negative mito-tagged CREB, decreased the expression of mitochondrial genes, down-regulated complex I-dependent mitochondrial respiration and increased susceptibility to the plant toxin, 3-nitropropionic acid (3-NP), an inductor of Huntington disease in an experimental model [95]. Ryu et al. [96] found also that activation by the antioxidant iron chelator deferoxamine of the PKA localized in the mitochondrial matrix [53], promoted CREB binding to the mtDNA D-loop.

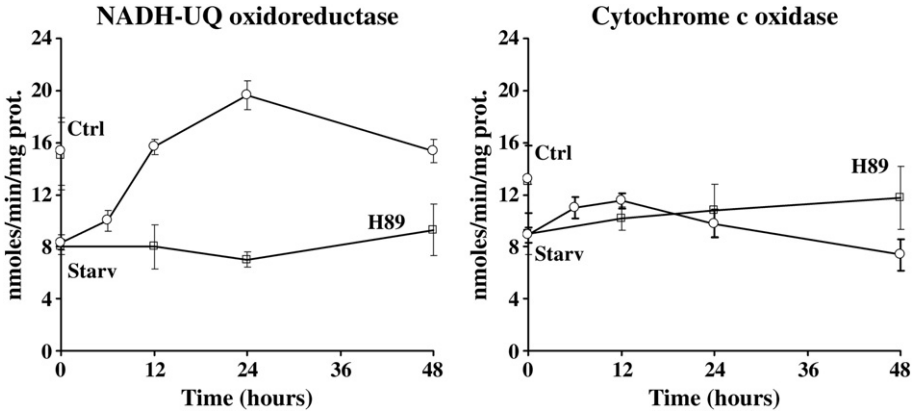

Fig. 6. Promotion by serum supplementation of the activity of complex I and prevention of the effect by H89, in fibroblast culture. NADH–ubiquinone oxidoreductase and cytochrome *c* oxidase activities in mitoplasts from NHDFn fibroblasts grown in standard medium, with 10% FBS (Ctrl), after 72 h serum limitation, 0.5% FBS (Starv), (points on the ordinate) followed by serum induction, (addition of 10% FBS) in the absence (circles), or in the presence of 5 μM H89 (squares). Experimental details as the legend of Fig. 1 and [6].

Results from work in the author's laboratory (Fig. 8), show that [^{35}S] methionine-labelled CREB, produced "in vitro" by expression in the reticulocyte lysate system of the full-length CREB cDNA, enters into the inner compartment of isolated rat liver mitochondria by a $\Delta\psi$ and Tom-20 dependent import process [97]. The imported CREB does not undergo N-terminal processing as has also been observed for other nuclear encoded mitochondrial matrix-targeted proteins [74,98]. The imported CREB promotes the synthesis of subunits of respiratory chain complexes (in particular those of complex I), this effect being strongly potentiated when CREB is added together with cAMP or the catalytic subunit of PKA (Fig. 9). It can be noted that cAMP has per se some stimulatory effect. The promoting effect of CREB and cAMP or cPKA is completely abolished by the PKA inhibitor, H89.

Thus CREB, imported into mitochondria promotes, when phosphorylated by cAMP-dependent protein kinase, the synthesis of mitochondrially encoded subunits of OXPHOS complexes, in particular of complex I. CREB in the inner mitochondrial compartment can also be phosphorylated by the PKA present in the same compartment [53], where it can be activated by cAMP generated by a carbondioxide/bicarbonate regulated soluble adenylyl cyclase present inside the mitochondria [99–101].

