

CREB activation induced by mitochondrial dysfunction is a new signaling pathway that impairs cell proliferation

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We characterized a new signaling pathway leading to the activation of cAMP-responsive element-binding protein (CREB) in several cell lines affected by mitochondrial dysfunction. *In vitro* kinase assays, inhibitors of several kinase pathways and overexpression of a dominant-negative mutant for calcium/calmodulin kinase IV (CaMKIV), which blocks the activation of CREB, showed that CaMKIV is activated by a mitochondrial activity impairment. A high calcium concentration leading to the disruption of the protein interaction with protein phosphatase 2A explains CaMKIV activation in these conditions. Transcriptionally active phosphorylated CREB was also found in a p0 143B human osteosarcoma cell line and in a MERRF cybrid cell line mutated for tRNA^{Lys} (A8344G). We also showed that phosphorylated CREB is involved in the proliferation defect induced by a mitochondrial dysfunction. Indeed, cell proliferation inhibition can be prevented by CaMKIV inhibition and CREB dominant-negative mutants. Finally, our data suggest that phosphorylated CREB recruits p53 tumor suppressor protein, modifies its transcriptional activity and increases the expression of p21^{Waf1/Cip1}, a p53-regulated cyclin-dependent kinase inhibitor.

Keywords: calcium–calmodulin kinase IV/cell proliferation/cyclicAMP response element-binding protein/mitochondrial dysfunction/retrograde communication

Introduction

Cells can modulate the expression of nuclear genes in response to changes in the activity level of their organelles. For example, mitochondria dysfunction induces the activation of transcription factors in a response called ‘retrograde communication’ (Parikh *et al.*, 1987; Liao and Butow, 1993). Several lines of evidence previously established that alterations in the mitochondrial activity result in changes in nuclear gene expression (Wang and Morais, 1997). In yeast, numerous studies have shown that a retrograde signaling pathway can act as a homeostatic or stress response mechanism to adjust metabolic activities to the alterations of mitochondria activity. It has been particularly well characterized for the *CIT2* gene,

encoding a peroxisomal isoform of citrate synthase that catalyzes the first step in the glyoxylate cycle. In cells lacking mitochondrial DNA (mtDNA), increased expression of *CIT2* is dependent on the activation of *RTG1* and *RTG3* encoding basic helix–loop–helix leucine zipper (bHLH–Zip) transcription factors (Liao and Butow, 1993). In yeast, detailed mechanisms on the regulation of the mitochondria to nucleus signaling and metabolic control through Rtg proteins in response to mitochondria impairment have been described recently (Komeili *et al.*, 2000; Sekito *et al.*, 2000).

The interorganelle ‘retrograde communication’ pathways have been described not only in yeast (Liao and Butow, 1993; Komeili *et al.*, 2000; Sekito *et al.*, 2000) but also in plant (Mayfield, 1990) and mammalian cells (Biswas *et al.*, 1999). However, the signaling pathway(s) used by mitochondria to communicate the state of activity to the nucleus is still poorly understood in mammalian cells.

Mitochondrial activity plays a crucial role in cellular physiology, as emphasized recently by the discovery of a wide variety of human diseases caused by or associated with mitochondrial alterations such as genetic defects in mtDNA or nuclear DNA that affect the respiratory chain and ADP phosphorylation (Wallace, 1999). Among these diseases, myoclonus epilepsy with ragged-red fibers (MERRF) is a maternally inherited disorder characterized by point mutations in the mtDNA-encoded tRNA^{Lys} gene which impairs respiration and mitochondrial protein synthesis (Lombes *et al.*, 1989). Cells cultured with ethidium bromide (EtBr) partly or completely lose their mtDNA (mtDNA-depleted or p0 cells) and consequently cannot carry out oxidative phosphorylation (OXPHOS) and thus glycolysis is their only source of ATP (King and Attardi, 1989). Recently, these cells have been used to study mitochondrial disorders due to genetic defects such as deletions in mtDNA, depletion of mtDNA or reduction of mtDNA copy number (King and Attardi, 1989; Moraes *et al.*, 1991). These mtDNA-depleted cells usually have a proliferation defect characterized by a longer doubling time, as observed for mtDNA-depleted HeLa S3 and 143B cells (Vaillant and Nagley, 1995; Buchet and Godinot, 1998), but no molecular control of the cell cycle after inhibition of mitochondria has been evidenced so far.

Mitochondrial dysfunction also greatly modifies nuclear gene expression (Epstein *et al.*, 2001). Indeed, increased expression of ANT-1 and ATP synthase β -subunit mRNAs was described in skeletal muscle of patients with respiratory defects resulting from mtDNA point mutations (Heddi *et al.*, 1993), and increased mRNAs encoding cytochrome oxidase (COX IV and COX VIaL) were also observed in p0 cells (Li *et al.*, 1995). Biswas *et al.* (1999) recently described that in mouse C2C12 cells, the decrease of

mtDNA content initiates a signaling pathway leading to the activation of JNK and increased transcription of ryanodine receptor-1 Ca^{2+} release channel and COX Vb genes. While eukaryotic cells can respond to mitochondrial dysfunction by a modulation of nuclear gene expression, evidence for signaling pathways and transcriptional mechanisms are lacking in most instances. Interestingly, several genes encoding mitochondrial proteins contain CRE (cAMP response element) sequences such as some respiratory chain subunits (Scacco *et al.*, 2000), cytochrome *c* (Gopalakrishnan and Scarpulla, 1994), manganese superoxide dismutase (Kim *et al.*, 1999) or carnitine palmitoyl-transferase (Brady *et al.*, 1992).

In this study, we analyzed the effect of mitochondrial activity inhibition on the signaling and transcriptional machinery of mammalian cells. We identified CRE-binding protein (CREB) as a new transcription factor involved in the 'retrograde communication' pathways between impaired mitochondria and the nucleus. We used several cellular models: a mtDNA-depleted murine fibrosarcoma cell population (L929), a true $\rho 0$ human osteosarcoma cell line (143B), a cybrid cell line with a nucleotide mutation (A8344G) in the mitochondria-encoded tRNA^{Lys} gene and an acute inhibition of mitochondria in 293 cells treated with metabolic mitochondrial inhibitors. We found that mitochondrial activity impairment is sufficient to trigger CREB phosphorylation on Ser133 by calcium/calmodulin kinase IV (CaMKIV). In turn, phosphorylated CREB mediates a proliferation defect in mtDNA-depleted L929 cells by increasing p53 transcriptional activity that leads to the up-regulation of p21^{Waf1/Cip1}, a cyclin-dependent kinase inhibitor gene regulated by p53. Our results suggest that the activation of a 'retrograde communication' signaling pathway initiated by a mitochondrial dysfunction could be an important modulator of the cell cycle in cells submitted to energetic stress conditions.

Results

Functional and morphological characterization of the mtDNA-depleted L929 cell line

PCR analysis revealed that EtBr-treated L929 cells are dramatically depleted in but not completely devoid of mtDNA. As shown in Figure 1A, using specific primers for the D-loop fragment in mouse mtDNA, we amplified a fragment of the expected size (823 bp) in L929 cells, but only a very weak band was obtained for EtBr-treated cells. No amplification was obtained with nuclear DNA, confirming the specificity for mtDNA. Consistently, the mtDNA-encoded COX I protein was weakly stained in mtDNA-depleted L929 compared with parental cells (Figure 1B). The functional effect of EtBr treatment on oxidative phosphorylation was assessed further on *in situ* respiration as measured by the respiratory control ratio (RCR). EtBr treatment induces a complete inhibition of the oxidative respiration ($\text{RCR} = 1$) in mtDNA-depleted cells (see Supplementary data available at *The EMBO Journal Online*). The total cellular ATP measured in mtDNA-depleted L929 cells was reduced by 60% and was no longer sensitive to the uncoupler FCCP, suggesting that the residual ATP content in these cells is supplied by glycolysis (data not shown).

