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Research Article

Identification of tyrosine-phosphorylated proteins of the mitochondrial oxidative phosphorylation machinery

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Abstract. The role of some serine/threonine kinases in the regulation of mitochondrial physiology is now well established, but little is known about mitochondrial tyrosine kinases. We showed that tyrosine phosphorylation of rat brain mitochondrial proteins was increased by *in vitro* addition of ATP and H_2O_2, and also during *in situ* ATP production at state 3, and maximal reactive oxygen species production. The Src kinase inhibitor PP2 decreased tyrosine phosphorylation and respiratory rates at state 3. We found that the 39-kDa subunit of complex I was tyrosine phosphorylated, and we identified putative tyrosine-phosphorylated subunits for the other complexes. We also have strong evidence that the FoF1-ATP synthase α chain is probably tyrosine-phosphorylated, but demonstrated that the β chain is not. The tyrosine phosphatase PTP 1B was found in brain but not in muscle, heart or liver mitochondria. Our results suggest that tyrosine kinases and phosphatases are involved in the regulation of oxidative phosphorylation.

Key words. Mitochondria; oxidative phosphorylation; tyrosine phosphorylation; Src kinase; PTP 1B; oxidant.

Mitochondria are responsible for energy production and are also the main cellular site for reactive oxygen species (ROS) generation [1, 2]. However, the regulation of oxidative phosphorylation (OXPHOS) is still unclear, and the role played by ROS and protein phosphorylation remains elusive. For example, reversible protein phosphorylation has been investigated in the mitochondria [3–7], and results showed the existence of a cAMP-dependent protein kinase (PKA) in the inner mitochondrial membrane and matrix fraction. Of particular interest are data showing PKA-dependent phosphorylation of the 18-kDa (AQDQ) subunit of complex I [6] and of subunit I of cytochrome c oxidase [7] resulting in increased and decreased activity of the respective enzyme complexes. Recent studies have identified several new phosphoproteins involved in mitochondrial processes in plants [8], and specifically on a threonine residue for the NDUFA10 subunit of complex I in bovine heart mitochondria [9], as well as on serine residues in the ESSS and MWFE subunits of this complex [10].

Besides cAMP-dependent kinase activity, a mitochondrial tyrosine kinase activity has been described in transformed cells [11, 12]. Tyrosine-phosphorylated proteins have been found in mitochondria from human placenta [5], and also in brain mitochondria, resulting from the activity of tyrosine kinases of the Src family [13]. These tyrosine kinases have been shown to up-regulate cytochrome c oxidase

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activity in osteoclasts [14]. Another well-studied tyrosine-phosphorylated protein is the δ subunit of the FoF1-ATP synthase, phosphorylated in response to platelet-derived growth factor stimulation [15]. As kinase activity is generally reversed by phosphatases, the existence of such enzymes has also been investigated in mitochondria. The phosphatase PP2C was first characterized in the mitochondrial inner membrane and matrix fraction [16], and the presence of the tyrosine phosphatase SHP 2 in brain mitochondria has recently been reported [17]. These enzymes contain a cysteine residue in their active site, whose oxidation by H_2O_2 results in their inhibition, leading to increased phosphorylation [18–20]. Thus our hypothesis is that free radical production in the mitochondrial compartment could directly affect the tyrosine phosphorylation of mitochondrial proteins, and thereby regulate OXPHOS. Given the recent interest in mitochondrial tyrosine phosphorylation and the great amount of evidence indicating that oxidative stress can play a role in pathology, the aim of this report was to study phosphorylation mediated by tyrosine kinases and the influence of H_2O_2, using rat brain mitochondria as a model system. Phosphorylation was studied in mitochondria under different energy states, first using an *in vitro* approach with direct exposure of mitochondria to ATP and H_2O_2, but also during their *in situ* mitochondrial production. We also investigated the effect of inhibiting phosphorylation using PP2, a specific inhibitor of the Src tyrosine kinase family, and found a decrease in tyrosine phosphorylation accompanied by a decrease in respiratory rates at state 3. We detected the prototypic tyrosine phosphatase PTP 1B in brain mitochondria, but not in muscle, heart or liver mitochondria. Then, we studied each respiratory chain complex as well as FoF1-ATP synthase by blue native polyacrylamide gel electrophoresis (BN-PAGE), second-dimension sodium dodecyl sulfate (SDS)-PAGE, western blotting and liquid chromatography-tandem mass spectroscopy (LC-MS/MS). We found that the 39-kDa subunit of complex I was tyrosine-phosphorylated, in contrast to the FoF1-ATP synthase β chain, while we have strong evidence that the α chain and some subunits of the other respiratory complexes were probably tyrosine-phosphorylated. This work constitutes a first attempt to reveal the involvement of tyrosine phosphorylation in mitochondrial OXPHOS.

Materials and methods

Chemicals

The Micro-BCA Assay Protein quantitation kit was from Pierce (Rockford, Ill.). PP2, n-dodecyl-β-D-maltoside and the monoclonal antibody to porin were from Calbiochem (La Jolla, Calif.). Calyculin A was from Biomol (Plymouth Meeting, Pa). The mouse monoclonal antibody to phosphotyrosine (PY20) was from Transduction Laboratories (Lexington, Ky.) and the agarose-conjugated form was from Santa Cruz Biotechnology (Santa Cruz, Calif.). The antiphosphoserine antibody was from Zymed Laboratories (San Francisco, Calif.). The anti-MnSOD antibody came from Stressgen Biotechnology Inc. (San Diego, Calif.). The monoclonal antibody to PTP 1B, and the rabbit polyclonal antibody to GRP 78 were from Oncogene (Calbiochem-Novabiochem, San Diego, Calif.) and Santa Cruz Biotechnology, respectively. The polyclonal rabbit antibodies to the α, β and γ chains of FoF1-ATP synthase were a generous gift from Dr. J. Velours (Institut de Biochimie et Génétique Cellulaires du CNRS, Bordeaux, France). The horseradish peroxidase-conjugated seondary antibody [$F(ab')_2$ fragments of antimouse and antirabbit, $F(ab')_2$ fragment specific], were from Jackson Immuno-Research (West Grove, Pa.). All other chemicals were from Sigma (St Louis, Mo.).