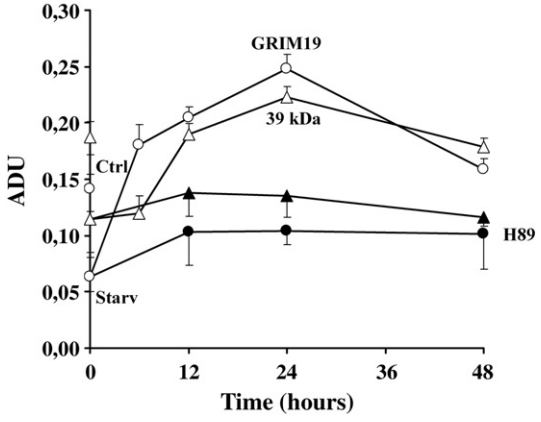

Fig. 7. Effect of serum supplementation on the level of the 39 kDa and GRIM-19 subunits of complex I and prevention of the effect by H89 in fibroblast culture. The level of the 39 kDa (triangles) and GRIM 19 (circles) complex I subunits normalized vs beta-actin level was immunodetermined with specific antibodies in the total cell lysate of NHDFn fibroblasts grown in standard medium, with 10% FBS (Ctrl), 72 h after serum limitation, 0.5% FBS (Starv), (points on the ordinate) and in 48 h after 10% FBS addition in the absence (empty symbols) and in the presence of 5 μM H89 (filled symbols). Experimental details as in [6].

4. Conclusions

In fibroblast cultures increase of the cAMP concentration, induced by the β-adrenergic agonist isoprotenerol, promotes, within an hour, the catalytic activity of complex I and lowers the ROS level. PKA dependent phosphorylation of the NDUFS4 subunit of complex I (see Table 1B) promotes in the same time span import/maturation in mitochondria of the precursor of this protein, an effect which appears to be associated with the stimulation of the catalytic activity of the

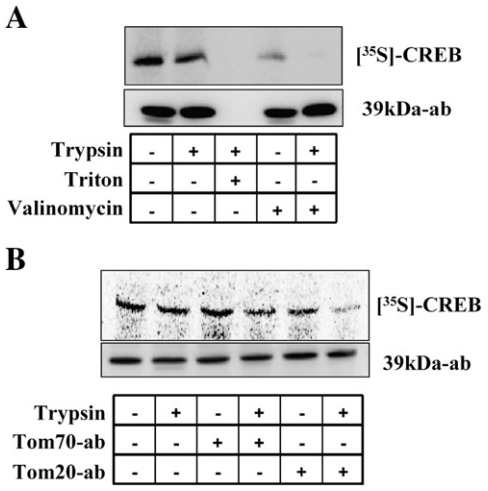

Fig. 8. Import in rat liver mitochondria of the [^{35}S] methionine-labelled human CREB protein. [^{35}S] methionine-labelled CREB was synthesized in the RRL translation system and added to the import mixture containing isolated rat liver mitochondria and an ATP energy supplying system. After 60 min incubation at 30 °C mitochondria were spun down and analyzed by SDS/PAGE and autoradiography. Where indicated mitochondria were treated, before pelletting, with trypsin (1 μg per 50 μg mitochondrial proteins) in the absence or in the presence of 0.2% Triton X-100 for 35 min at 0 °C. Panel A, import incubation for 60 min, in the absence or in the presence of valinomycin (0.1 μg per mg mitochondrial proteins) as specified in the figure. Panel B, mitochondrial import in the absence or in the presence of 3 μg of the antibody against Tom70 or against Tom20 as specified in the figure. The SDS-PAGE slabs were also blotted with an antibody against the 39 kDa subunit of complex I. Reproduced with permission from [97]. For experimental details see [97] and text.