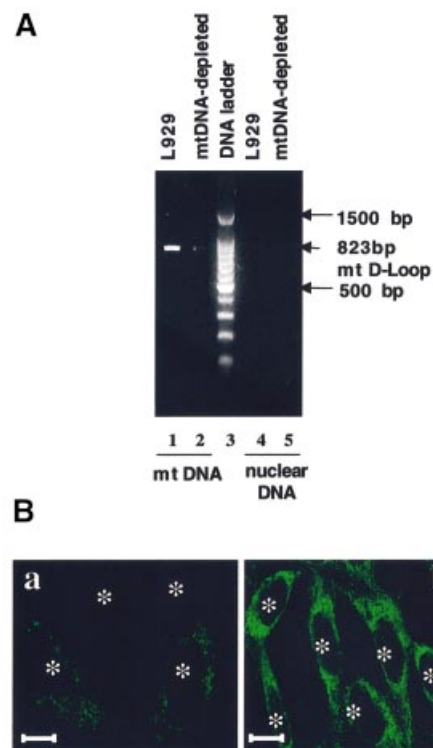


Fig. 1. Effects of EtBr-treatment on mitochondria in L929 cells. (A) PCR amplification of a specific mtDNA fragment in the mitochondrial D-loop performed on purified mtDNA (lanes 1 and 2) and nuclear DNA (lanes 4 and 5). (B) Immunofluorescence patterns of cells stained with a monoclonal antibody against the COX I subunit: (a) mtDNA-depleted cells; (b) L929 cells. Asterisks indicate the localization of nuclei. Scale bars = 10 μm .

CREB is constitutively activated by phosphorylation on Ser133 in mtDNA-depleted L929 cells

To address the question of whether or not the inhibition of mitochondrial activity modulates the transcriptional activity, L929 or mtDNA-depleted L929 cells were transiently co-transfected with a plasmid encoding β -galactosidase and different luciferase reporter constructs transactivated by various transcription factors. As illustrated in Figure 2A, the luciferase activity was increased specifically for the constructs driven either by the authentic α -inhibin promoter (Pei *et al.*, 1991) or by the authentic *c-fos* promoter, both containing CRE sites. CREB transactivation is also observed in a $\rho 0$ 143B cell line or clonal cybrids obtained from $\rho 0$ 143B cells that have been repopulated with mtDNA from a MERRF patient harboring a tRNA^{Lys} mutation (A8344G) (King and Attardi, 1989). Luciferase activity was compared in L929, mtDNA-depleted L929 cells, cybrids containing either 100% mutated or wild-type mtDNA, and 143B and $\rho 0$ 143B cells after transient transfection with the luciferase reporter construct driven by the α -inhibin promoter (Figure 2B). The different cell lines associated with severe defects in respiratory chain activity show a significant increase in CREB-dependent luciferase activity. This effect seems to be specific for mitochondrial inhibition, as glycolysis inhibition with 2-deoxy-D-glucose is without any effect on CREB activation (data not shown). In mtDNA-depleted L929 cells, the overexpression of

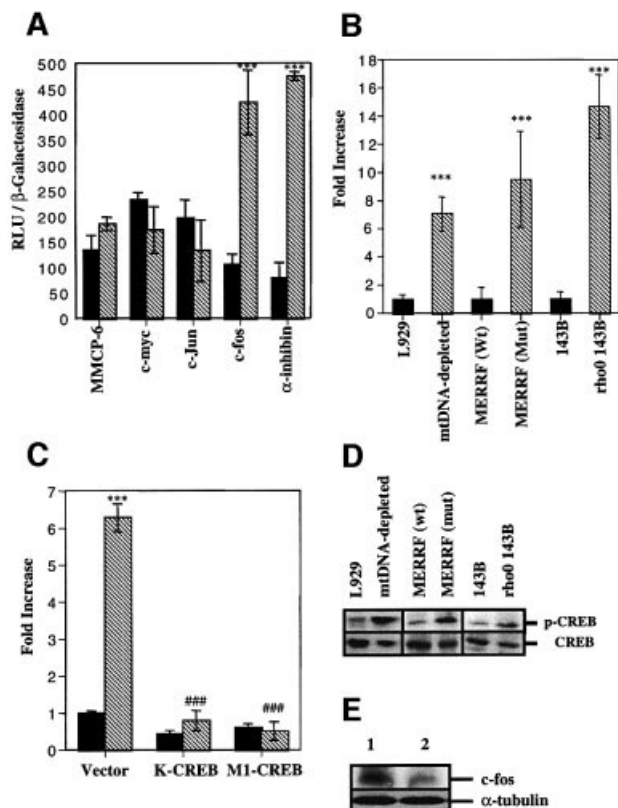


Fig. 2. (A) Luciferase activity in transiently transfected L929 control and mtDNA-depleted cells with luciferase constructs driven by *c-myc*, *c-Jun*, authentic MMCP-6, *c-fos* and α -inhibin promoters. L929 (black) and mtDNA-depleted L929 (hatched) cells, were transiently co-transfected with β -galactosidase and the luciferase constructs. (B) CREB-dependent luciferase activity in L929, mtDNA-depleted L929, wild-type (wt) MERRF cybrid, mutated (mut) MERRF cybrid, 143B and p0 143B cells transiently transfected with the luciferase reporter construct driven by the α -inhibin promoter and an expression plasmid encoding β -galactosidase. (C) CREB-dependent luciferase activity in L929 (black) and mtDNA-depleted L929 (hatched) cells transiently transfected with K-CREB or M1-CREB, together with the CREB-sensitive luciferase reporter construct and an expression plasmid encoding β -galactosidase. Luciferase activity was determined 24 h post-transfection. Results are expressed in relative light units (RLU) after normalization for β -galactosidase activity (A) or in fold increase of control cells (B and D) as means \pm 1 SD (***, significantly different from L929 and mtDNA-depleted L929 cells with $P < 0.001$). (D) Western blot analysis of phospho-CREB (p-CREB) and CREB assessed for the different cell lines used in (B). (E) Western blot analysis for *c-fos* and α -tubulin assessed for L929 (lane 2) and mtDNA-depleted L929 (lane 1).

K-CREB and M1-CREB, two dominant-negative mutants for CREB (Bonni *et al.*, 1999), completely inhibited the luciferase activity driven by the α -inhibin promoter (Figure 2C). Western blotting analysis revealed that CREB phosphorylation on Ser133 is induced in mtDNA-depleted L929 cells, mutated MERRF cybrids and p0 143B cells (Figure 2D). Confocal microscopy and subcellular fractionation-western blot analyses show that phosphorylated CREB is found mainly in nuclei of the different cell lines associated with a mitochondrial dysfunction (data not shown). To make sure that phosphorylated CREB is able to modify gene expression, we checked the amount of *c-fos* expression, a CREB-regulated immediate-early gene (Hall *et al.*, 2001). As