Preparation of brain mitochondria and submitochondrial fractions

Male Wistar rats were sacrificed by cervical shock and decapitation. Brain mitochondria were isolated from whole brain according to the method described by Clark and Nicklas [21] with a final Ficoll density gradient step to eliminate synaptosomes. Cytosolic contaminations, as detected by lactate dehydrogenase (LDH) activity never exceeded 0.5% relative to total activity in homogenates. Microsomal contamination was assayed by NADPH-cytochrome c reductase activity [13] and was 0.26 ± 0.05 nmol/min per milligram in mitochondria compared to 16.16 ± 1.24 nmol/min per milligram (n = 3) in homogenate. Rat muscle and heart mitochondria were isolated as detailed by Morgan-Hughes et al. [22], and liver mitochondria by Jumelle-Laclau et al. [23].

Submitochondrial fractionation of brain mitochondria was performed as already described [24], with mitoplast sonication (three times at 10 W for 10 s) performed with a Vibracell 72446 sonicator (Biobloc, Strasbourg, France). Western blotting with antibodies to GRP 78 (a protein marker of the endoplasmic reticulum), to MnSOD (a matrix protein), and to subunit Va of complex IV (an inner membrane protein) was performed to check for contamination and to verify the purity of the different fractions, in addition to enzymatic assay for complex IV activity [25].

In vitro studies of tyrosine phosphorylation of mitochondria and protein solubilization

Mitochondria (0.5–1 mg in 500 µl) were preincubated at 30 °C for 3 min in the kinase buffer as described in Struglics et al. [4] containing 10 mM $MnCl_2$ to stimulate tyrosine kinase activity [11], 20 µM oligomycin and 30 µg/ml rotenone as in Papa et al. [6] to inhibit mitochondrial energy metabolism and when indicated, 100 µM H_2O_2 or 2 mM orthovanadate. Then, 200 µM ATP was added (except in the control samples) and incubation was

allowed to continue for 10 min. Samples were immediately frozen in liquid nitrogen and conserved at –80 °C. Samples were thawed, centrifuged at 4 °C for 30 min at 14,000 g, and the pellet was resuspended in phosphate saline buffer containing n-dodecyl-β-D-maltoside (1.5 mg/mg protein) in the presence of 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µM PMSF, 2 mM orthovanadate, 10 mM NaF and 0.2 µM calyculin A. After 30 min at 4 °C, the sample was centrifuged at 4 °C for 30 min at 14,000 g and protein concentration in the supernatant was determined using the Micro-BCA assay.

Studies of protein phosphorylation during *in situ* ATP production

Measurements of oxygen uptake were performed with a Clark-type oxygen electrode in a respiratory buffer (75 mM mannitol, 25 mM sucrose, 100 mM KCl, 10 mM Tris-phosphate, 10 mM Tris-HCl, pH 7.4, 50 µM EDTA). Mitochondria (500 µg/ml) were incubated for 5 min at 30 °C in the presence of 10 µM PP2, a Src kinase inhibitor [13] in an oxygraphic cuvette, after which 10 mM pyruvate plus 10 mM malate were added as substrates. After approximately 2 min, 2 mM ADP was added and the oxygen consumption rate was followed for about 3 min (state 3). Then, mitochondria were taken from the cuvette and immediately frozen in liquid nitrogen. Samples were subsequently processed for protein solubilization with n-dodecyl-β-D-maltoside as described above. Similar experiments were performed without the addition of ADP to analyze the tyrosine phosphorylation pattern at state 4. Control experiments were performed with 0.1% DMSO in place of PP2.

Measurements of ATPase activity [26] in mitochondria incubated with or without 10 µM PP2 ruled out the possibility that PP2 could inhibit ATP synthase (169 ± 8 U for control and 170 ± 5 U for 10 µM PP2, n = 3).

Tyrosine phosphorylation during in situ generation of hydrogen peroxide

Hydrogen peroxide production was measured with the p-hydroxyphenylacetate fluorescent method as described in Hyslop and Sklar [27] with 100–250 µg of mitochondrial protein per assay at 30°C in the respiration buffer, using 25 mM succinate as substrate, 2 µg/ml antimycin to inhibit complex III, and pure H_2O_2 (Fisher Scientific, Illkirch, France) as standard. We found a rate of 125 ± 42 (n=5) pmol H_2O_2/min per milligram protein, in good agreement with Esposito et al. [28].

For determination of tyrosine-phosphorylated proteins during a kinetic assay of H_2O_2 generation, mitochondria (1 mg/ml, in a volume of 1.5 ml) were preincubated at 30 °C for 3 min in the respiration buffer with 25 µM oligomycin, 200 µM ATP and 25 mM succinate. Then, 2 µg/ml antimycin was added and samples (250 µl) were taken at 0, 5, 10, 15 and 20 min after the addition of antimycin, and immediately frozen in liquid nitrogen. Samples were subsequently processed for protein solubilization with n-dodecyl-β-D-maltoside as described above.

Immunoprecipitation

Mitochondria were preincubated in the kinase buffer in the presence of 200 µM ATP and 2 mM orthovanadate, pelleted, and solubilized with n-dodecyl-β-D-maltoside as described above. Then, detergent-solubilized samples (0.4–1.0 mg protein) were incubated with 25 µl PY20-agarose conjugated in 500 µl 2X-immunoprecipitation buffer (10 mM Tris-base, 150 mM NaCl, 20 mM NaF, 0.2 mM orthovanadate, 1 mM EGTA, 1 mM EDTA, 0.2 mM PMSF, 1% Triton X-100, 0.5% Nonidet P-40, pH 7.4) and distilled water in 1 ml total volume. After 30 min incubation at 4 °C with occasional stirring, the sample was centrifuged at 4 °C for 15 min at 14,000 g. The pellet was washed twice with 1 ml of 1X-immunoprecipitation buffer, and incubated at 40–50 °C for 1 h in 30–50 µl in 2X-sample buffer (1.16 ml 0.5 M Tris, pH 6.8, 8.34 ml H_2O, 0.5 ml glycerol, 0.4 g SDS, 0.2 g bromophenol blue) containing 4% β-mercaptoethanol. Supernatant was separated from the agarose by centrifugation and then analyzed by SDS-PAGE (12–18% polyacrylamide gradient). The total detergent to protein ratio during immunoprecipitation was in the 15–39 mg/mg range, and probably too high to keep subunits associated [29], and therefore only tyrosine-phosphorylated proteins were immunoprecipitated.