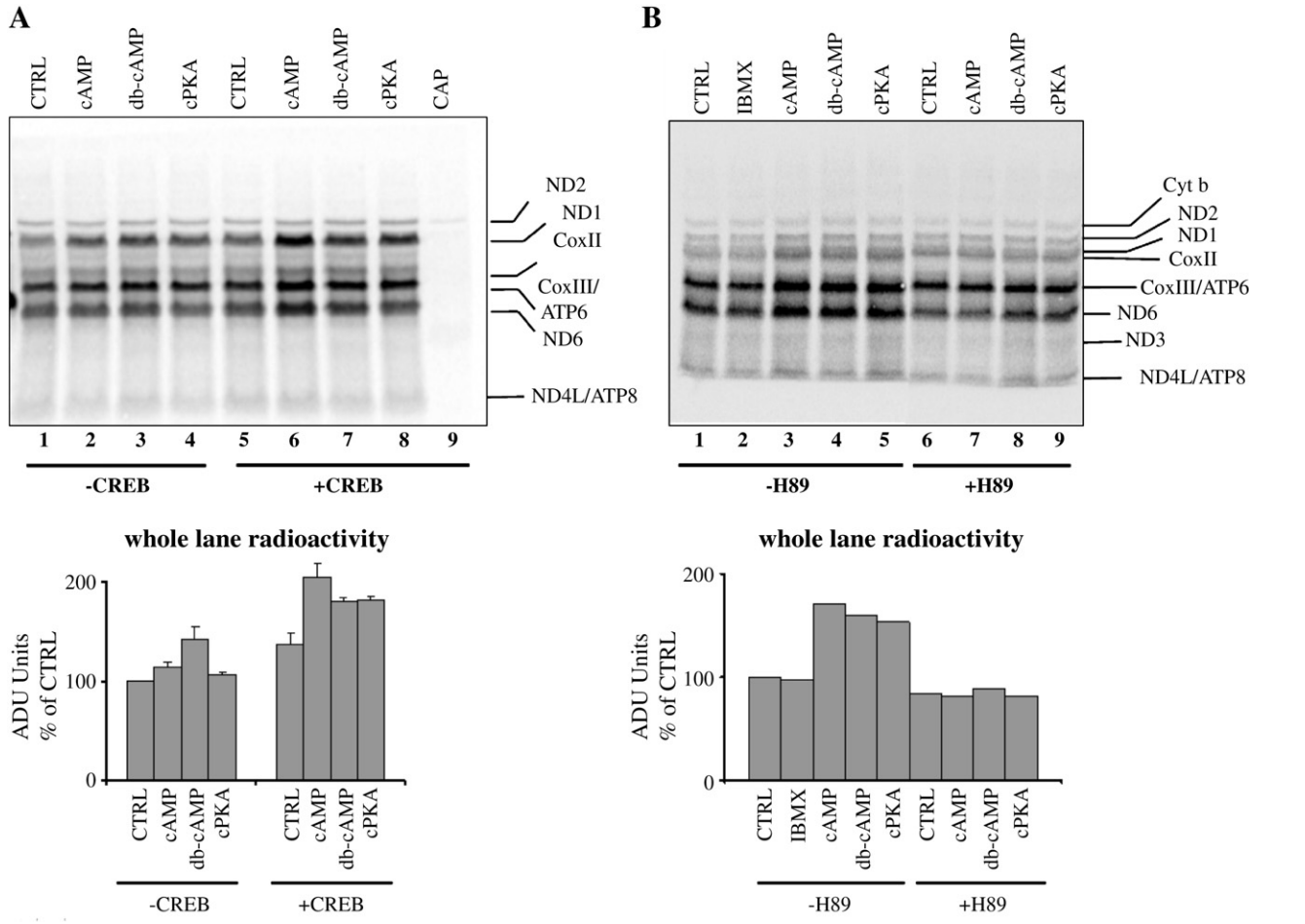

Fig. 9. Effect of in vitro-synthesized CREB, cAMP, cPKA and H89 on the synthesis of mtDNA-encoded subunits of respiratory chain and ATP synthase in the rat liver mitochondria. Mitochondrial protein synthesis was performed in a mixture containing rat liver mitochondria, aminoacid mixture with [^{35}S] methionine, cycloheximide plus the addition of the RRL translation mixture with or without cold synthesized CREB, for 60 min at 30 °C. After incubation mitochondria were spun down and subjected to SDS/PAGE and autoradiography. Panel A, mitochondrial protein synthesis was performed in the presence of the RRL translated mixture without the cold synthesized CREB (lanes 1–4), or in the presence of the RRL mixture with the cold synthesized CREB (lanes 5–9). Lane 1: control. Lane 2: 50 μM cAMP plus 50 μM IBMX. Lane 3: 50 μM db-cAMP plus IBMX. Lane 4: cPKA (1 U per 10 lg of mitochondrial protein). Lane 5: no addition. Lane 6: cAMP plus IBMX. Lane 7: db-cAMP plus IBMX. Lane 8: cPKA. Lane 9: chloramphenicol (CAP) (3 mg/ml). Histograms showing the mean ADU (as percentage of control) of the whole gel lane radioactivity of the [^{35}S] methionine-labelled mitochondrial proteins. Mean values of three separate experiments. Panel B, the RRL mixture with cold synthesized CREB was present in all the lanes, including the control, where indicated H89 (100 nM) was present during the mitochondrial protein synthesis incubation. Histograms showing the mean ADU (as percentage of control) of the whole gel lane radioactivity of the [^{35}S] methionine-labelled mitochondrial proteins. Reproduced with permission from [97]. For experimental details see [97] and text.