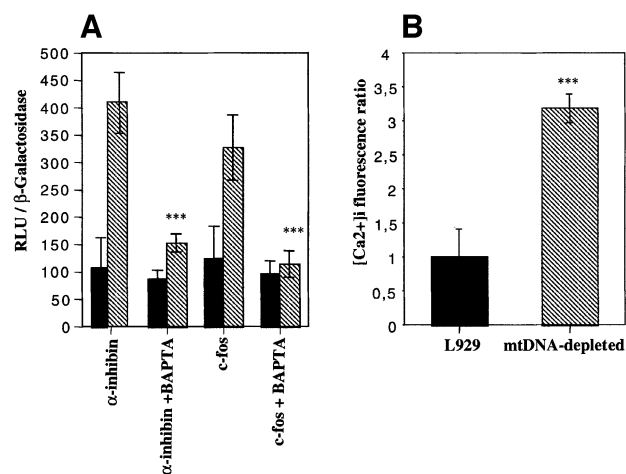


Fig. 3. (A) Effect of a 6 h treatment with 20 μ M BAPTA-AM on the CREB-sensitive luciferase activity after transfection of L929 (black) or mtDNA-depleted L929 (hatched) cells with luciferase reporter genes driven by either CREB-regulated *c-fos* or α -inhibin promoters. Luciferase activity was determined and expressed as RLU after normalization for β -galactosidase activity ($n = 6$; ***, significantly different from mtDNA-depleted cells with $P < 0.001$). (B) Intracellular calcium concentration ratio in L929 and mtDNA-depleted cells loaded with 10 μ M Fluo-3-AM. Results are expressed in fold increase of control cells, and represent the mean \pm SD for $n = 3$ (***, significantly different from L929 with $P < 0.001$).

can be seen in Figure 2E, *c-fos* protein is more abundant in mtDNA-depleted cells.

Identification of the kinase responsible for the phosphorylation of CREB

We next examined the activity of several kinase cascades leading to CREB phosphorylation in mtDNA-depleted L929 cells. We first measured the *in vitro* cAMP-dependent protein kinase (PKA) activity in cell homogenates, but no difference was observed between L929 and mtDNA-depleted cells, while PKA is activated in a time-dependent manner in cells treated with 100 μ M dibutyryl-cAMP (see Supplementary data). We then tested whether CREB phosphorylation was mediated by a Ca²⁺-dependent mechanism. Cell treatment with BAPTA, an intracellular Ca²⁺ chelator, inhibits luciferase activity observed in mtDNA-depleted L929 cells on both CREB reporter constructs (Figure 3A), while BCECF, an unrelated probe tested to rule out the potentially toxic effect, is without any effects (data not shown). We also confirmed that intracellular calcium concentration in cells loaded with Fluo-3, a fluorescent calcium indicator, is higher in mtDNA-depleted L929 cells (Figure 3B).

As calcium is able to activate several kinase pathways leading to CREB phosphorylation on Ser133 such as CaMKI, II or IV, p70S6K, rsk2, MSK, PKCs, Ras/Raf/MAPK and MAPKAP-K2, allowing the interaction with the CREB-binding protein (CBP/p300) (De Cesare *et al.*, 1999), we used different inhibitors to modulate the CREB-dependent luciferase activity. As shown in Figure 4A, only KN-93, a CaMK inhibitor, and W-7, a calmodulin antagonist, were able to inhibit CREB-dependent transcription significantly, while inhibitors of the PKA (H-89), PKC (calphostin C), mitogen-activated protein kinase (PD 95089) or phosphatidylinositol 3-kinase (wortmannin)

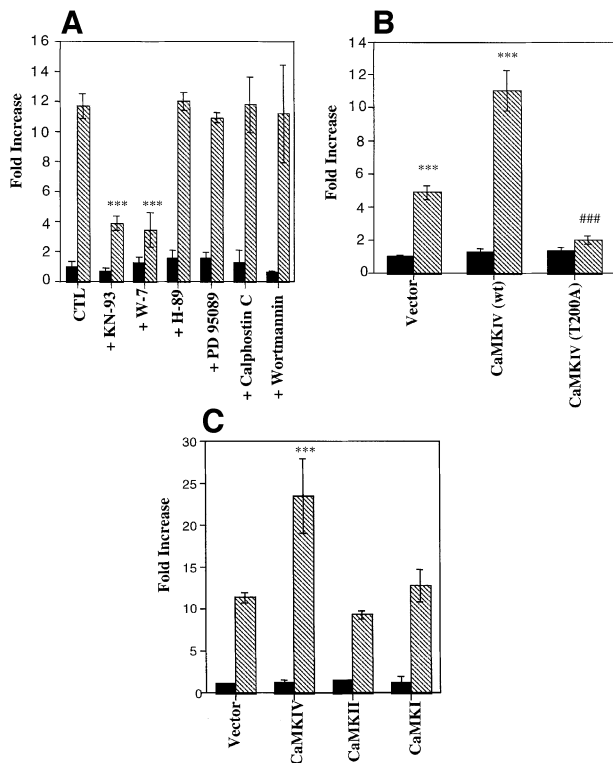


Fig. 4. (A) Effect of kinase pathway inhibitors on CREB-dependent luciferase activity in L929 (black) and mtDNA-depleted (hatched) cells. After transfection, cells were incubated for 16 h before the assay with 50 μ M KN-93, 50 μ M W-7, 10 μ M H-89, 20 μ M PD 95089, 1 μ M calphostin C or 1 μ M wortmannin. (B) L929 (black) and mtDNA-depleted L929 (hatched) cells were transiently co-transfected with a plasmid encoding β -galactosidase, the CREB luciferase reporter construct driven by the α -inhibin promoter and an expression vector encoding wild-type CaMKIV or the dominant-negative form CaMKIV T200A. (C) Effect of overexpression of CaMKI, CaMKII and CaMKIV on the activation of the CREB luciferase reporter construct driven by the α -inhibin promoter. L929 (black) and mtDNA-depleted L929 (hatched) cells were transiently co-transfected as described in (B) with an expression vector encoding wild-type CaMKI, II or IV. Results are normalized for β -galactosidase activity, are expressed in fold increase of luciferase activity of the L929 control cells and represent the mean \pm SD for $n = 3$ (***, significantly different from L929 with $P < 0.001$; ###, significantly different from mtDNA-depleted L929 cells with $P < 0.001$).

pathway have no effect. We thus investigated the possible role of CaMKI, II, and IV in CREB activation. Expression of a dominant-negative form of CaMKIV (CaMKIV T200A) (Chatila *et al.*, 1996) completely suppressed the activation of the CREB reporter construct in transfected mtDNA-depleted L929 cells, while overexpression of the wild-type enzyme enhanced the luciferase activity only in mtDNA-depleted cells (Figure 4B). When these cells are transiently transfected with plasmids encoding the wild type of different isoforms (CaMKI, II and IV), only CaMKIV overexpression is able to enhance the CREB-dependent luciferase activity by an additional 2-fold increase (Figure 4C).

