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Contribution of the Phosphorylable Complex I in the Growth Phase-Dependent Respiration of C6 Glioma Cells in Vitro

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The energy metabolism of rat C6 glioma cells was investigated as a function of the growth phases. Three-dimensional cultures of C6 cells exhibited diminished respiration and respiratory capacity during the early growth phase, before reaching confluence. This decrease in respiration was neither due to changes in the respiratory complex content nor in the mitochondrial mass per se. Nevertheless, a quantitative correlation was found between cellular respiration and the rotenone-sensitive NADH ubiquinone oxidoreductase (i.e. complex I) activity. Immunoblot analysis showed that phosphorylation of the 18 kDa-subunit of this complex was associated with the growth-phase dependent modulation of complex I and respiratory activity in C6 cells. In addition, by using forskolin or dibutyryl cAMP, short-term activation of protein kinases A of C6 cells correlated with increased phosphorylation of the 18-kDa subunit of complex I, activated NADH ubiquinone oxidoreductase activity and stimulated cellular respiration. These findings suggest that complex I of C6 glioma cells is a key regulating step that modulates the oxidative phosphorylation capacity during growth phase transitions.

KEY WORDS: Complex I; NADH ubiquinone oxidoreductase; cAMP-dependent phosphorylation; respiration; C6 glioma.

INTRODUCTION

During aerobic metabolism, adaptation of ATP synthesis to satisfy energy demand is a key feature of cell energetics. The response of cells to physiological and pathological conditions undergoes changes in respiratory activity to reach a new steady state where ATP synthesis matches ATP utilization. It is well documented that the short-term adaptation of oxidative phosphorylation activity to fluctuations in ATP demand involves: (i) changes in the thermodynamic forces, namely the NADH redox potential (i.e. NAD/NADH ratio), the phosphorylation potential (i.e. ATP/ADPxPi ratio) and the proton electrochemical difference across the mitochondrial inner membrane (i.e. $\Delta\mu_H^+$) and/or (ii) changes in the kinetic constraints exerted by rate-controlling enzymes (e.g. Ca^{++} activation of Krebs cycle dehydrogenases) (see for reviews: Balaban, 1990; Brown, 1992). Moreover, in vitro studies have demonstrated that complex I (i.e. NADH ubiquinone oxidoreductase) and complex IV (i.e. cytochrome *c* oxidase) of the respiratory chain, two of the major controlling steps for ATP synthesis in isolated mitochondria (Davey *et al*., 1998; Rossignol *et al*., 1999), can be phosphorylated respectively on the 18-kDa subunit (i.e. AQDQ subunit) (Papa *et al*., 2002 for review) and on subunit II (Bender and Kadenbach, 2000). These covalent modifications, which are under the control of mitochondrial protein kinases A and phosphatases (Signorile *et al*.,

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Key to abbreviations: Bt_2cAMP, dibutyryl cAMP; ClCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; DIV, day in vitro; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; IBMX, 3-isobutyl-1-isobutyl-1-methylxanthine; J_o, oxygen consumption rate in nanomol of atoms of oxygen per min and per million of viable cells (nat. O/min/10^6 cells); PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

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2002; Technikova-Dobrova *et al*., 2001), modulate the kinetic properties of the complexes. Indeed, data obtained on isolated heart mitochondria and on cultured cells have shown that cAMP-induced phosphorylation of the 18-kDa subunit increased the turnover number of complex I and therefore increases the cellular respiration (Papa *et al*., 2001; Scacco *et al*., 2000; Technikova-Dobrova *et al*., 2001). Recently, a human inherited mutation in the phosphorylation site of this subunit was associated with a fatal neurological syndrome characterized by a partial complex I deficiency (Papa *et al*., 2001). However, the occurrence of this phosphorylation under physiological conditions and its functional role in cellular energetics are not clearly understood.

In addition to the short-term control mechanisms, a long-term adaptation of eukaryotic cells to changes in ATP demand may be achieved by modulating the quantity of functional units of oxidative phosphorylation. Indeed, in animal models, an increase in the mitochondrial enzyme content has been described for brown adipocytes (Butow and Bahassi, 1999), myocytes (Moyes *et al*., 1998), and hepatocytes (Cuezva *et al*., 1997; Nogueira *et al*., 2001) in response to chronic environmental and neurohormonal stimuli. Such an induction of mitochondrial enzymes, as well as the molecular protagonists involved, have also been extensively studied in cultured mammalian cells (for review see Scarpulla, 2002). Conversely, a down-modulation of the mitochondrial enzyme content, of the respiratory activity, and other aberrant mitochondrial phenotypes have been described during neoplastic transformation of mammalian cells (for review: Cuezva *et al*., 1997 and references therein; Pedersen, 1978). Moreover, a decrease in the respiratory activity of tumor cells has also been reported as a function of the growth phase. Indeed, the respiratory activity of cultured C6 glioma cells has been shown to decrease during the transition from early- to mid-growth phases (Martin *et al*., 1998). This evolution pattern seems to be a general phenomenon since it was also observed in C6 cells differentiated toward the oligodendrocyte phenotype (Martin *et al*., 1998) and in other cell lines growing under different conditions (Bredel-Geissler *et al*., 1992; Walenta *et al*., 1989). Two concomitant phenomena could be responsible for the decrease in oxygen uptake as a function of the growth period, i.e., a decrease in ATP turnover and/or a decrease in the respiratory capacity of these cells.

The aim of this paper was to analyze the cause of the decrease in the respiratory activity of C6 glioma cells as a function of growth status. Firstly, the evolution of C6 cell oxygen uptake was shown to correlate with that of the specific activity of complex I. Secondly, the evolution of complex I specific activity occurred without any change in the complex I protein content. Finally, we present evidence of a causal link between the phosphorylation level of the 18-kDa subunit and the specific activity of complex I, on one hand, and the respiratory activity of C6 cells, on the other. These data show that the phosphorylation of complex I is part of a regulatory pathway that controls the respiration of proliferating C6 glioma cells.

MATERIALS AND METHODS

Cell Culture and Counting

C6 cells were initially plated at a density of 2.5×10^4 cells/cm^2 in Falcon dishes (10 cm diameter), in 10 mL Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (100 U/mL), streptomycin (0.1 mg/mL), fungizone (0.25 mg/mL), sodium bicarbonate (2 mg/mL), and foetal calf serum (FCS) (5%). Cultures were performed for 5 days in a humidified 5% CO_2/95% air atmosphere at 37°C. Before cells reached confluence, they were seeded on gelatin microcarrier beads (Cultispher-GL, Percell Biolytica AB, Sweden) (1.25×10^6 cells per 300 mg wet beads and 10 mL of the same culture medium). Culture was maintained for various times before experiments and the culture medium was replaced every day.