complex. These effects of PKA are counteracted by promotion of protein phosphatase activity.

In addition to short-term post-translational modulation of complex I catalytic activity, PKA plays a longer-term role in the biosynthesis of complex I subunits. Serum starvation causes arrest of fibroblast growth (in the G0 phase), growth being restored by serum supplementation which reintroduces cells in the protein biosynthetic G1 phase of the cycle. The functional capacity and the subunit level of complex I markedly decrease in serum starvation and increases upon serum supplementation. Both serum promotion of fibroblast growth and restoration of the level and activity of complex I are completely suppressed by the PKA inhibitor H89. In addition to PKA a role in the promotion of biosynthesis of complex I subunits is played by the transcription factor CREB, whose activity depends on phosphorylation by PKA. Phosphorylation by PKA and/or CaMKs of CREB and TORC family members (transducer of regulated CRE-binding proteins) induces transcription of PGC-1α a master gene regulator of mitochondrial biogenesis (Fig. 10). PGC-1α activates a transcriptional regulatory cascade, involving in a down-stream sequence NRF1, NRF2 and TFAM. Promotion by phosphoCREB of this regulatory cascade activates at nuclear and mitochondrial level the concerted expression of nuclear and mitochondrial encoded subunits of complex I and other respiratory chain proteins. In this way the cAMP cascade can modulate adaptive plasticity of mitochondrial oxidative phosphorylation system in mammalian cells.