The crucial role of CaMKIV in the phosphorylation of CREB in mtDNA-depleted L929 cells was evidenced further by the fact that overexpression of dominant-negative CaMKIV T200A or KN-93 treatment dramatically inhibited CREB phosphorylation, as observed by

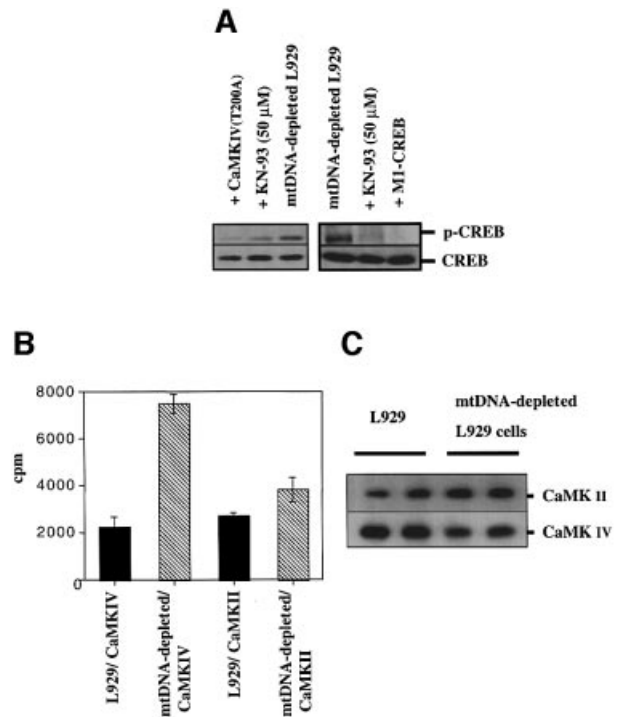


Fig. 5. (A) Effect of dominant-negative CaMKIV T200A or M1-CREB overexpression and the calmodulin kinase inhibitor KN-93 on the CREB phosphorylation pattern in mtDNA-depleted L929 cells. mtDNA-depleted L929 cells were either transfected with a plasmid encoding CaMKIV T200A or M1-CREB or treated with 50 μ M KN-93 for 24 h before phospho-CREB and CREB expression were analyzed by western blotting. (B) Endogenous CaMKIV or CaMKII activity in L929 and mtDNA-depleted cells was measured after immunoprecipitation. Representative results of one experiment in duplicate are shown and are expressed as c.p.m. (C) The amounts of immunoprecipitated kinases were controlled by western blotting.

western blot analysis (Figure 5A). The overexpression of the dominant-negative M1-CREB also inhibited phosphorylation of endogenous CREB, probably by competing for binding and subsequent phosphorylation (Figure 5A). Finally, endogenous CaMKII and CaMKIV were immunoprecipitated and their activity was determined *in vitro*. The results shown in Figure 5B and C clearly indicate that the CaMKIV activity is increased by 3- to 4-fold in mtDNA-depleted L929 cells while CaMKII activity seems to be unaffected. The slight increase observed in the CaMKII activity is most likely to be the result of the higher amount of the immunoprecipitated kinase used for the assay (Figure 5C). In conclusion, these results clearly support the hypothesis that CREB phosphorylation in mtDNA-depleted L929 cells is mediated by CaMKIV.

CREB phosphorylation is induced by inhibitors of mitochondrial activity

In order to determine whether CREB activation is induced directly by a mitochondrial activity impairment, we tested the effect of metabolic mitochondrial inhibitors on CREB activation. Phospho-CREB localization in cell lines treated with FCCP, oligomycin or antimycin A is almost exclusively nuclear, as shown for L929 cells in Figure 6A. Phosphorylated CREB is also transcriptionally active in cells treated with metabolic inhibitors, as demonstrated by

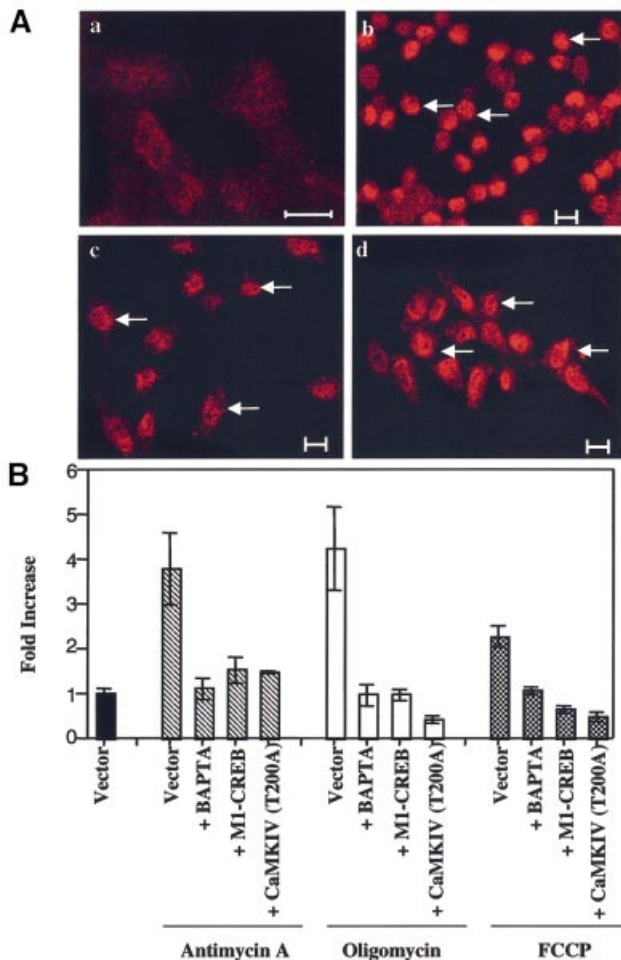


Fig. 6. (A) Immunolocalization of phospho-CREB on Ser133 in L929 cells treated or not (a) for 6 h with 10 μ M FCCP (b), 8 μ M oligomycin (c) and 1 μ M antimycin A (d) and analyzed the next day by immunofluorescence and confocal microscopy. Bars = 10 μ m. Arrows indicate a nuclear localization. (B) CREB-dependent luciferase activity in 293 cells triggered by treatment with 1 μ M antimycin A (hatched bars), 8 μ M oligomycin (white bars) or 10 μ M FCCP (black bars) is inhibited by 20 μ M BAPTA for 6 h, or by expression of a dominant-negative form of CREB (M1-CREB) or a dominant-negative mutant of CaMKIV (CaMKIV T200A). Results are expressed as fold increase of control cells (vector) after normalization. The results are representative of two experiments in triplicate and are expressed as means \pm 1 SD.

the activation of the CREB reporter gene construct (Figure 6B). Cell incubation with BAPTA and over-expression of dominant-negative form M1-CREB or CaMKIV T200A almost completely inhibited the luciferase activity triggered by the different mitochondrial inhibitors (Figure 6B). Using the Ca^{2+} -sensitive photoprotein apoaequorin as a calcium probe, we confirmed that free cytosolic $[\text{Ca}^{2+}]_{\text{cyt}}$ is increased in 293 cells treated with mitochondrial inhibitors while mitochondrial $[\text{Ca}^{2+}]_{\text{mt}}$ is decreased (see Supplementary data). Endogenous CaMKIV activity is also increased (5–7 times) in 293 cells after treatment with oligomycin, antimycin A or FCCP (see Supplementary data). Altogether, these results suggest that mitochondrial inhibitors are also able to trigger CREB phosphorylation through a CaMKIV-dependent mechanism and they demonstrate that CREB

activation induced by mitochondria dysfunction is not cell specific as it is observed in two different cell lines.

CaMKIV activity is differentially regulated by PP2A in cells affected by mitochondria dysfunction

Once activated by phosphorylation on a single threonine residue by the calmodulin-dependent kinase kinase (CaMKK), CaMKIV activity becomes independent of both calcium and calmodulin (Park and Soderling, 1995). However, CaMKIV is known to be inactivated by protein phosphatase 2A (PP2A) (Westphal *et al.*, 1998). To determine whether these enzymes exist as a complex in L929 and mtDNA-depleted L929 cells, we immunoprecipitated endogenous CaMKIV from cell lysates and analyzed the immune complexes by immunoblotting with an antibody specific for the PP2A catalytic C subunit. As shown in Figure 7A, PP2A catalytic C subunit co-immunoprecipitated with CaMKIV in L929 cells (lane 1), but the interaction is dramatically weaker in mtDNA-depleted L929 cells (lane 2). To make sure the differential interaction is caused by mitochondrial inhibition, we compared the co-immunoprecipitated PP2A in L929 cells treated with the different inhibitors. As shown in Figure 7A, antimycin A, FCCP and oligomycin are all able to impair the interaction between both enzymes. These results suggest that PP2A interaction with CaMKIV is weaker in mtDNA-depleted L929 cells and in cells submitted to the metabolic inhibitors. A very important question to answer is whether or not the weaker association of CaMKIV with PP2A observed in mtDNA-depleted cells is caused by CaMKIV's increased activity as a result of the higher calcium concentration or if the impaired association with PP2A is responsible for causing the increase in CaMKIV. To address this question, we performed co-immunoprecipitation experiments between CaMKIV and PP2A in L929 cells treated with ionomycin, a calcium ionophore, or in mtDNA-depleted cells treated with KN-93 to inhibit CaMKIV activity. The results presented in Figure 7B clearly show that ionomycin treatment inhibits the interaction while the association is increased in mtDNA-depleted L929 cells treated with KN-93. These results support the first hypothesis that the interaction can be modulated by the CaMKIV activity status.