For cell counting, an aliquot of bead suspension was collected at the end of experiments, centrifuged, and rinsed with phosphate-buffered saline (PBS) medium. Cells were dissociated from the substratum during 10–15 min of bead digestion with 0.25% trypsin in EDTA-supplemented PBS medium, containing phosphatase inhibitors [5 mM NaF, 1 mM sodium orthovanadate, and 0.5 μM okadaic acid]. Then, enzymatic action was stopped by adding 20% FCS and cell detachment was mechanically achieved with a small-bore Pasteur pipette. Blue trypan labelling was used to count cell suspensions.

Respiration Assays

The oxygen consumption of C6 cells was measured polarographically at 37°C using a Clark-type large diameter Orbisphere oxygen electrode in a 2-mL thermostatically controlled chamber (Oroboros Oxygraph, Paar, Graz, Austria). The respiration medium was the culture medium containing the beads and maintained in a 5% CO_2/95% O_2 atmosphere. Data were recorded at sampling intervals of 1 s (DatLab Acquisition software, Oroboros, Innsbruck, Austria). First, the endogenous respiratory rate (J_O endogenous) was determined from the slope of a plot of

O_2 concentration versus time. Then, the maximal oxygen uptake ($J_{O\ max}$) that can be sustained by cells was determined from a titration curve of the protonophoric uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone (ClCCP), from 0.1 to 2 μM. Both oxygen consumption rates were expressed as per nanomoles of atoms of oxygen consumed per min per million cells (natom O/min/10^6 cells).

To permeabilize cells, freshly prepared cells (5×10^6 cells per mL) were suspended in the oxygraph chamber at 37°C in the following respiration medium: 75 mM sucrose, 50 mM KCl, 5 mM potassium phosphate, 3 mM $MgCl_2$, 0.5 mM EDTA, and 30 mM Tris-HCl (pH 7.4) (Scacco *et al*., 2000). After 1 min incubation, 100 μg of digitonin were added. Integrity of the outer and inner mitochondrial membrane during permeabilization was checked with cytochrome c and NADH, respectively. Five minutes after digitonin addition, oxygen consumption measurements were initiated by the addition of either 10 mM glutamate + malate or 10 mM succinate-Tris, 1 mM ADP-Tris, 12.5 μg/mL oligomycin, and finally 0.75 μM ClCCP.

Enzymatic Assays

Pellets of either freshly prepared or frozen cells (3×10^6) were suspended in 250 μL of 33 mM potassium phosphate (pH 7.2) and were exposed to ultrasound energy (110 W) for 15 s at 0°C. When needed, Tween 20 [1% (vol/vol) final concentration] was added to the suspension prior to measuring citrate synthase and complex IV activity.

Citrate synthase was measured according to the procedure of Martin *et al*. (1998), one unit of citrate synthase (E.C. 2.3.3.1 formerly E.C. 4.1.3.7) being considered equal to the reduction of 1 μmole of 5-5′-dithiobis-2-nitrobenzoic acid per min. Activity of complex III (decylubiquinol cytochrome *c* oxidoreductase, E.C. 1.10.2.2) and complex IV (cytochrome *c* oxidase, E.C. 1.9.3.1) was measured spectrophotometrically according to Rustin *et al*. (1994). One unit of complex III was taken to be equal to the reduction of 1 μmole of ferrocytochrome *c* per min and 1 unit of complex IV was considered equal to the oxidation of 1 μmole of ferricytochrome *c* per minute. Complex I activity (NADH:decylubiquinone oxidoreductase; E.C. 1.6.5.3) was measured spectrophotometrically at 37°C according to Rustin *et al*. (1994). Activity was corrected for rotenone-insensitive NADH consumption. One unit of complex I was taken to be equal to the oxidation of 1 μmole of NADH per minute.

cAMP Assay

C6 cells were precipitated by adding perchloric acid [6.5% (v/v) and 5 mM EDTA, final concentration] to the bead suspensions. The protein fraction was eliminated by centrifugation at 9000 × g for 4 min at 4°C. The supernatant was neutralized with 2 N KOH/0.3 M Mops and the precipitate was eliminated by centrifugation. The cAMP content was measured by using the [^{3}H]-cAMP binding protein assay system (Amersham, code TRK432) according to the supplier's instructions.

Mitoplast Isolation and Complex I Immunoprecipitation

Mitoplasts were prepared from C6 cells according to the procedure described by Klement *et al*. (1995) with minor modifications. Briefly, C6 cells were incubated for 10 min at 37°C in PBS solution (5×10^6 cells per mL) containing phosphatase inhibitors (5 mM NaF, 1 mM sodium orthovanadate, and 0.5 μM okadaic acid), an antiprotease mixture (complete EDTA-free; Boehringer Mannheim), and 40 μg of digitonin per 10^6 cells. Since optimal digitonin concentration is known to vary as a function of cell type, this concentration was set to allow a maximal release of the cytoplasmic lactate dehydrogenase with a minimal release of citrate synthase from the mitochondrial matrix. The suspension was then centrifuged at 2000 *g* for 10 min and the supernatant was centrifuged at 12,000 g for 10 min at 4°C. The pellet containing mitoplasts was suspended in the immunoprecipitation buffer [1% lauryl maltoside, 150 mM NaCl, and 50 mM Tris-HCl (pH 7.4)] supplemented with the phosphatase and protease inhibitor cocktails. Complex I immunoprecipitation was carried out according to Scacco *et al*. (2000). Briefly, protein A-Sepharose (20 mg) was washed in the immunoprecipitation buffer and resuspended in 50 μL of the same solution. Rabbit antibody against the 75-kDa subunit of complex I (20 μg) (a gift from Pr. J. E. Walker, Medical Research Council, Cambridge, U.K.) was added. After 6 h of incubation at room temperature, the antibody-Sepharose complex was centrifuged and washed twice with the buffer to eliminate any unbound antibody. Then, mitoplasts (20–50 μg of proteins) were added to the antibody-Sepharose complex. After overnight incubation, the protein A-Sepharose immunoprecipitate was collected by centrifugation, washed twice with the buffer and dissolved in 20 μL of lysis buffer [5% SDS, 2% β-mercaptoethanol, 15% glycerol, 50 mM Tris-HCl (pH 6.8)].

Electrophoresis and Immunoblotting

Immunoprecipitated proteins or cell lysates were subjected to 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose (Hybond C Extra, Amersham). Membranes were incubated with either: (i) a mouse monoclonal antibody against the 19.6-kDa subunit of complex IV (dilution 1:2500); (ii) the 39-kDa subunit of complex I (dilution 1:1000) (Molecular Probe); (iii) a mouse antiphosphoserine monoclonal antibody (dilution 1:500) (Sigma); or (iv) a polyclonal antibody against the 24-kDa subunit of complex I (dilution 1 : 5000) (a gift from Pr. J. E. Walker, Cambridge, U.K.). After incubation with the primary antibody, blots were incubated with anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibody (dilution 1:10000) (Jackson) and were visualized using the ECL Plus western blotting detection system, according to the supplier's instructions (Amersham). Densitometric analysis was performed using ImageQuant software (Molecular Dynamics).

Statistical Analysis

The results are expressed as the mean ± SD. Statistical analysis was carried out using a nonparametric Mann–Whitney test (Statsdirect).