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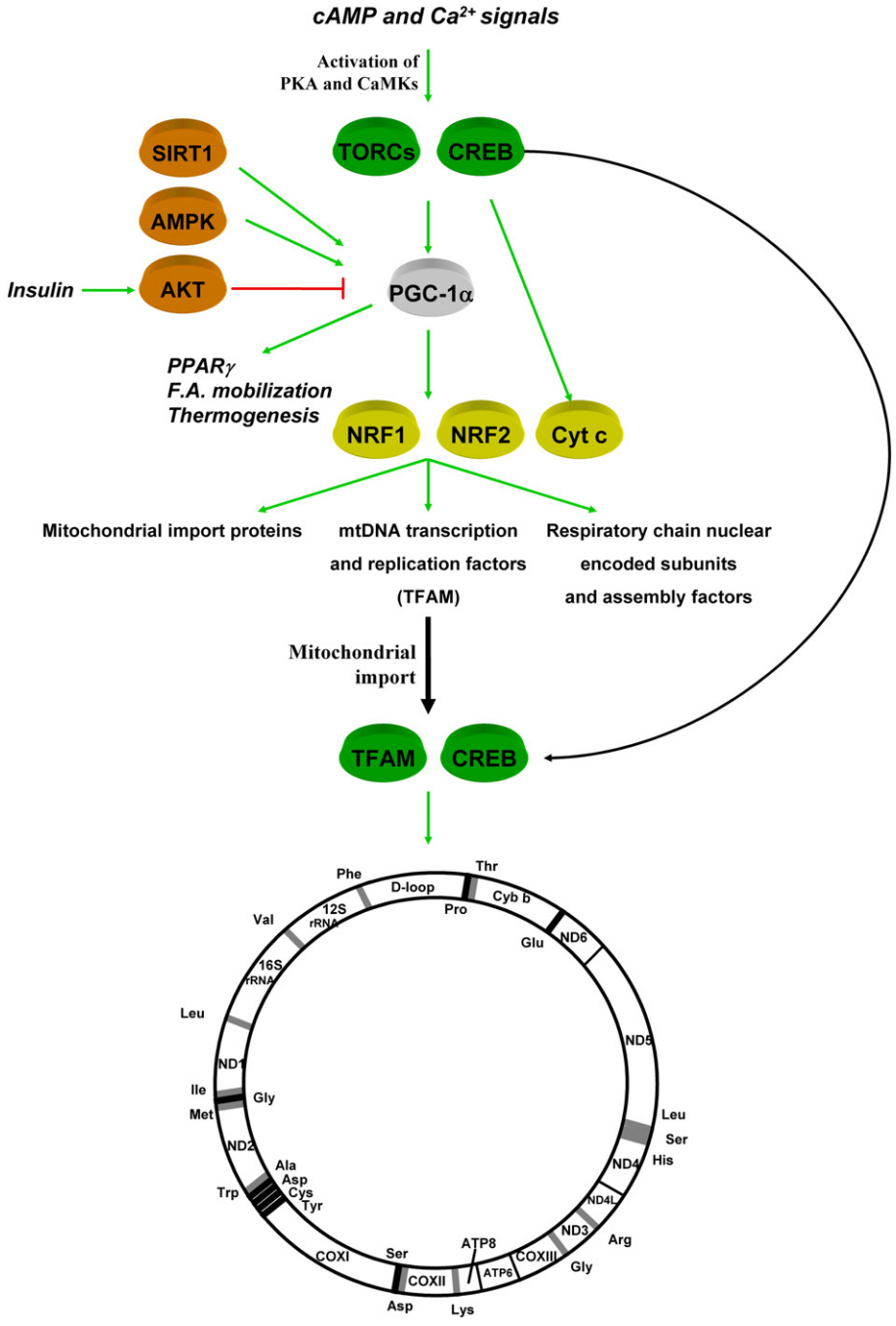

Fig. 10. Schematic representation of CREB mediated pathways which control mitochondrial biogenesis. Activation of protein kinases by cAMP and/or Ca^{2+} results in phosphorylation of CREB and members of the transducer of regulated CREB binding protein family (TORCs) [102]. The phosphorylation of TORCs proteins causes the translocation of these proteins from the cytoplasm to the nucleus where they bind to the CREB complex on gene promoters and exert their transcriptional coactivator action [102,103]. CREB and TORC together promote the transcription of PGC-1α and cytochrome *c* genes. PGC-1α is also activated by post-translational modifications, like deacetylation by SIRT1 [104], phosphorylation by AMPK [105] and inhibited by phosphorylation by AKT [105,106] which is activated by insulin. PGC-1α in combination with other factors, activates the expression of enzymes of fatty acid oxidation and of the nuclear respiratory transcription factors, NRF1 and NRF2. NRF1 and NRF2 activate, in turn, the expression of the mitochondrial transcription and replication factors (TFAM, TFB1, TFB2 and MRP RNA) and nuclear genes coding for structural proteins and assembly factors of OXPHOS complexes. These include cytochrome *c*; complex I TYKY subunit; complex II subunits B, C, and D; complex III ubiquinone-binding protein, core protein I; complex IV subunits IV, Vb, VIaL, VIc, VIIc and VIIaL; FoF1-ATP synthase, subunits c, γ and β; assembly factors, SURF-1, chaperonin 10 and COX17; mitochondrial import, ion channel and shuttle proteins (glycerol phosphate dehydrogenase); Tom 20, Tom 70, VDAC3, and VDACI. The mTFA and CREB proteins, after mitochondrial import, promote the expression of mtDNA [82].

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