Involvement of phosphorylated CREB in the cell proliferation defect

A slower proliferation of mtDNA-depleted cells than parental cells has been observed in several cell lines and is usually attributed to the lower ATP content in these cells (King and Attardi, 1996; Morais, 1996), but the molecular pathway is unknown. As stimulation of the cAMP pathway inhibits proliferation of some tumoral cell lines (Yokozaki *et al.*, 1992), we hypothesized that phosphorylated CREB contributes to the lower proliferation of the mtDNA-depleted L929 or L929 cells treated with an inhibitor of mitochondrial activity. As shown in Figure 8A, the $[\text{H}^3]$ thymidine incorporation into mtDNA-depleted L929 cells and in oligomycin-treated L929 cells was strongly reduced as compared with L929. Therefore, we assessed the role of phosphorylated CREB on cell entry into S phase. L929 cells were transiently transfected with

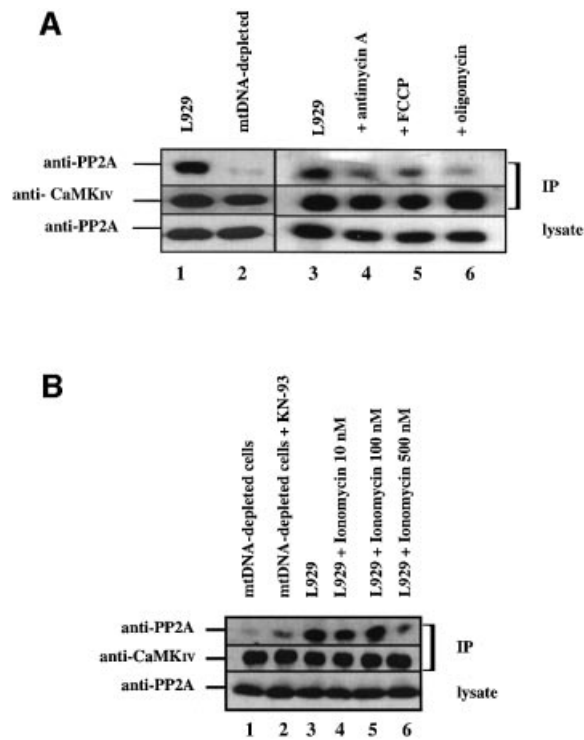


Fig. 7. (A) Co-immunoprecipitation of PP2A catalytic C subunit with CaMKIV. CaMKIV was immunoprecipitated (IP) from L929 cells (lanes 1 and 3) treated or not with mitochondrial inhibitors (8 μ M oligomycin, 1 μ M antimycin A and 10 μ M FCCP) (lanes 4–6) or from mtDNA-depleted L929 cells (lane 2). (B) Co-immunoprecipitation of PP2A catalytic C subunit with CaMKIV. CaMKIV was immunoprecipitated from L929 cells (lane 3) treated or not with ionomycin at 10, 100 or 500 nM for 16 h (lanes 4–6) or from mtDNA-depleted L929 cells treated or not with 50 μ M KN-93 for 16 h (lanes 1 and 2). Immune complexes were analyzed by immunoblotting with an anti-PP2A C subunit antibody (anti-PP2A) or an anti-CaMKIV antibody (anti-CaMKIV). Expression levels of the PP2A C subunit in the lysates are also shown.

a control vector or with constructs encoding CREB dominant-negative mutants K-CREB and M1-CREB, 24 h before the addition of 8 μ M oligomycin for 6 h. Oligomycin decreases [3 H]thymidine incorporation by almost 40–50% in both untransfected control cells and transfected control cells. However, this effect is largely reduced when cells overexpressed either K-CREB or M1-CREB (Figure 8A). Similarly, mtDNA-depleted L929 cells incorporated only 50% of [3 H]thymidine compared with parental cells, and the decrease can be significantly reduced by K-CREB or M1-CREB overexpression (Figure 8A). A reduced decrease in the [3 H]thymidine incorporation was also observed when mtDNA-depleted L929 cells were transiently transfected with dominant-negative CaMKIV or treated with KN-93 (Figure 8B), two conditions that reduce the phosphorylation of CREB on Ser133 (Figure 5A). As a positive control, we showed that M1-CREB is also able to reduce the decrease of [3 H]thymidine incorporation in forskolin-treated L929 cells (Figure 8B). In conclusion, mtDNA depletion or mitochondrial inhibitors such as oligomycin appear to inhibit cell cycle entry into S phase through the activation of CREB transcription factor.

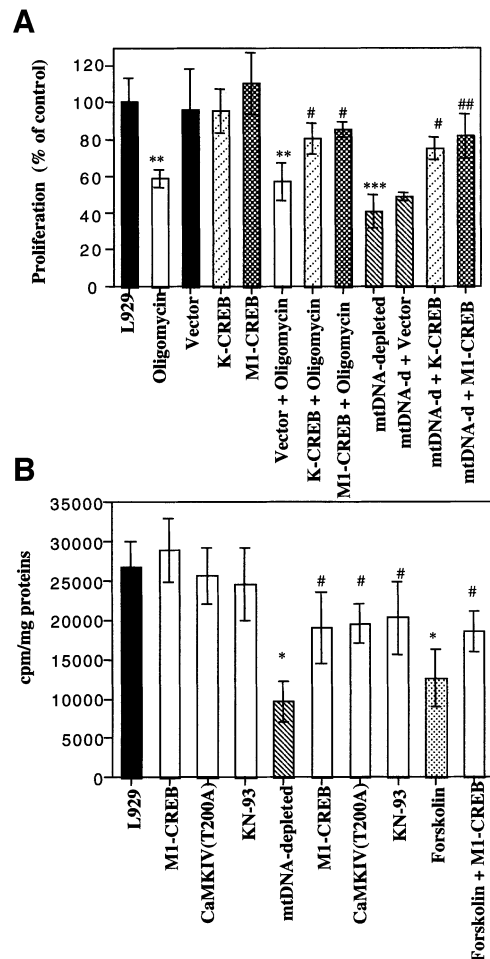


Fig. 8. (A) Incorporation of [3 H]thymidine into DNA was analyzed in L929 cells (treated or not with oligomycin) and compared with the incorporation for mtDNA-depleted L929 cells. In some conditions, these cells were transiently transfected before treatment with an empty vector or plasmids encoding dominant-negative CREB (K-CREB and M1-CREB). At 16 h post-transfection, oligomycin (8 μ M) was added or not for 6 h and, the next day, cells were labeled with 2 μ Ci of [3 H]thymidine for 4 h and processed accordingly. (B) Effect of M1-CREB and CaMKIV T200A overexpressions or KN-93 treatment on [3 H]thymidine incorporation. L929 and mtDNA-depleted L929 cells were transiently transfected with an empty vector or plasmids encoding M1-CREB or CaMKIV T200A. The vector-transfected cells were treated or not with 50 μ M KN-93 for 24 h before the [3 H]thymidine incorporation assay. A control was performed with forskolin (10 $^{-5}$ M) for 24 h with or without M1-CREB overexpression. Values were determined and are expressed either as c.p.m./mg of proteins (B, $n = 3$) or as a percentage of [3 H]thymidine incorporation for control L929 cells (A, $n = 4$). (*, **, *** or #, ##, ###, significantly different from L929 control cells or mtDNA-depleted L929 cells with $P < 0.05$, $P < 0.01$ or $P < 0.001$).