Two dimensional electrophoresis

After their solubilization with n-dodecyl-β-D-maltoside (2.5 mg/mg protein), the mitochondrial proteins (in 100 µl) were processed for electrophoresis according to Schägger et al. [30]. A linear 5–13% polyacrylamide gradient was used for first-dimensional BN-PAGE in a vertical apparatus at 4–7 °C, running first at 100 V, and continuing with voltage limited to 150 V, using the Mini PROTEAN 3 electrophoresis Biorad system. After electrophoresis of mitochondrial proteins, the lanes were cut from the BN-PAGE and used as follows: one for Coomassie blue coloration, and four for catalytic staining by histochemical reactions for complex I, II, IV and FoF1-ATP synthase [31]. The other lanes were used for the second dimension SDS-PAGE: each complex and FoF1-ATP synthase were excised from two lanes, denatured and finally processed in duplicate for the second-dimension SDS-PAGE on 12.5% polyacrylamide as in Williams et al. [32]. One lane was used for immunoblotting, the other for LC-MS/MS after Coomassie blue coloration.

Western blotting

Samples subjected to SDS-PAGE (on a 12–18% polyacrylamide gradient for immunoprecipitates, 12.5% for the second dimension after BN-PAGE and 10–18% for

the other experiments) were transferred onto a nitrocellulose membrane (Hyper film, Amersham, Little chaffont, UK). Tyrosine-phosphorylated proteins were labeled with PY20, a specific monoclonal mouse antibody against phosphotyrosine.

Membranes were stripped at 60 °C for 30 min in a buffer containing 62 mM Tris-HCl, pH 6.8, 100 mM β-mercaptoethanol, 2% (w/v) SDS, extensively washed, and reprobed with another antibody. An antiphosphoserine antibody was used to check the specificity of the antiphosphotyrosine antibody by comparing both labelings in the orthovanadate sample after stripping the membrane.

Analysis by densitometry on western blots was carried out using Densitometric analysis GelPlot2 macros in Scion Image software, an adaptation of the public NIH Image program (developped at the U.S. National Intitutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image).

Analysis by liquid chromatography and MS/MS

To identify proteins, nano LC-MS/MS analysis of tryptic peptides was performed by means of an ion trap mass spectrometer operated in the electrospray mode (LCQ DecaXP^{Plus}; ThermoFinnigan, San Juan, PR) and interfaced with a Dionex–LC Packings chromatographic system (C18 column, 75 µm ID, 150 mm long). After analysis, the TurboSequest program was used to assign peptide sequences from their fragment ions. Identification was considered positive when at least two peptides matched the protein.

Statistical analysis

Means ± SD were calculated and comparison of two sets of data was performed using paired Student's t test and the difference considered significant when $p < 0.05$ with $n \geq$ three independent experiments.