RESULTS

Respiratory Activity and Mitochondrial Enzyme Equipment of C6 Glioma Cells Grown on 3D Support

C6 glioma cells were grown on microcarrier beads to minimize morphological and functional disturbances that could occur when separating cells from their support. On this substratum, cells are able to enter and proliferate in the beads so that growth may be tridimensional. Figure 1 shows a typical growth curve of C6 cells cultured in the presence of glutamine and 5% SVF. After a 24-h lag, C6 cells entered their growth phase and reached a plateau at about the fifth day after seeding. This growth arrest was rather due to confluence in the microcarrier beads than to substrate depletion, since the culture medium was replaced every 2 days (day 1 and 3) and then every day (from day 4 to 7). To compare the different cultures, the measured metabolic and enzymatic activities were expressed as a function of the density of cells instead of culture time.

Figure 2 shows the evolution of oxygen consumption rate of C6 cells during cell growth. It is worth noting

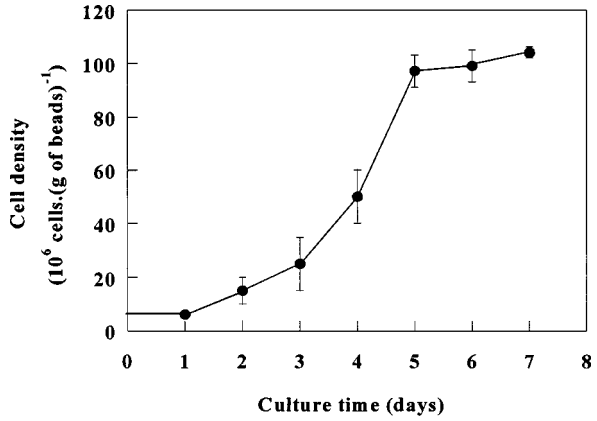

Fig. 1. Growth curve of C6 cells cultured in microcarrier beads. Cells were seeded at 5×10^6 cells for 300 mg beads and 10-mL DMEM medium supplemented with 5% FCS and containing 4 mM glutamine, as described by Martin *et al*. (1998).

that the oxygen uptake measured under all conditions was totally sensitive to the classical mitochondrial inhibitors (e.g. myxothiazol, cyanide), so the major contribution of oxygen consumption originated from the mitochondrial respiratory chain (data not shown). For cell density values below 40×10^6 cells /g of beads (from day 1 to 4), the respiratory activity of growing cells decreased from about 8 to 4 nat. O/min/10^6 cells. This decrease was not due to a substrate depletion in the culture medium, since the latter was replaced as mentioned above. For cell densities higher than 40×10^6 cells/g of beads (for culture times longer than 4 days), the endogenous respiratory activity remained quite constant at a low value of about 3.5 nat. O/min/10^6 cells.

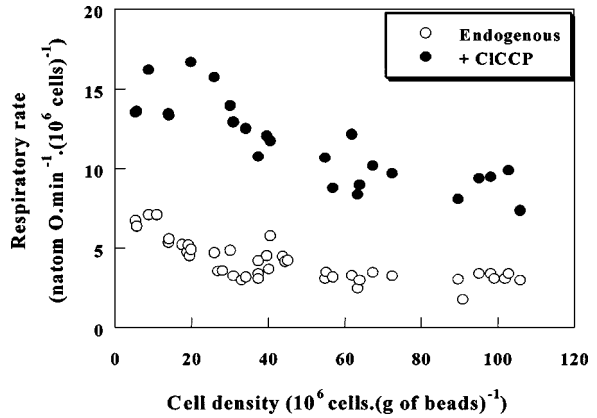

Fig. 2. Evolution of oxygen consumption rate of C6 cells as a function of cell density. (○), Endogenous respiratory rate; (●), Respiratory capacity. C6 cells were grown in microcarrier beads and the oxygen uptake was measured as described in Materials and Methods and reported as a function of the number of cells per gram of beads (g of beads).

To investigate the respiratory capacity of C6 cells, oxygen uptake was measured in the presence of a protonophoric uncoupler, ClCCP, to dissipate the proton electrochemical difference across the inner mitochondrial membrane, and thereby to stimulate the respiratory chain activity to its maximal value (Martin *et al*., 1998). Like the endogenous respiration, the respiratory capacity declined during the early stages of culture (cell density below 50×10^6 cells/g of beads or culture from day 1 to day 4), and remained at a constant value during the remaining culture time (Fig. 2). Taken together, these data are consistent with what has previously been observed for the same type of cells growing on the same substratum (Martin *et al*., 1998).

To analyze whether the respiratory capacity was somehow related to changes in the mitochondrial enzyme properties, the specific activity of different mitochondrial enzymes was measured. Figure 3 shows that the activity of a soluble matrix enzyme, citrate synthase, which is usually measured to probe changes in the mitochondrial mass (James *et al*., 1996), did not significantly vary during growth. No significant change in the specific activity of complexes III and IV was observed. In contrast, Fig. 3 shows that the specific activity of complex I decreased continuously as the cell density increased from about 5 to 40×10^6 cells/g of beads. Above this density, the specific activity remained constant. The data in Figs. 2 and 3 show that the lower the complex I activity, the lower the endogenous respiration and the respiratory capacity. Moreover, the respiratory activity of C6 cells was shown to be linearly correlated with the specific activity of complex I (Fig. 4).