Phosphorylated CREB modifies transactivation of p53 leading to p21 overexpression in mtDNA-depleted L929 cells

Finally, we delineated the mechanism by which phosphorylated CREB could control the cell cycle of mtDNA-depleted L929 cells. Inspired by the recent findings that phosphorylated CREB strongly and directly interacts with the N-terminus of the p53 tumor suppressor protein (Giebler *et al.*, 2000), we tested the interaction between p53 and CREB as well as the transcriptional activity of p53 in mtDNA-depleted L929 cells. As shown in Figure 9A,

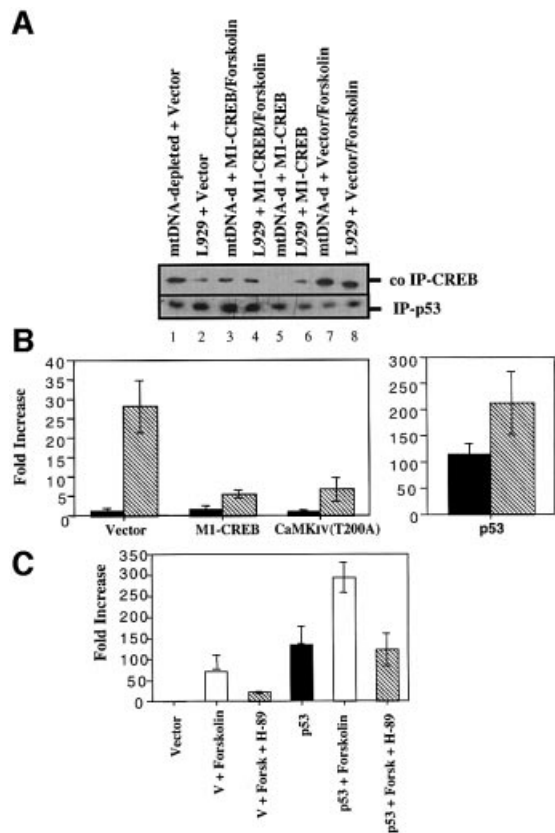


Fig. 9. (A) CREB directly interacts with p53 tumor suppressor protein in a phosphorylation-dependent manner. p53 protein was immunoprecipitated (IP) from L929 and mtDNA-depleted L929 cells transiently transfected or not with a plasmid encoding M1-CREB. When indicated, cells were treated with 10 μ M forskolin for 24 h. Bound CREB protein was detected by western blot analysis. (B) p53-dependent transcription is increased in mtDNA-depleted L929 cells. The p53-responsive pG13-Luc reporter was co-transfected into L929 (black) or mtDNA-depleted L929 cells (hatched) with the β -galactosidase construct, together with 1 μ g of an empty vector, of a plasmid encoding M1-CREB and CaMKIV T200A (left panel) or a p53 expression plasmid (pC53-SN3) (right panel). (C) p53-dependent transcription is stimulated by forskolin treatment in an H-89 inhibitable manner. The p53-responsive pG13-Luc reporter was co-transfected into L929 cells with the β -galactosidase construct, together with 1 μ g of an empty vector or a plasmid encoding wild-type p53 (pC53-SN3). Cells were then stimulated with 10 μ M forskolin with or without 10 μ M H-89 for 16 h. Luciferase activity was measured 48 h post-transfection. Reported values are expressed in fold increase of L929 control cells transfected with an empty vector as means \pm SD ($n = 3$).

CREB physically interacts with p53 in a phosphorylation-dependent manner. p53 tumor suppressor protein was immunoprecipitated from L929 and mtDNA-depleted L929 cells and the amount of co-immunoprecipitated CREB was analyzed by western blotting. The total CREB co-immunoprecipitated in mtDNA-depleted L929 cells is more abundant than in L929 control cells (Figure 9A, lanes 1 and 2). This protein-protein interaction is mediated by the phosphorylation status of CREB, as overexpression of M1-CREB, a non-phosphorylatable CREB protein, completely abolished the interaction in mtDNA-depleted L929 cells (lane 5). In both cell lines, forskolin treatment to induce the phosphorylation of CREB results in an increase of CREB associated with p53 (lanes 7 and 8) while the overexpression of M1-CREB before forskolin stimulation decreases CREB protein binding to p53

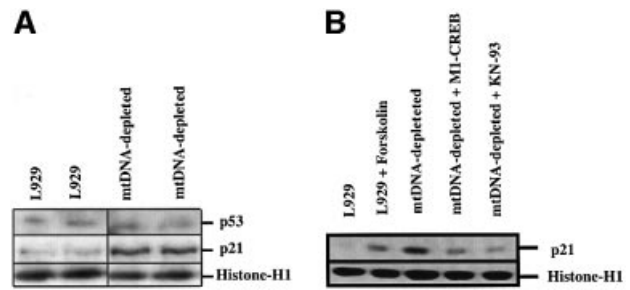


Fig. 10. (A) p21 is overexpressed in mtDNA-depleted L929 cells. Western blotting analysis for p53 and p21 derived from L929 and mtDNA-depleted L929 cells. (B) p21 expression is stimulated in L929 cells treated with 10 μ M forskolin for 16 h (lanes 1 and 2) while its expression is reduced in mtDNA-depleted cells transiently transfected with M1-CREB or incubated with 50 μ M KN-93 for 16 h (lanes 3–5). Protein loading was controlled by the expression of histone H1.

(lanes 3 and 4). The physical interaction between phosphorylated CREB and p53 tumor suppressor protein observed in mtDNA-depleted L929 cells led us to address the question of whether or not the binding of phosphorylated CREB to p53 facilitates the p53-dependent transcription. We thus performed transient transfection assays with a p53-dependent luciferase reporter gene. L929 or mtDNA-depleted L929 cells were transiently transfected with a reporter plasmid carrying 13 copies of a p53-responsive promoter driving expression of the luciferase gene (pG13-Luc). As observed in Figure 9B, the luciferase activity is strongly increased in mtDNA-depleted L929 cells (28-fold increase) while co-transfection of either M1-CREB or CaMKIV (T200A) significantly inhibited p53-dependent luciferase activity. As expected, transfection of a p53 expression plasmid dramatically stimulated luciferase in L929 cells but, interestingly, the overexpression of p53 in mtDNA-depleted L929 cells resulted in an additional 2-fold stimulation (Figure 9B). As forskolin enhances CREB interaction with p53, we tested its effect on the expression of the p53 reporter gene in L929 cells. As observed in Figure 9C, forskolin also triggers p53-dependent luciferase expression (69-fold increase) and the stimulation can be partially inhibited by H-89, a PKA inhibitor. These effects are even stronger when L929 cells are transiently transfected with a plasmid encoding wild-type p53 before the stimulation with forskolin (Figure 9C).