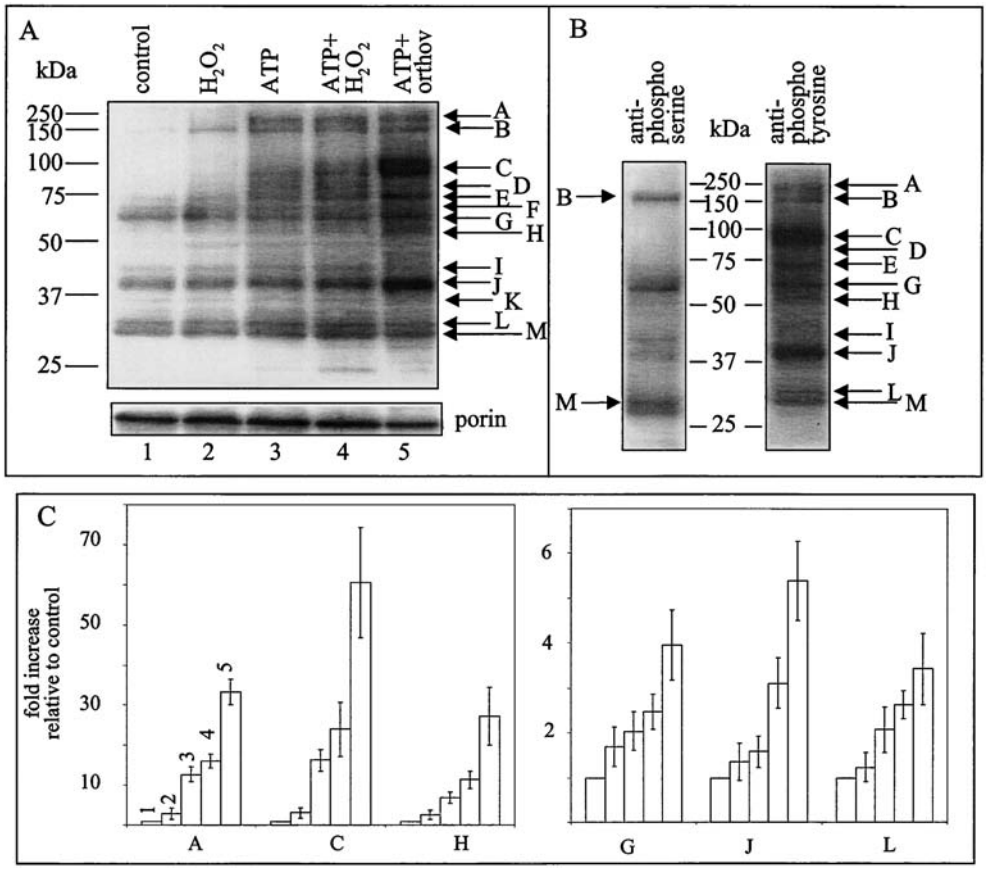

Figure 1. Detection of tyrosine-phosphorylated proteins. (*A*) Brain mitochondria were untreated (lane 1) or incubated for 3 min at 30 °C in the kinase buffer with 100 µM H_2O_2 (lanes 2 and 4), or 2 mM orthovanadate (lanes 5). Then, 200 µM ATP was added alone (lane 3) or in the samples preincubated with H_2O_2 (lane 4) or orthovanadate (lane 5) and incubation allowed to continue for 10 min, after which samples were processed for solubilization as described in Materials and methods. Proteins were assayed using the Micro-BCA method, and 34 µg was loaded per lane for SDS-PAGE on a 10–18% polyacrylamide gradient, and immunoblotting with antiphosphotyrosine antibody. The membrane was stripped and reprobed with an antiporin antibody (below). Data are representative of at least three experiments. (*B*) The membrane was stripped and reprobed with an antiphosphoserine antibody, and labeling was compared for the orthovanadate sample. (*C*) Tyrosine-phosphorylated bands were quantified by densitometry and normalized to the porin signal and results expressed as fold increase relative to control. 1 to 5 refer to the different treatments. For clarity, bands were separated into two groups.

Results

In vitro tyrosine phosphorylation of mitochondrial proteins

To reveal mitochondrial tyrosine-phosphorylated proteins and their ROS dependence, an *in vitro* study was performed with ATP added, alone or in the presence of either H_2O_2 or orthovanadate, and western blotted with an antiphosphotyrosine antibody (fig. 1A). The treatment with orthovanadate was performed to ensure optimal tyrosine kinase activity by inhibiting tyrosine phosphatases.

The membrane was stripped and reprobed with an antibody to porin, a mitochondrial protein which can be used as an internal standard (fig. 1A, bottom). This allows the quantification of the band intensity relative to control, with correction for protein loading (fig. 1C). Results show that all treatments increased tyrosine phosphorylation levels above that observed in control mitochondria, the greatest increases found in the samples incubated in the presence of orthovanadate, and particularly for band A, C and H (32.20 ± 3.11, 60.49 ± 13.77 and 27.28 ± 7.25, respectively, fold increase relatively to control, n = 3), while band G, J and L exhibited a lower increase (3.96 ± 0.79, 5.38 ± 0.88 and 3.42 ± 0.79, respectively, fold increase relative to control, n = 3).

To check the specificity of the phosphotyrosine antibody, the membrane was stripped and reprobed with an anti-phosphoserine antibody (fig. 1B), and labelings were compared for the orthovanadate sample. Results showed that only band B and M were aligned in both immunoblots, suggesting that these bands could contain co-migrating phosphotyrosine and phosphoserine proteins and/or double-labeled proteins, while the other bands were exclusively tyrosine-phosphorylated proteins.

Presence of the tyrosine phosphatase PTP 1B in mitochondria

As the additional increase in protein phosphorylation induced by orthovanadate, but also by H_2O_2 (fig. 1), was suggestive of the presence of tyrosine phosphatases in brain mitochondria, we investigated the presence of PTP 1B, a prototypic member of the non-receptor tyrosine phosphatase family [33], in mitochondrial subfractions (fig. 2). This tyrosine phosphatase has been shown to dephosphorylate and activate Src kinase [34] and can also be phosphorylated by this kinase [35]. Cytosolic and microsomal contaminations of our mitochondrial preparation were low, as verified by measurements of LDH and NADPH-cytochrome c reductase activity, respectively (see Materials and methods). MnSOD was revealed mainly in