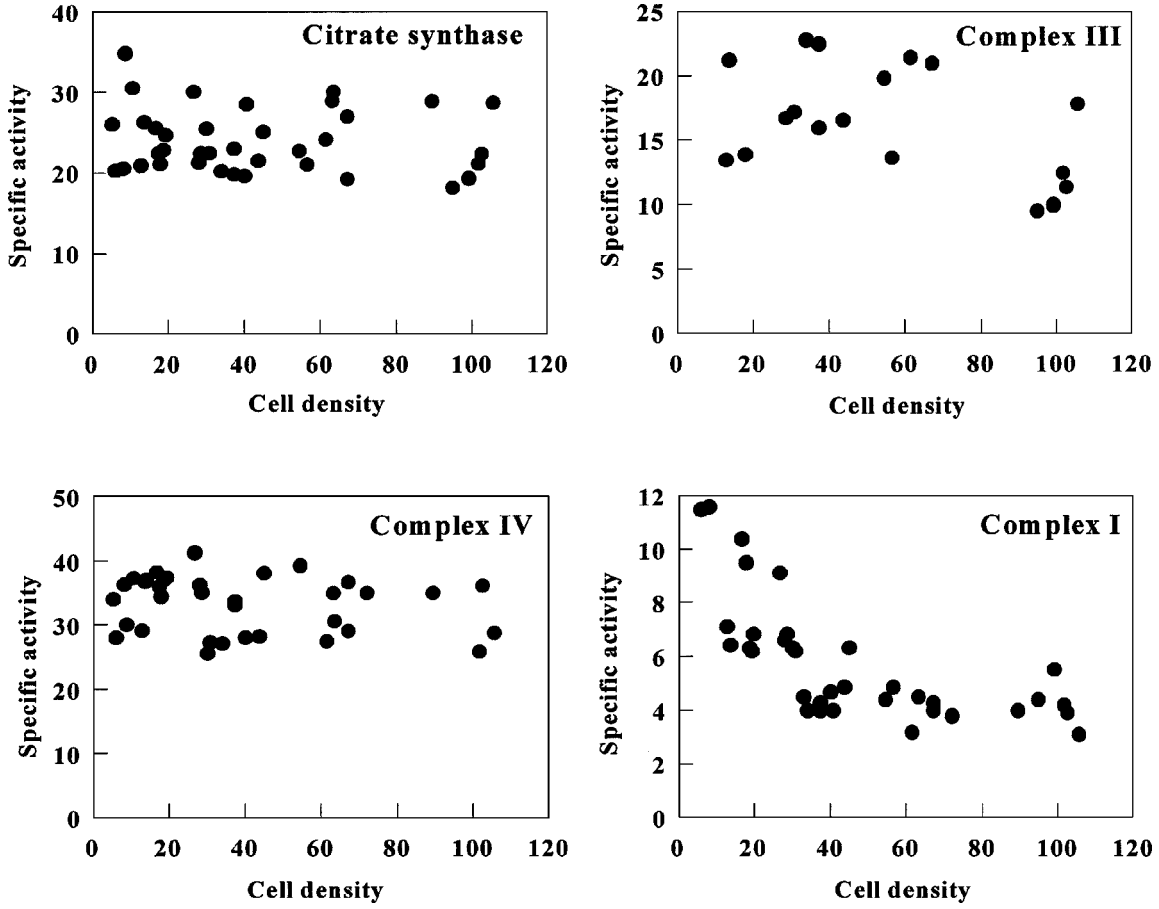

Fig. 3. Evolution of citrate synthase and respiratory complex activity as a function of cell density. One Unit (U) is defined as the amount of enzyme catalyzing the conversion of 1 μmol of substrate or product per minute. Specific activities are expressed as Unit per 10^6 cells per g of beads and cell density as 10^6 cells per g of beads. Each experimental point is the average of three enzymatic measurements carried out on cell homogenates (see Materials and Methods) provided from 20 independent cultures and on different days in vitro. All enzymatic assays were performed with the same cells tested for their respiratory activity.

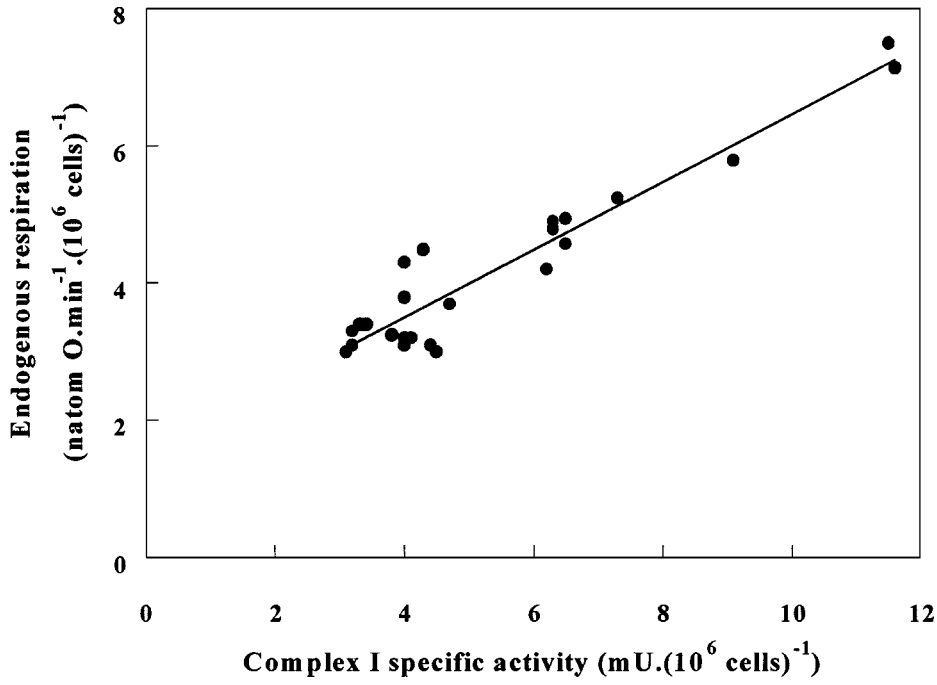

Fig. 4. Relationship between endogenous respiration of C6 cells and specific activity of complex I. Data are compiled from Fig. 2 and 3. Linear regression shows a correlation with an R^2 value equal to 0.91.

To check whether changes in the specific activity of complex I were related with changes in complex I protein content, western blots of the 24-kDa subunit of complex I (subunit 4) and the 19.6-kDa subunit of complex IV (subunit 4) were performed on cell lysates. Figure 5 shows that the content of the 19.6-kDa subunit did not vary significantly as a function of the culture time. This is in agreement with the constant cytochrome *c* oxidase activity presented in Fig. 3. Surprisingly, the content of the 24-kDa subunit of complex I did not significantly vary with respect to the culture time, thus making the ratio between the 19.6- and the 24-kDa blot nearly constant throughout the growth period (from day 1 to 6) (Fig. 5 and legend therein).

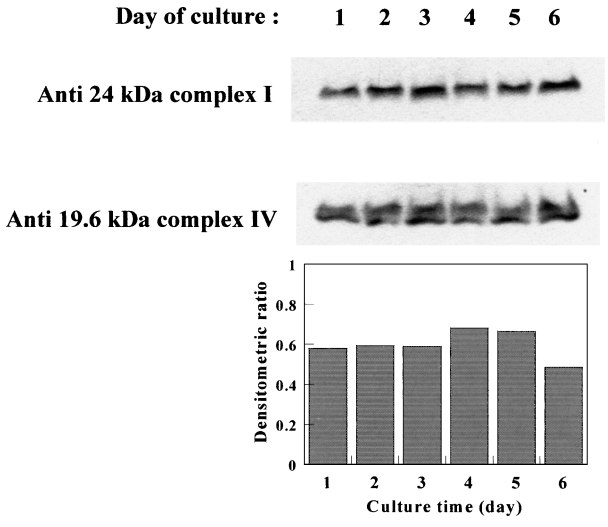

Fig. 5. Immunodetection of the 24-kDa subunit of complex I and of the 19.6-kDa subunit of complex IV as a function of day in culture. The 24-kDa subunit (i.e. subunit 4) of complex I and 19.6-kDa subunit (i.e. subunit 4) of complex IV were detected by immunoblotting with specific anti-24-kDa subunit (*Anti 24 kDa*) and anti-19.6-kDa subunit (*Anti 19.6 kDa*) antibodies on SDS-polyacrylamide gel electrophoresis of solubilized proteins of C6 cells (1×10^6 cells per well) from the first to the sixth day of culture (*lanes 1–6*). Molecular weights were assigned from the migration of prestained standards (not shown). The lower panel represents the densitometric ratio between Anti 24-kDa and Anti 19.6-kDa blots as a function of day in culture.