To address the biological relevance of the phosphorylated CREB pathway on p53-dependent transcription function, we analyzed the expression of an endogenous p53-responsive gene. We selected p21^{Waf1/Cip1}, a cyclin-dependent kinase inhibitor gene, and examined its expression in L929 and mtDNA-depleted L929 cells. Western blot analysis revealed that the p21 protein level is increased in mtDNA-depleted L929 cells while little or no change in p53 tumor suppressor protein level was observed (Figure 10A). Furthermore, the level of p21 expression can be modulated by conditions that affect CREB phosphorylation status. Indeed, as observed in Figure 10B, forskolin stimulation increases p21 expression in L929 cells while inhibition of CaMKIV activity by KN-93 or overexpression of dominant-negative M1-CREB dramatically decreases p21 abundance in

mtDNA-depleted cells. These results support a link between CREB activation and p21 expression.

Discussion

In this study, we report a new signaling pathway initiated by a dysfunction of the mitochondria and we analyzed its effect on transcription activation and cell proliferation. We used several models to study the cellular response to inhibition of mitochondrial activity: mtDNA-depleted L929 and $\rho 0$ 143B cell lines chronically treated with EtBr and a homoplasmic cybrid cell line relevant for MERRF disease which is mutated in a single nucleotide (A8344G) in the mtDNA-encoded tRNA^{Lys} (King and Attardi, 1989). In all these cell lines, we found that CREB was phosphorylated on Ser133, localized in the nucleus and was able to trigger the transcription of a luciferase reporter gene driven by the authentic α -inhibin promoter containing several CRE consensus binding sites.

We confirmed the direct role of a mitochondrial activity impairment in CREB activation by testing the effects of FCCP, oligomycin and antimycin A. All these metabolic mitochondrial inhibitors were able to trigger CREB-dependent luciferase activity. In the different models used to study mitochondrial dysfunction, CREB-sensitive luciferase expression is completely inhibited by K-CREB and M1-CREB, two CREB dominant-negative mutants (Bonni *et al.*, 1999). Nuclear localization of phosphorylated CREB on Ser133 induced by metabolic inhibition was confirmed by confocal microscopy, while CREB expression was unchanged by the treatments, as analyzed by western blotting (data not shown).

Besides PKA, several kinases such as CaMKs, p70S6K, rsk2, MSK and MAPKAP-K2 are able to phosphorylate CREB on its transcriptional regulatory site, allowing interaction with the CREB-binding protein (CBP/p300) (De Cesare *et al.*, 1999). Mitochondrial inhibition is not known to have any effect on the cAMP pathway and we did not observe any PKA activation in mtDNA-depleted L929 cells. Our results suggest an important role for a CaMK in CREB activation. Identification of the specifically activated CaMKIV by mitochondrial dysfunction and the fact that it is responsible for CREB phosphorylation was based on the results obtained from direct *in vitro* kinase assays and from the correlated inhibition of both CREB phosphorylation and luciferase activity by the overexpression of a dominant-negative mutant for CaMKIV.

Biswas *et al.* (1999) recently showed that elevated cytosolic calcium concentration observed after mitochondrial activity inhibition leads to increased transcription of the ryanodine receptor-1 Ca²⁺ release channel and COX Vb genes. We also found an increase in the [Ca²⁺]_{cyt} in cells showing a mitochondrial activity impairment, and data obtained with mitochondrial aequorin overexpression suggest that the cytosolic increase induced by metabolic inhibitors is accompanied by a decrease in the mitochondrial calcium pool. Interestingly, hereditary defects in mitochondrial proteins such as pyruvate dehydrogenase (PDH) that decrease aerobic metabolism have been linked to reduced mitochondrial Ca²⁺ uptake and sequestration (Padua *et al.*, 1998).

How do we explain the sustained CaMKIV activity observed in both mtDNA-depleted L929 cells and cells treated with metabolic inhibitors? The hypothesis of pre-existing complexes containing active protein kinases and phosphatases suggests an attractive mechanism by which the appropriate phosphorylation state of the kinase is regulated. Phosphorylation of CaMKIV is required to generate autonomous activity, and PP2A is a constitutively active phosphatase that turns down the activity of CaMKIV (Westphal *et al.*, 1998). Immunoprecipitation of CaMKIV and analyses of the immune complexes by immunoblotting with an anti-PP2A antibody indicate that PP2A interaction with CaMKIV is very weak in both mtDNA-depleted L929 cells and cells treated with inhibitors. However, as the interaction can be inhibited by increasing the calcium concentration with ionomycin, it seems that the association is calcium dependent. Furthermore, KN-93 treatment that inhibits CaMKIV activity allows a stronger interaction in mtDNA-depleted cells. One can thus imagine that the physical interaction between PP2A and CaMKIV is modulated by calcium and the activity of CaMKIV.

CREB family members have been described as important transcription factors regulating cell proliferation and other nuclear responses (De Cesare *et al.*, 1999). Stimulation of the cAMP/PKA signal transduction pathway inhibits proliferation of some tumoral cell lines (Yokozaki *et al.*, 1992) and inhibits proliferation of activated hepatic stellate cells (Houglum *et al.*, 1997). In culture, mtDNA-depleted cells or PDH-deficient fibroblasts replicated far more slowly than normal cells (Buchet and Godinot, 1998; Padua *et al.*, 1998).

We first examined the role of CREB on cell proliferation by treating cells with forskolin, which inhibits L929 cell proliferation by 40–50%. We then confirmed that inhibition of mitochondrial respiratory function, induced by either oligomycin or mtDNA depletion, decreases cell [³H]thymidine incorporation and thus inhibits cell progression to G₁ (Sweet and Singh, 1995). Here, we found that cells transfected with dominant-negative mutants K-CREB or M1-CREB are almost insensitive to cell proliferation inhibition induced by oligomycin. Furthermore, these mutants suppress the cell proliferation defect observed in mtDNA-depleted L929 cells. These results suggest that phosphorylated CREB contributes to the control of cell cycle progression. CREB activation could thus explain why doubling time is higher for mtDNA-depleted L929 cells, an observation commonly reported for other mtDNA-depleted cell lines (Buchet and Godinot, 1998).

Although little is known about the numerous mechanisms by which CREB affects the cell cycle, they could be mediated through molecular interactions between phosphorylated CREB and CBP, a key integrator of cellular functions (Kamei *et al.*, 1996), or transcription factors such as p53 tumor suppressor protein (Giebler *et al.*, 2000). Here, we demonstrate that, in mtDNA-depleted L929 cells, phosphorylated CREB physically interacts with p53 tumor suppressor protein and increases p53 transcriptional activity as assessed in a p53-dependent luciferase assay. p53 transcriptional activation in mtDNA-depleted cells can be suppressed by either M1-CREB or CaMKIV T200A overexpression. However, p53 protein

expression is equal in both L929 and mtDNA-depleted cells. These results suggest that the increase of p53 transcriptional activity in mtDNA-depleted L929 cells is mediated through phosphorylated CREB, which recruits p53 protein and modifies its transcriptional activity. Among p53-inducible genes, the p21^{Waf1/Cip1} gene encodes a protein that inhibits cyclin-dependent kinase activity and leads to G₁ growth arrest (el-Deiry *et al.*, 1993). Indeed, as evidence of p53 activation in mtDNA-depleted L929 cells, we found that p21^{Waf1/Cip1} protein is more abundant in this cell line. The mechanism by which phosphorylated CREB triggers the transactivation of p53 in mtDNA-depleted cells, while p53 protein abundance is equal, is unknown. However, CBP acetylates not only histones but also transcription factors such as p53, c-Myb, E2-F and GATA-1 and increases their DNA sequence-specific binding capacity (Sano and Ishii, 2001).