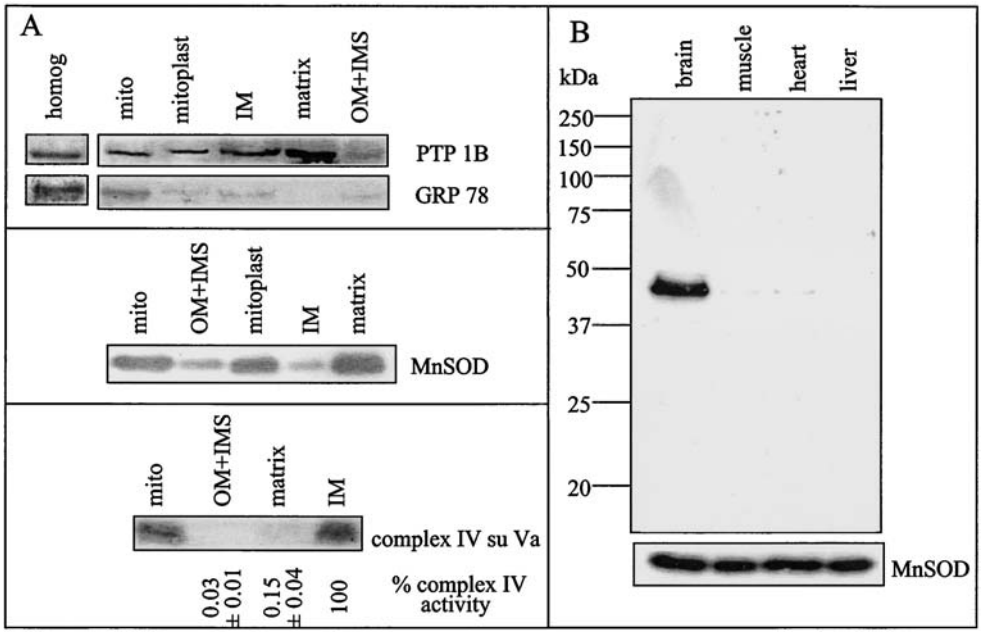

Figure 2. Detection of the tyrosine phosphatase PTP 1B in mitochondria. (*A*) Immunodetection of PTP 1B in brain mitochondria and submitochondrial fractions. Labeling in homogenate lysates is also shown for comparison. Protein content in the different fractions was assayed by the Micro-BCA method, and 50 µg protein of each fraction, of isolated mitochondria and of homogenate were loaded per lane for SDS-PAGE on 12% polyacrylamide and immunoblotting with anti-PTP 1B antibody. The membrane was stripped and reprobed with an antibody to GRP 78, a specific marker of the endoplasmic reticulum. Western blotting was also performed with an antibody to MnSOD, a matrix protein, and with an antibody to subunit Va of complex IV, an inner membrane protein, to assess the purity of the mitochondrial fractions. Also shown are the % complex IV activities calculated in the different fractions, assuming 100% activity for the inner membrane fraction. Results are representative of three independent experiments. IM, inner membrane; OM, outer membrane; IMS, intermembrane space. (*B*) Immunodetection of PTP 1B in rat brain, muscle, heart and liver mitochondria (50 µg per lane). The membrane was stripped and labeled with an anti-MnSOD antibody (below).

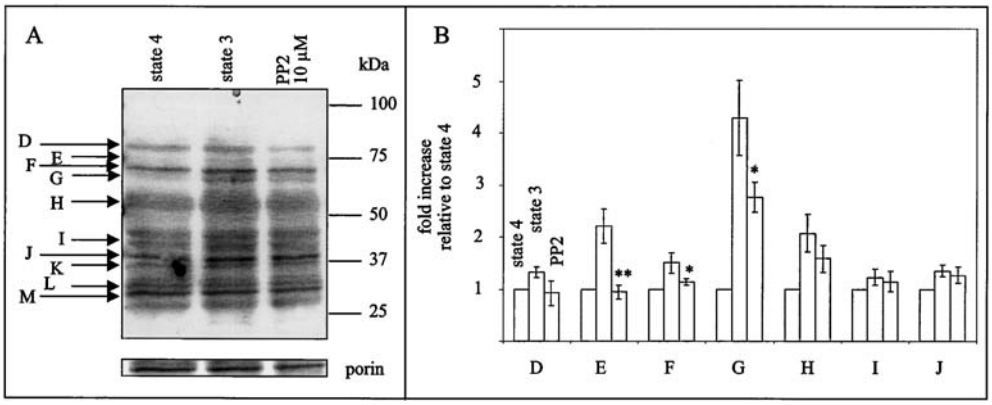

Figure 3. Detection of tyrosine-phosphorylated proteins at different energetic states. (*A*) Mitochondria (0.5 mg) respiring with 10 mM pyruvate and 10 mM malate in the oxygraphic cuvette were taken before (state 4) or at 3 min after the addition of 2 mM ADP (state 3), pelleted and immediately frozen at –80 °C. Samples were thawed and detergent-solubilized as described in Materials and methods. PP2 (10 µM) or DMSO (0.1%) was preincubated with mitochondria for 5 min at 30 °C before the oxygraphic experiment. Proteins (50 µg per lane) were loaded for SDS-PAGE on a 10–18% polyacrylamide gradient, and immunoblotted with an antiphosphotyrosine antibody. Data are representative of at least three experiments. (*B*) Bands were quantified by densitometry and normalized to porin signal and results expressed as fold increase relative to state 4. **p<0.01, *p<0.05.

the matrix fraction, as expected, and weakly in inner membranes and outer plus intermembrane space (fig. 2). Western blotting with an antibody to subunit Va of complex IV, an inner membrane enzyme of the respiratory chain, revealed that the matrix and the outer plus intermembrane space subfractions were apparently pure. This result was confirmed by an enzymatic assay for complex IV activity (fig. 2A), showing a negligible contamination of this enzyme in the matrix fraction (0.15 ± 0.04% relative to 100% in the inner membrane). More important, the virtual absence of contamination by the endoplasmic reticulum where PTP 1B is also localized [36] was verified with an antibody to GRP 78, a marker of endoplasmic reticulum [13]. As shown in figure 2A, GRP 78 was present in the homogenate and mitochondrial lysates, and to a lesser extent in the inner membranes, but not in the matrix fraction, where PTP 1B is particularly enriched (fig. 2A).

This original result prompted us to study the presence of PTP 1B in mitochondria from other rat tissues, namely muscle, heart and liver (fig. 2B). Results showed the virtual absence of PTP 1B in these tissues, suggesting a specific distribution for this tyrosine phosphatase in brain mitochondria.

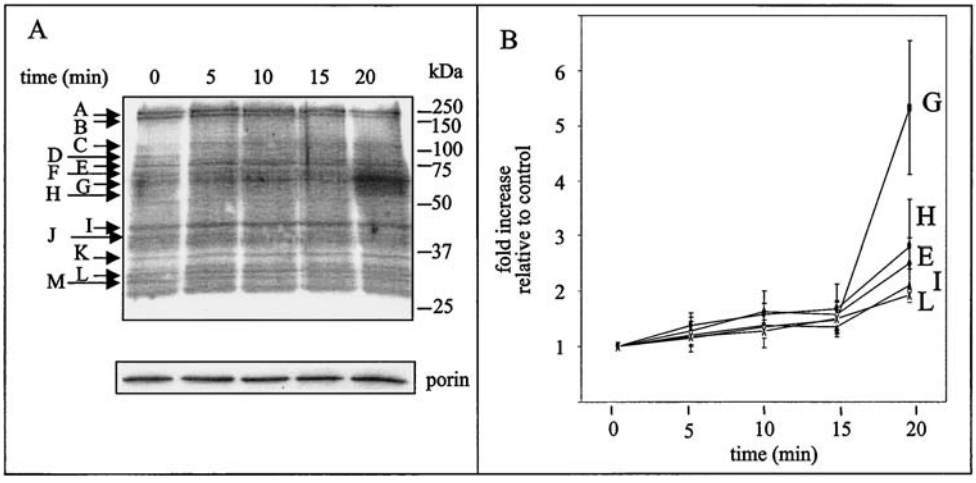

Figure 4. Detection of tyrosine-phosphorylated proteins during *in situ* H_2O_2 mitochondrial production. (*A*) Samples (250 µl) of mitochondria (1 mg/ml) respiring with succinate (25 mM) were extracted at different time intervals after the addition of antimycin (2 µg/ml), pelleted, and processed for solubilization with n-dodecyl-β-D-maltoside (1.5 mg/mg protein) as described in Materials and methods. Protein contents were assayed with the Micro-BCA method, and 20 µg mitochondrial protein was loaded per lane for SDS-PAGE on a 10–18% polyacrylamide gradient, and immunoblotted with an antiphosphotyrosine antibody. (*B*) Bands were quantified by densitometry and normalized to the porin signal, and results expressed as fold increase relative to control. Data are representative of three independent experiments.