Phosphorylation of the 18-kDa Subunit of Complex I During Growth

It is well documented that the mature 18-kDa AQDQ subunit of the mammalian complex I can be phosphorylated on a single serine residue (Papa *et al*., 2002 for review). Therefore, under our conditions, changes in the specific activity of complex I could be somehow related to changes in the phosphorylation level of the 18-kDa subunit. Thus, the phosphorylation state of complex I of

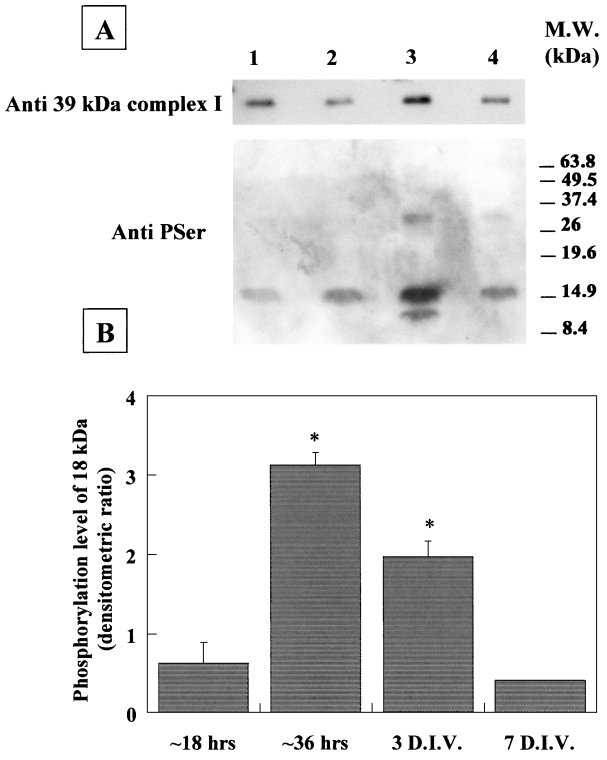

Fig. 6. Phosphorylation of the 18-kDa subunit of complex I as a function of day in culture. (A) Typical immunodetection of phosphoserine and 39-kDa subunit of immunoprecipitated complex I. Mitoplasts were isolated from C6 cells using the digitonin permeabilization procedure and complex I was subsequently immunoprecipitated using the polyclonal antibody against 75 kDa, as described in Materials and Methods. Phosphoserine-containing proteins and the 39-kDa subunit of complex I were detected by immunoblotting with an antiphosphoserine (*Anti Pser*) and the monoclonal anti-39-kDa (*Anti 39 kDa*) antibodies on SDS-polyacrylamide gel electrophoresis of immunoprecipitated proteins. Molecular weights were assigned from the migration of prestained standards. *Lane 1*: 18 h of cell culture; *lane 2*: 36 h of cell culture; *lane 3*: 3 days of culture; *lane 4*: 7 days of culture. (B) Phosphorylation level of 18-kDa subunit of complex I as a function of day in culture. Phosphorylation level (in arbitrary subunit) of the 18-kDa subunit was calculated from the densitometric quantitation of the phosphoserine blot relative to that of the 39-kDa blot; 18 h: about 18 h of cell culture (below 5×10^6 cells/g of beads); 36 h: about 36 h of culture ($5–10 \times 10^6$ cells/g of beads); *3 D.I.V.*: 3 days in vitro culture ($15–30 \times 10^6$ cells/g of beads) and *7 D.I.V.*: 7 days in vitro culture (100×10^6 cells/g of beads). Data are expressed as means $\pm$ SD of three to five independent experiments. * : P value ≤ 0.02 versus 18 h of cell culture.

C6 cells has been analyzed according to the procedure of Scacco *et al*. (2000). Figure 6(A) shows a typical immunodetection of the phosphoserine-containing proteins of immunoprecipitated complex I. To correct for loading variations, the 39-kDa subunit of this complex was also immunodetected. Figure 6(A) shows that a major protein band with an apparent molecular mass of about 16 kDa reacted strongly with the antiphosphoserine. This band, with an apparent Mr of 16–18 kDa depending on the electrophoretic procedure, has already been ascribed to the 18-kDa subunit of complex I (Papa *et al*., 1996; Sardanelli, *et al*., 1995; Technikova-Dobrova *et al*., 1994). When the protein loading was high, several minor bands were also revealed by the antiphosphoserine (Fig. 6(A), lane 3). In previous reports, these phosphoproteins ($\sim$40, $\sim$30, and $\sim$10 kDa) were detected on whole mitochondrial lysates, and two of them have already been ascribed as contaminating proteins that are loosely associated to the immunoprecipated complex I (Sardanelli *et al*., 1996; Technikova-Dobrova *et al*., 2001). Similar experiments were carried out throughout the culture period of C6 cells and the antiphosphoserine blot of the 18-kDa subunit was normalized by using the antisubunit 39-kDa blot. Figure 6(B) shows that the phosphorylation level of 18-kDa subunit varied with respect to the culture period. Indeed, whereas the phosphorylation level was low during the lag phase (i.e. about 18 h of culture), it became maximal in the early growth phase (i.e. about 36 h of culture) (Fig. 6(B)). Then, it decreased significantly during the transition from early- to mid-growth phase (i.e. 3 days in vitro) and was even lower in the plateau phase (i.e. 7 days in vitro) (Fig. 6(B)).

Short-Term Effect of Intracellular cAMP Increase on Cellular Respiration and Complex I Phosphorylation

It has already been shown on cultured myoblasts and fibroblasts that the cAMP-dependent phosphorylation of 18-kDa subunit activates the NADH ubiquinone oxidoreductase specific activity of complex I (Papa *et al*., 2001; Scacco *et al*., 2000; Technikova-Dobrova *et al*., 2001). The activity of cAMP-dependent protein kinases was increased in two different ways: (i) by adding 100 μM of the plasma-membrane permeant cAMP analogue, dibutyryl cAMP (Bt_2cAMP), which has previously been shown in C6 cells to raise the cAMP intracellular concentration to a value of about 100 pmol per 10^6 cells [table 1 in Nicolaï *et al*. (1996)]; and (ii) by adding 10 μM forskolin, an activator of the adenylate cyclase. Under both conditions, the phosphodiesterase activity was inhibited by 100 μM of 3-isobutyl-1-methyl xanthine (IBMX). The effects of Bt_2cAMP and forskolin treatment on cellular respiratory activity were analyzed as a function of time with C6 cells grown for 3 days. Figure 7 shows that the maximal effect of 100 μM Bt_2cAMP was reached after 1 h of incubation, whereas the stimulation was optimal after 20–30 min incubation with 10 μM forskolin. In contrast to the Bt_2cAMP stimulation, the activation was only transient