In conclusion, the results show that CaMKIV may be involved in the calcium-dependent transcriptional regulation of genes in mitochondria-deficient cells through phosphorylation of CREB. This transcription factor could mediate calcium-induced transcription of immediate-early genes or genes encoding mitochondrial proteins and containing CRE sequences (Soderling, 1999). Indeed, CREB has been suspected to play a role in the regulation of the expression of several mitochondrial proteins such as respiratory chain subunits (Scacco *et al.*, 2000), cytochrome *c* (Gopalakrishnan and Scarpulla, 1994), manganese superoxide dismutase (Kim *et al.*, 1999) and carnitine palmitoyltransferase (Brady *et al.*, 1992). In this study, we demonstrated that phosphorylated CREB induced by a mitochondrial dysfunction is not only involved in transcriptional regulation of a target gene such as *c-fos* but can also be seen as a factor that causes a cell cycle defect in cells with impaired OXPHOS. CREB, a regulator of the cell cycle and cellular differentiation program, is thus a new factor involved in the 'retrograde signaling' and molecular communication between functionally impaired mitochondria and the nucleus in mammalian cells.

Materials and methods

Cell cultures

The 143B, p0 143B, wild-type MERRF cybrids, mutated (A8344G) MERRF cybrids, L929 and mtDNA-depleted L929 cell lines have been described previously (King and Attardi, 1989; Schulze-Osthoff *et al.*, 1993). Human embryonic kidney 293 cells were from ATCC. The different cell lines were grown in high glucose (4.5 g/l) Dulbecco's modified Eagle's medium (DHG) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL) in a 5% CO₂ incubator. mtDNA-depleted L929 and p0 143B cells were grown in the presence of 400 ng/ml EtBr (Sigma), 50 µg/ml uridine and 1 mM pyruvate.

Immunofluorescence confocal laser scanner microscopy

Cells were fixed and permeabilized with methanol/acetone (20/80%), rinsed three times with phosphate-buffered saline (PBS) and unspecific sites were blocked with 1% bovine serum albumin (BSA) in PBS. For phospho-CREB, cells were immunostained overnight at 4°C with a primary rabbit polyclonal antibody against phosphorylated CREB on Ser133 (NEBL) at 1:1000, while for COX I detection, cells were incubated for 1 h with a monoclonal antibody against mouse COX I protein (Molecular Probes) at a 1:20 dilution, washed three times with PBS, incubated with (1:1000) anti-rabbit or anti-mouse Alexa antibodies (Molecular Probes) for 1 h at 37°C and processed for confocal microscopy (Leica).

mtDNA analysis

Subcellular fractionation to obtain nuclear- and mitochondria-enriched fractions was performed and genomic DNA or mtDNA was prepared as previously described (Wiesner *et al.*, 1991). A specific mitochondrial genome fragment of 823 bp was amplified by PCR using forward (5'-TTTGGGAAGTACTAGAATTGATCA-3') and reverse (5'-GCTGGT-ATTCTAATTAAGTACTT-3') primers specific for the mouse mitochondrial D-loop. PCR was performed using 10 ng of mtDNA or genomic DNA for 30 cycles at the hybridization temperature of 50°C.

Luciferase assay

293, 143B, p0 143B, wild-type MERRF cybrids, mutated (A8344G) MERRF cybrids, L929 and mtDNA-depleted L929 cells seeded in 12-well plates were transiently transfected by the calcium phosphate method or Superfect reagent (Qiagen) with a luciferase reporter construct (0.5 µg/well) and a β-galactosidase (0.25 µg/well) expression vector. In some experiments, cells were treated with 20 µM BAPTA-AM (Molecular Probes) for 6 h or co-transfected with 0.5 µg/well of an expression vector encoding K-CREB, M1-CREB, full-length CaMKIV or a dominant-negative form, CaMKIV T200A. For p53 activity, cells were transiently co-transfected with 0.5 µg/well of a p53-responsive (pG13-Luc) reporter with the β-galactosidase construct, together with 1 µg of an empty vector, or of a plasmid encoding M1-CREB, CaMKIV T200A or a p53 expression plasmid (pC53-SN3). In some experiments, cells were treated with 10 µM forskolin for 16 h in the presence or absence of 10 µM H-89. Cells were then harvested and luciferase activity was determined in cleared lysates using the commercial Reporter™ assay system (Promega) and normalized for β-galactosidase activity. Inhibitors were added for 16 h.

Cell proliferation index

L929 and mtDNA-depleted L929 cells were seeded in 12-well dishes at 50 000 cells/well. After a 24 h period of serum starvation, proliferation was triggered by the addition of fresh medium supplemented with 10% FBS, and the next day cells were incubated with 2 µCi of [³H]thymidine for 4 h. Cells were harvested and [³H]thymidine incorporation was determined as previously described (Buck *et al.*, 1994) and expressed as c.p.m./mg of proteins. In some conditions, 24 h before the addition of 8 µM oligomycin for 6 h or 10 µM forskolin for 24 h, L929 and mtDNA-depleted L929 cells were transiently transfected using Superfect reagent with 0.5 µg/well of a control vector, with constructs encoding M1-CREB, K-CREB or CaMKIV T200A. KN-93 (50 µM) was added to empty vector-transfected L929 and mtDNA-depleted L929 cells for 24 h.

Western blotting analysis

Immunoblotting analyses were performed with 10 µg of protein of cleared lysates after separation on a 10 or 12% SDS-polyacrylamide gel. PVDF membranes were blocked overnight at 4°C in 5% non-fat dry milk or BSA-TBST. Primary antibodies were either anti-c-fos (Santa Cruz) at 1:1000, anti-CaMKIV or anti-CaMKII (Santa Cruz) at 1:100, anti-PP2A C subunit (Santa Cruz) at 1:1000, anti-phospho-CREB (Upstate Biotechnology), anti-CREB (NEBL) (1 µg/ml) or anti-p21 (Santa Cruz) at 1:2500, anti-histone-1 (Santa Cruz) at 1:1000 and anti-p53 (PharMingen) at 2 µg/ml. For loading controls, immunoblotting for α-tubulin or histone H1 was performed on the same membranes after stripping or not, followed by incubation with horseradish peroxidase-coupled secondary antibodies (Dako), and were detected by chemiluminescence (NEN).

Co-immunoprecipitation experiments

Endogenous CaMKII and IV were immunoprecipitated respectively with 10 µg of an anti-CaMKII or IV antibody (Santa Cruz) from clear lysates of mtDNA-depleted L929 cells or L929 cells treated or not for 6 h with 1 µM antimycin A, 8 µM oligomycin or 10 µM FCCP. Immune complexes were immobilized by adding 75 µl of a protein G + protein A-agarose mixture (Oncogene), washed three times and 40 µl of sample buffer was added. Western blot analyses for the PP2A C subunit in lysates, immunoprecipitated CaMKIV and co-immunoprecipitated PP2A C subunit were performed. p53 was immunoprecipitated from the lysates of L929 and mtDNA-depleted cells with 10 µg of an anti-p53 (Ab-1/ epitope amino acids 371–380) before p53 and CREB were analyzed by western blotting.

In vitro kinase assays

CaMKII or IV activity was determined after immunoprecipitation by measuring its ability to phosphorylate a synthetic CREB phosphopeptide

with the sequence KRREILSRRPS(p)YR (NEBL) according to the procedure described in the Supplementary data.

Statistical analysis

All experiments were performed at least twice and one set of representative results is shown. Results are expressed as means \pm SD. Statistical significance was determined by Scheffé's contrasts after analysis of variance (ANOVA1).

Supplementary data

Supplementary data for this paper are available at *The EMBO Journal* Online.

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