Variation in phosphorylation levels during *in situ* ATP production

The increased phosphorylation by *in vitro* addition of ATP in brain mitochondria (fig. 1) led us to examine whether *in situ* ATP production could activate protein tyrosine phosphorylation. To this end, tyrosine phosphorylation was studied in mitochondria respiring with pyruvate and malate at state 3, in which mitochondria synthesize ATP with added ADP, and compared to state 4 (no ADP). This enabled us to detect protein bands sensitive to *in situ* produced ATP, as a physiologic approach. As shown in figure 3, mitochondria in state 4 contained a basal level of tyrosine-phosphorylated proteins, and the labeling increased in state 3, except for bands K, L and M (fig. 3B), the greater increase concerning bands G, E and H (4.29 ± 0.72, 2.21 ± 0.33, and 2.07 ± 0.36, respectively $p < 0.05$, n = 3).

As the Src family of tyrosine kinases has been reported in mitochondria [13], we investigated tyrosine phosphorylation and respiration rates at state 3, using PP2, a specific membrane-permeable inhibitor of Src family kinases [37]. Western blot analyses indicated that treatment with 10 µM PP2 reduced tyrosine phosphorylation of several bands (fig. 3A, B, lane 3). Furthermore, this was accompanied by a small but significant decrease in state 3 respiration rates (88.01 ± 4.70% of control values, n = 6, $p < 0.05$).

Variation in phosphorylation level during *in situ* free radical production

As *in vitro* treatment of mitochondria with H_2O_2 leads to enhanced phosphorylation (fig. 1), we determined whether maximal *in situ* mitochondrial free radical production, obtained by antimycin inhibition of complex III in mitochondria respiring on succinate [2], could change the phosphorylation pattern. To achieve this, we performed a kinetic study of tyrosine phosphorylation on mitochondria respiring with succinate upon addition of antimycin. Results (fig. 4A, B) showed the increased labeling after 20 min antimycin addition of several bands, particularly bands G, H and E (3.33 ± 1.22, 2.81 ± 0.84, and 2.50 ± 0.46, respectively, relative to control values at time zero, $p < 0.05$, n = 3).

Phosphorylation of FoF1-ATP synthase

Since each phosphorylated band can contain several proteins (figs 1, 3 and 4), the next step was to study tyrosine phosphorylation of the different respiratory chain complexes separated by BN-PAGE and second-dimension SDS-PAGE. To assign their position in the BN-PAGE accurately, we used histochemical specific staining corresponding to their enzymatic activities (fig. 5). To optimize resolution in the second dimension, complexes were excised from BN-PAGE before processing for the second SDS-PAGE and western blotting.

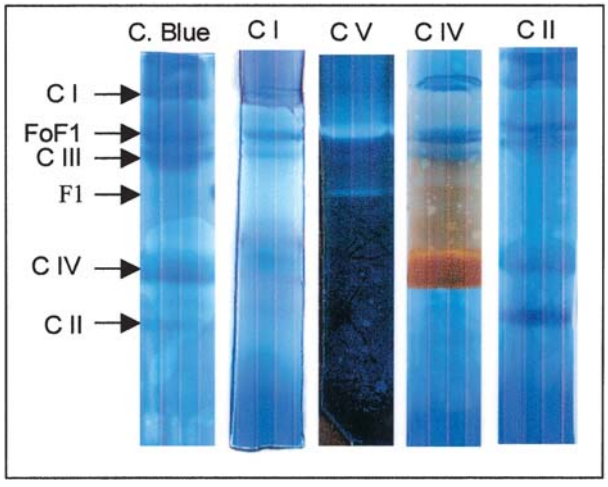

Figure 5. BN-PAGE and histochemical labeling of enzyme activities. Mitochondria were incubated in the kinase buffer in the presence of 2 mM orthovanadate and 200 µM ATP for 10 min at 30 °C, the pellet was solubilized with n-dodecyl-β-D-maltoside (2.5 mg/mg protein), proteins assayed by the Micro-BCA method, and BN-PAGE (5–13% polyacrylamide gradient) was processed with 100 µg protein per lane. Each lane was cut and individually treated: one for Coomassie Blue staining, the others for histochemical detection of specific complex activity for complex I, II and IV, and for FoF1-ATP synthase (C V). Data are representative of three independent experiments.

For FoF1-ATP synthase, western blots revealed three tyrosine-phosphorylated bands (fig. 6A, left). The gel was stripped and reprobed, first with an antibody to the β subunit (fig. 6A, middle), then restripped and labeled with both antibodies to the α and γ subunits (fig. 6A, right). Careful alignment of the three blots clearly showed that the α and γ subunits were aligned with phosphorylated bands while the β subunit was not, thus showing that the β chain was not phosphorylated on tyrosine residues.

To further confirm that the β chain of FoF1-ATP synthase was not phosphorylated, mitochondrial tyrosine-phosphorylated proteins were immunoprecipitated with the antiphosphotyrosine antibody PY20 directly coupled to agarose. Immunoprecipitated proteins were then Western blotted either with PY20, or the antibody to the β chain of FoF1-ATP synthase (fig. 6B). Major bands labeled with the antiphosphotyrosine antibody were G and H. The antibody to the β-chain did not label any band in the antiphosphotyrosine immunoprecipitate, in contrast to mitochondrial lysate where a strong band was detected at about 50 kDa (fig. 6B), thus confirming the preceding results (fig. 6A) that the FoF1-ATP synthase β chain is not tyrosine-phosphorylated.