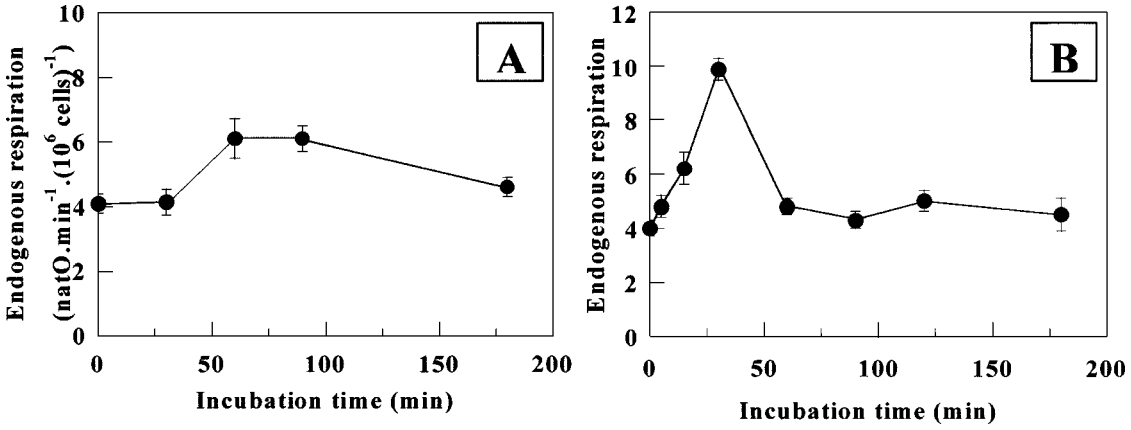

Fig. 7. Time course effect of dibutyryl cAMP and forskolin treatment on the endogenous oxygen uptake of C6 glioma cells. C6 glioma cells grown in microcarrier beads for three days. The oxygen uptake was measured after different incubation times with either 100 μM Bt_2cAMP + 100 μM IBMX (A); or 10 μM forskolin + 100 μM IBMX (B).

in the presence of forskolin (Fig. 7), thus demonstrating a stronger negative feedback on the signaling pathway. This forskolin-induced stimulation is in agreement with the rise in intracellular cAMP concentration from 17 ± 1.7 to 400 ± 50 pmol per 10^6 viable cells after 15 min, and 320 ± 20 pmol per 10^6 viable cells after 30 min incubation.

Further experiments were carried out on C6 cells treated with 100 μM Bt_2cAMP for 1 h and subsequently permeabilized by digitonin. This procedure makes it possible to study in situ different steady states of oxidative phosphorylation of mitochondria: (i) ATP synthesis (+ADP); (ii) no ADP phosphorylation (+oligomycin); and (iii) uncoupled respiration (+ClCCP). Figure 8(A) shows that Bt_2cAMP treatment induced a significant increase in the oxygen consumption rate sustained by glutamate + malate, under conditions of either ATP synthesis (i.e. state 3) or uncoupling (Fig. 8(B)). In contrast, the succinate-driven respiration was not significantly changed by Bt_2cAMP treatment regardless of the steady state (Fig. 8(A)). Finally, the oxygen consumption rate measured in the presence of oligomycin (i.e. state 4) was unaffected by Bt_2cAMP regardless of the respiratory substrate used (Figs. 8(A) and and (B)). It should be noted that measurements of other mitochondrial activities showed that the stimulation of respiration took place without any significant change in the specific activity of cytochrome *c* oxidase (complex IV) and citrate synthase (data not shown). This suggests that neither an activation of complex IV nor an increase in the mitochondrial biogenesis was responsible for the cAMP-induced activation of respiration.

Therefore, the NADH ubiquinone oxidoreductase activity was subsequently measured after either Bt_2cAMP

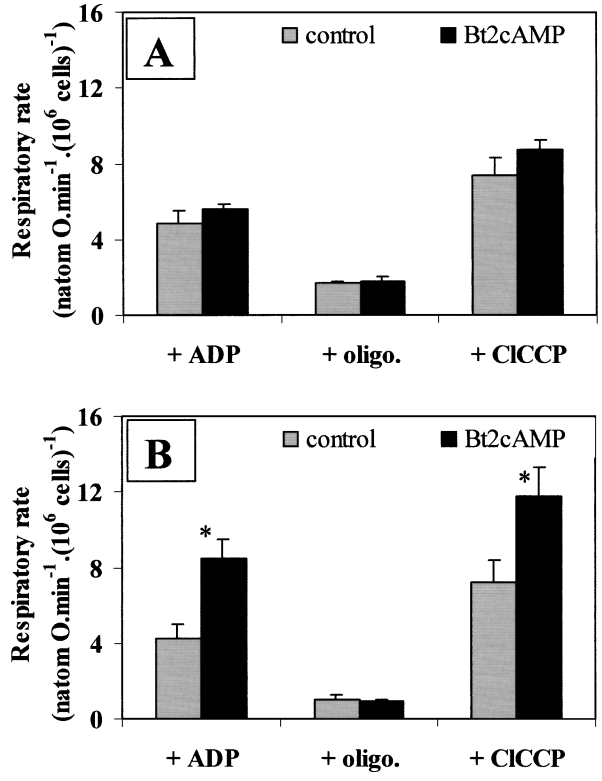

Fig. 8. Effect of dibutyryl cAMP treament on digitonin-permeabilized C6 glioma cells. C6 cells grown on microcarrier beads for 3 days were treated with 100 μM Bt_2cAMP + 100 μM IBMX for 1 h. Cells were harvested in the presence of phosphatase inhibitors and immediately permeabilized with digitonin as described in Materials and Methods. Oxygen uptake was measured either in the presence of succinate (A) or of glutamate + malate (B) as respiratory substrates. When added, ADP was 1 mM, oligomycin 12.5 μg/mL and ClCCP 0.75 μM. * : P value ≤ 0.025 versus untreated control cells.

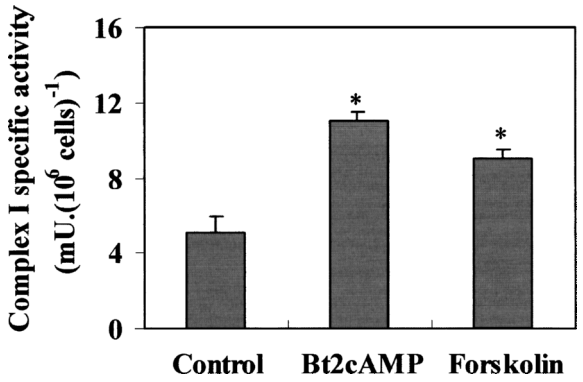

Fig. 9. Effect of dibutyryl cAMP and forskolin treatment on complex I activity of C6 cells. C6 cells grown on microcarrier beads for 3 days were treated either with 100 μM Bt_2cAMP + 100 μM IBMX for 1 h or with 10 μM forskolin + 100 μM IBMX for 30 min. Cells were harvested in the presence of phosphatase inhibitors and immediately sonicated. The rotenone-sensitive NADH ubiquinone oxidoreductase (complex I) activity was measured as described in Materials and Methods section. * : P value $\leq$ 0.01 versus untreated control cells.

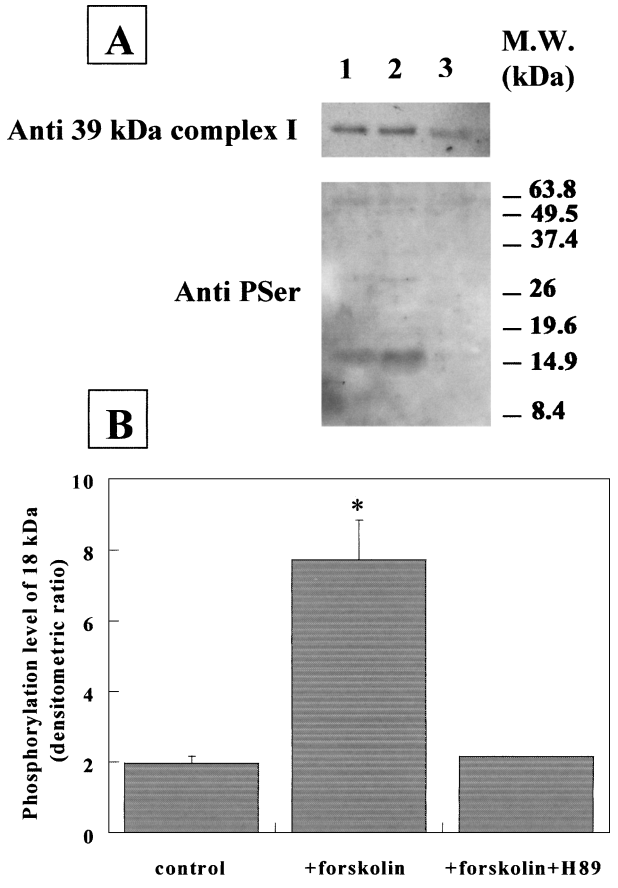

Fig. 10. Effect of forskolin treatment of the phosphorylation level of 18-kDa subunit of complex I. C6 cells were grown on microcarrier beads for 3 days and treated for 30 min with 10 μM forskolin + 100 μM IBMX, in the presence or not of 10 μM of the protein kinase A inhibitor, H89. Mitoplast preparation, complex I immunoprecipitation, immunoblotting, quantitation and statistical analysis were performed as described in Fig. 6. (A) Typical immunodetection of phosphoserine and 39-kDa subunit of immunoprecipitated complex I. *Lane 1*: control cells; *lane 2*: cells treated with forskolin + IBMX; *lane 3*: cells treated with forskolin + IBMX + H89. (B) Relative phosphorylation level of the 18-kDa subunit of complex I. * : P value $\leq$ 0.05 versus untreated control cells.

or forskolin treatment. Figure 9 shows that the treatment of C6 cells with Bt_2cAMP (for 1 h) and with forskolin (for 30 min) stimulated complex I activity by a factor 2.1 and 1.8, respectively. Immunodetection of the phosphoserine (Fig. 10(A)) showed that treatment of C6 cells by forskolin for 1 h was associated with a marked increase in the phosphorylation of the 18-kDa subunit of complex I (Fig. 10(A)). Quantitations showed that the relative phosphorylation level of this subunit was increased by a factor of about 4 (Fig. 10(B)). Moreover, the stimulation of this phosphorylation by forskolin completely vanished when the treatment was carried out in the presence of the protein kinase A inhibitor, H89 (Fig. (10)).

DISCUSSION

Long-Term Modulation of Cellular Respiration, Complex I Activity and Its Phosphorylation During the Growth of C6 Glioma Cells

Since Warburg's discovery in 1926, in vitro and in vivo studies have shown that tumor cells are characterized by a high number of deviations with regard to their differentiation status, proliferation capacity, and metabolic orientation (Pedersen, 1978 for review; Warburg *et al*., 1926). Indeed, elevated lactate production and low mitochondrial content are the major features of fast-growing neoplastic cells. While ATP production in these rapidly dividing cells was assumed to be more dependent on glycolysis than on oxidative phosphorylation (Pedersen, 1978 for review), it has been shown for slow-growing hepatoma (Kovacevic and Morris, 1972) and glioma cells (Martin *et al*., 1998) that mitochondrial oxidative phosphorylation is the major ATP-producing pathway. For the latter cells, our previous study showed that the glycolytic and respiratory activities are subjected to modulation throughout a short subculture period, i.e., during the early growth phase. This decrease in metabolic activity, reflecting a global decrease in the ATP production rate and in the respiratory capacity, has no consequence on the energy status of these cells because the ATP/ADP ratio remains almost constant throughout the entire culture period (Martin *et al*., 1998).

A similar evolution of oxygen uptake has already been described for other mammalian cell lines during the transition from growth to confluence phase. It was found that a decrease in the mean number of mitochondria per cell obviously accounted for the reduction in respiratory activity (Bredel-Geissler *et al*., 1992; Walenta *et al*., 1989). Nevertheless, the major question was therefore raised as to the origin of the decrease in the respiratory capacity of C6 glioma cells in the early stage of culture, before reaching confluence (Martin *et al*., 1998).

In this paper, we have extended the work of Martin and colleagues performed on C6 glioma cells that were able to proliferate and to differentiate on a three-D substratum. Indeed, the respiratory rate of C6 cells grown on microcarrier beads was shown to markedly decrease during the early growth phase, i.e., between day 1 and day 3 of culture, corresponding to a cell density comprised between 10 and 40×10^6 cells per g of beads (Fig. 2). This result is in agreement with our previous published data on the same cells (Martin *et al*., 1998) and with those obtained with other cultured cell lines (Bredel-Geissler *et al*., 1992; Walenta *et al*., 1989). One interesting feature is that the respiratory capacity of C6 cells, as assessed by means of a protonophoric uncoupler, underwent a similar decrease during the transition from early growth to late growth phase, before confluence was reached (Fig. 2 and see also Martin *et al*., 1998). Meanwhile, the activity of citrate synthase, a matrix enzyme usually used as a mitochondrial mass marker, and that of the respiratory complexes (e.g. complexes III and IV) remained nearly constant throughout the entire growth period (Fig. 3). This demonstrates that a change in the mean number of mitochondria per cell or in the mitochondrial enzyme content was not responsible for this decrease in respiratory capacity. Nevertheless, one of the major observations of the present report is that the rotenone-sensitive NADH ubiquinone reductase (i.e. complex I) activity of C6 cells declined in parallel to the cellular respiration, in such a way that a positive correlation could be drawn between these two parameters (Fig. 4). This relationship illustrates the key role played by complex I activity in the control of oxidative phosphorylation of C6 glioma cells during growth. This is in agreement with the quantitative correlations between respiration and the level of complex I impairment, that have previously been established out using metabolic control analysis (Davey *et al*., 1998). Thus, in PC12 neuronal cells, a 40% decrease in complex I was accompanied by a 20% respiration decrease. However, the threshold effect of complex I impairment on cell respiration may depend on the cell line. Indeed, in human osteosarcoma cells, respiratory activity begins to decrease by 5–10% only when complex I is inhibited by 35–40% (Barrientos and Moraes, 1999). Under our conditions, it is worth noting that the variation in complex I and respiratory activity of C6 cells occurred during the growth phase, well before confluence was reached. This is in agreement with the fact that cell proliferation is relatively insensitive to complex I impairment, probably because of the compensatory effect of glycolysis on cellular energetics. For instance, partial complex I deficiencies caused by either inherited human mutations (James *et al*., 1996; Papa *et al*., 2001; Petruzzella and Papa, 2002) or in vitro genetic experiments (Barrientos and Moraes, 1999) were shown to have little impact on growth. Moreover, complex I inhibition by rotenone has been shown to affect growth to a larger extent under conditions where cells derive much of their ATP from oxidative phosphorylation (i.e. galactose versus glucose as substrate) (Barrientos and Moraes, 1999). Concerning the C6 glioma cells, our previous work showed that, under our culture conditions (i.e. in the presence of glucose, pyruvate, and glutamine), the energy metabolism of C6 cells switched from aerobic glycolysis in the early growth phase to fermentative glycolysis in the late growth and confluence phases. Nevertheless, the contribution of oxidative phosphorylation to overall ATP synthesis remained high regardless of the culture period (figure 5 in Martin *et al*., 1998).