Phosphorylation of complex I, II, III and IV

The second-dimension SDS-PAGE was performed in duplicate for each of the other complexes excised from the BN-PAGE; one was used for immunoblotting with the antiphosphotyrosine antibody, and the second

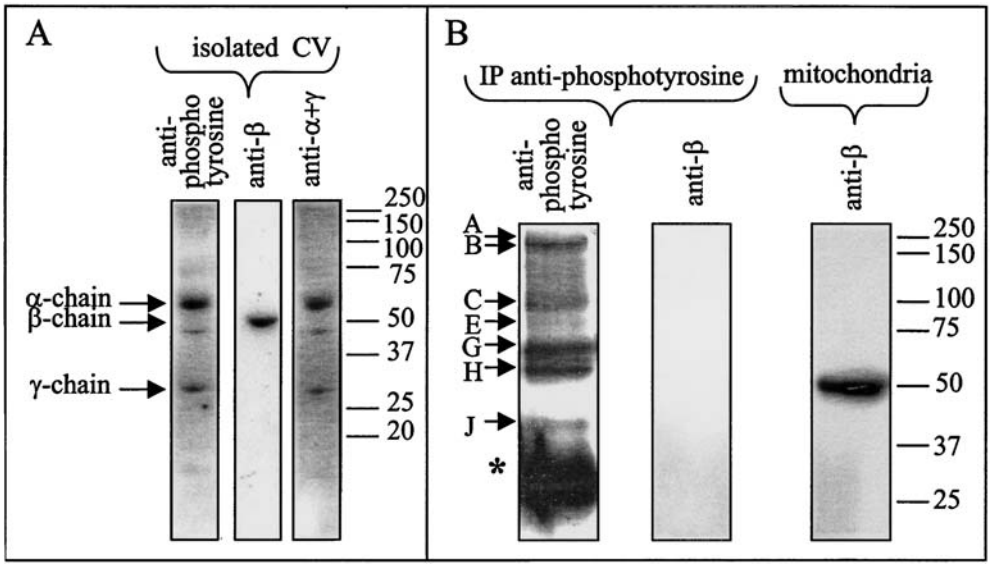

Figure 6. Evidence that the β chain was not tyrosine-phosphorylated (*A*) Identification of tyrosine-phosphorylated subunits of FoF1-ATP synthase by second-dimension SDS-PAGE on 12.5% polyacrylamide. The membrane was stripped and labeled successively with rabbit polyclonal antibody to the β chain, and with a mixture of α and γ chain antibodies. (*B*) Mitochondrial proteins, immunoprecipitated with the antiphosphotyrosine antibody coupled to agarose, were submitted to SDS-PAGE on a 12–18% polyacrylamide gradient, and subsequently western blotted with PY20 or an antibody to the FoF1-ATP synthase β chain. Also shown is immunolabeling of the FoF1-ATP synthase β chain in lysates of whole mitochondria. Data are representative of three independent experiments. *, antibody light chain.

reserved for analysis by LC-MS/MS after Coomassie blue coloration.

For complex I (fig. 7A), results showed two tyrosine-phosphorylated polypeptides (band 1 and 2). Analysis was performed on these bands excised from the companion Coomassie blue gel superimposed on the Western blot. Results showed the exclusive presence of FoF1-ATP synthase α and β chains in band 1 (22 matched peptides with 33% sequence coverage and 10 matched peptides and 33% sequence coverage, respectively). This is not surprising due to the close proximity of the two complexes in the BN-PAGE (fig. 5, lane C. Blue) and since FoF1-ATP synthase exists in oligomeric form, as seen by the smear in the corresponding histochemical band (fig. 5, top in lane C V). The protein content of band 2 was the 39-kDa subunit of NADH-ubiquinone oxidoreductase (10 matched peptides with 28% sequence coverage), with only FoF1-ATP synthase β chain as a contaminant peptide (4 matched peptides with 10% sequence coverage). As our previous results clearly showed that the ATP synthase β chain is not tyrosine phosphorylated, we can deduce that tyrosine-phosphorylation of band 2 concerned the 39-kDa subunit of complex I.

Application of this methodology to the other complexes revealed one tyrosine-phosphorylated band (arrows in fig. 7B), which was identified by LC-MS/MS as core protein 2 at 48 kDa for complex III (13 matched peptides with 28% sequence coverage), subunit II at 26 kDa for complex IV (4 peptides with 15% coverage) and the flavoprotein at 72 kDa for complex II (6 matched peptides with 12% sequence coverage). However, the presence of some associated proteins makes it difficult to be conclusive.

Discussion

Evidence that protein kinases regulate mitochondrial activity is now well established [6, 7, 38], but little is known about the role played by tyrosine kinases. In

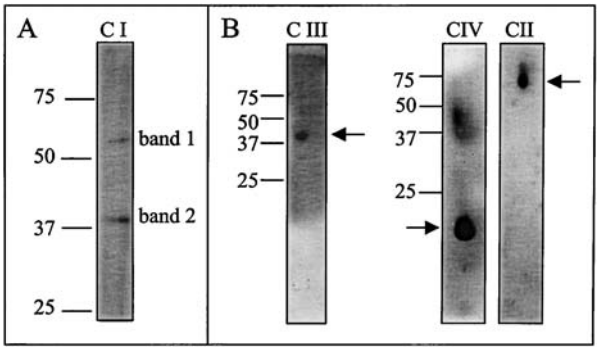

Figure 7. Identification of tyrosine-phosphorylated subunits for complex I, II, III and IV by second-dimension SDS-PAGE. The complexes were excised from the BN-PAGE and individually analyzed for tyrosine phosphorylation by immunolabeling after SDS-PAGE on 12.5% polyacrylamide for complex I (*A*), and complex III, IV and II (*B*). Bands 1 and 2 for complex I were identified by LC-MS/MS. Arrows indicate bands of the other complexes whose analysis by LC-MS/MS showed associated proteins (see text). Data are representative of three independent experiments.

addition, although considerable evidence has accumulated to show that oxidative stress is involved in mitochondrial pathologies, no studies have linked the effect of oxidants on tyrosine phosphorylation in OXPHOS.