Concerning the origin of the decrease in complex I activity during the transition from early- to late-growth phase, our immunoblot analysis showed that these changes were not associated with variations in the complex I protein level (Fig. 5) but rather to a change in the phosphorylation level of the 18-kDa subunit of this complex (Fig. 6). In sum, our data point to the regulation of the respiratory capacity of C6 cells by phosphorylation of complex I during the early stage of cell proliferation.

Short-Term Modulation of Complex I Activity and Cellular Respiration by the cAMP-Induced 18-kDa Subunit Phosphorylation

The 18-kDa AQDQ subunit is one of the 35 nuclear-encoded subunits of the mammalian complex I, whose carboxy-terminal region contains a cAMP-dependent protein kinase phosphorylation site (for review see Papa *et al*., 2002). In vitro studies performed on isolated bovine heart mitochondria have demonstrated the existence of an extension of the cAMP/protein kinase A signaling pathway in the mitochondrial compartment. Indeed, the phosphorylation of the 18-kDa subunit is under the control of: (i) the catalytic and regulatory subunits of the cAMP-dependent protein kinase located in the inner membrane and in the mitochondrial matrix (Technikova-Dobrova *et al*., 2001); and (ii) the Mg^{2+}-dependent and Ca^{++}-inhibitable PP2C-type

phosphatase, localized in the mitochondrial matrix (Signorille *et al*., 2002). Activation of the cAMP signaling pathway of serum-starved cultured myocytes and fibroblasts, either by up-regulating the adenylate cyclase (e.g. forskolin or cholera toxin treatment) or the protein kinase A (e.g. treatment with a cell permeant cAMP analogue), promoted the phosphorylation of the 18-kDa subunit and enhanced complex I activity (Papa *et al*., 2001; Scacco *et al*., 2000; Technikova-Dobrova *et al*., 2001).

The short-term effect of up-regulation of the cAMP/protein kinase A pathway in C6 glioma cells was then investigated. Direct exposure of C6 cells to dibutyryl cAMP for 1 h or forskolin for 30 min resulted in the stimulation of oxygen uptake. Further experiments carried out on digitonin-permeabilized cells demonstrated that the stimulation of C6 cell respiration was linked to an increase in NADH-linked respiration, rather than to succinate-driven respiration, and to a subsequent enhancement of the rotenone-sensitive NADH ubiquinone activity. Finally, immunodetection strongly suggested that the activation of complex I is at least a consequence of a protein kinase A-dependent phosphorylation of the 18-kDa AQDQ subunit of this respiratory complex.

In permeabilized cells allowing the in situ examination of mitochondria, activation of complex I due to protein kinase A overactivation enhanced the respiration only in the presence of ADP (state 3 of ATP synthesis) or of ClCCP (uncoupled state), and not in the presence of oligomycin. These results suggest that complex I is an important rate-controlling step in C6 mitochondria when the respiratory flux is elevated. This is in agreement with the high flux control coefficient of complex I, which has been measured on isolated brain mitochondria during ATP synthesis (Davey *et al*., 1998; Rossignol *et al*., 1999).

This study demonstrates for the first time in a glial cell line that: (i) 18-kDa subunit phosphorylation is at least under the control of the cAMP signaling pathway; (ii) phosphorylation of the 18-kDa subunit enhances the complex I activity; and (iii) activation of complex I increases the respiratory activity of C6 cells. However, the physiological importance of the cAMP cascade, on one hand, and of the covalent modification of complex I, on the other, in the regulation of the respiration of C6 cells during growth remains questionable. During growth, we were unable to evidence a direct link between the cAMP level and the phosphorylation level of the 18-kDa AQDQ subunit. cAMP content analysis during the entire culture period showed that the concentration of this second messenger did not significantly vary (i.e. about 17 pmol $\times$ $(10^6$ cells$)^{-1}$ see also Pianet *et al*., 1989), whereas the covalent modification of complex I underwent a biphasic phenomenon (see Fig. 6(B)). The marked increase in phosphorylation at the beginning of growth (during the first day of culture) followed by a decrease (between day 1 and 3) suggests the contribution of signals other than cAMP. Nevertheless, the steady state level of complex I phosphorylation may depend on the mitochondrial phosphatase activity, which is controlled by other signal transduction pathways, particularly that of Ca^{++} (Signorille *et al*., 2002). The lack of cAMP change during growth did not appear abnormal. In fact, a prolonged rise in intracellular cAMP (from 24 to 72 h) is known to inactivate the MAP kinase pathway, to stop proliferation and also to induce phenotypic changes in various cell lines (Stork and Schmitt, 2002 for review), including C6 glioma (Chen *et al*., 1998; Wang *et al*., 2001).

CONCLUSION

The significant role of complex I in the maintenance of energy homeostasis is beginning to be better understood. This is particularly true for nerve cells, where in contrast to other respiratory chain complexes, complex I has been found to vary considerably from one cell type to another (see e.g. Stewart *et al*., 1998). In addition, the reduction in respiratory chain activity due to complex I defects is one of the characteristics of neurodegenerative diseases such as Leber's hereditary optic neuropathy, focal dystonia, and Parkinson's disease (Barrientos and Moraes, 1999 and references therein). In the light of the large physiological variations in ATP turnover observed during proliferation, differentiation and/or oncogenesis of cultured cells (Cuezva *et al*., 1997 and references therein; see for review Pedersen, 1978), this study provides a comprehensive analysis of the reduction of respiratory activity in rat C6 glioma cells during the early growth phase. We propose that changes in the phosphorylation level of complex I are a major determinant of the long-term modulation of respiratory capacity in growing C6 cells. A major issue is now to investigate the signals and mechanisms allowing such large changes in complex I activity and in oxidative phosphorylation capacity.

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