The aim of the first set of experiments described here was to detect tyrosine-phosphorylated protein bands in brain mitochondria using an *in vitro* approach with added ATP, as generally performed [3, 8, 10, 13, 16]. The results demonstrated that tyrosine kinases are active in mitochondria and phosphorylate several proteins, named A–M (fig. 1). These bands were consistently found in all experiments, although with different relative intensities depending on the specific experiment or treatment. Labeling with an antiphosphoserine antibody (fig. 1B) showed that, except for band B and M which were double-labeled, and therefore could contain proteins phosphorylated both on serine and tyrosine residues, the other bands were exclusively tyrosine-phosphorylated.

The increased tyrosine phosphorylation pattern obtained in *in vitro* experiments by addition of ATP, with or without H_2O_2, constituted the first step of our study (fig. 1). The second step was to delineate experiments to reveal tyrosine-phosphorylated proteins and their variations under more physiological conditions. Thus we compared the phosphorylation pattern of mitochondrial proteins in state 3, where ATP is synthesized *in situ*, with that of state 4 (fig. 3). We also studied tyrosine phosphorylation of mitochondrial proteins during an *in situ* production of free radicals by antimycin-induced complex III inhibition in mitochondria respiring on succinate (fig. 4).

We found that state 3 respiration was accompanied by the increased tyrosine phosphorylation of several bands (fig. 3), suggesting that tyrosine kinases could use ATP synthesized by mitochondria as substrate for phosphorylation. Bands A–C were apparently either absent or sometimes only slightly labeled in western blots of oxygraphic experiments (fig. 3). ATP is progressively produced from ADP during oxidative phosphorylations in oxygraphic experiments. Therefore, we suggest that bands A–C required that mitochondria were incubated with ATP for a long time, as achieved in the *in vitro* experiment of figure 1, where FoF1-ATP synthase was further inhibited by the presence of oligomycin, thus preventing ATP hydrolysis (see Materials and methods).

We also found the increased labeling of several bands, especially band G, during endogenously formed free radicals by inhibiting electron transfer at complex III with antimycin (fig. 4). The different pattern of labeling compared to the *in vitro* experiment (fig. 1, lane 4) could result from mitochondrial production of primary free radical species (superoxide and hydroxyl radical) rather than the terminal product H_2O_2, in addition to their topologic local production. Furthermore, phosphorylation could be differently regulated in mitochondria according to their energetic state, since michondria were respiring on succinate in figure 4, while their metabolism was blocked by the presence of the inhibitors rotenone and oligomycin in figure 1.

To examine the functional consequence of H_2O_2, we investigated its effect on respiratory rates in mitochondria respiring with 10 mM pyruvate in the presence of 10 mM malate, and found that 100 μM H_2O_2 induced a significant decrease in respiratory rate (75.13 ± 14.01% of control values, $n = 5$, $p < 0.02$) and ATP synthesis (79.6 ± 8.6% of control values, $n = 4$, $p < 0.02$) at state 3, while there was no change at state 4, in accordance with a previous report [39]. However, further studies are required to link this functional consequence to H_2O_2-induced regulation of the tyrosine phosphorylation status by inhibiting tyrosine phosphatases [17–19], as H_2O_2 can also inhibit FoF1-ATP synthase [40].

The next step was to study the enzymes involved in tyrosine phosphorylation of mitochondrial proteins. As Src kinases have been reported in mitochondria [13], we used PP2, an inhibitor of the Src family kinases, to examine their participation in controlling respiration. We found that the intensity of some bands which increased at state 3, now decreased in the presence of 10 μM PP2 (fig. 3), with an inhibition of state 3 respiration rate (about 10%). The latter could result from inhibition of complex IV activity, since overexpression of Src in osteoclasts has been shown to result in stimulation of the activity of this complex [14]. However, further studies are required to disclose the relationship between tyrosine phosphorylation and mitochondrial energetic metabolism, due to the existence of different regulatory mechanisms localized at different levels [41].

Moreover, as the activity of tyrosine kinases can generally be reversed by that of tyrosine phosphatases, we looked for the presence of the prototypic non-receptor tyrosine phosphatase PTP 1B [33] in mitochondria, and found its localization in matrix and inner membrane fractions. To our knowledge, this is the first evidence of the presence of the tyrosine phosphatase PTP 1B in brain mitochondria, beside SHP 2, the former tyrosine phosphatase recently identified [17]. Interestingly, we further showed the absence of PTP 1B in mitochondria from rat muscle, heart and liver, indicating a specific expression of this tyrosine phosphatase in brain, the significance of which remains to be studied.

To identify mitochondrial proteins in the overall pattern of tyrosine phosphorylated bands, we analyzed tyrosine phosphorylation of each respiratory complex and FoF1-ATP synthase by BN-PAGE, second-dimension SDS-PAGE, followed by western blotting.

We have experimental evidence that the FoF1-ATP synthase β chain was not tyrosine-phosphorylated (fig. 6), a result confirmed by the absence of labeling with the antibody to the β chain of an antiphosphotyrosine immunoprecipitate from mitochondrial proteins. However,

phosphorylation on tyrosine of the β chain has been detected in a radiation-induced fibrosarcoma cell line in response to heat shock [42], and in a murine fibroblast cell line after tumor necrosis factor stimulation [43], suggesting that this phosphorylation could be a response to a specific signal transduction pathway, contrary to our experimental conditions.

In contrast, our data did not allow conclusions to be drawn about tyrosine phosphorylation of the FoF1-ATP synthase α chain, although the antibody to the α chain labeled a protein in the antiphosphotyrosine immunoprecipitate (result not shown), as several proteins could co-migrate in the same band.

For complex I, subunit separation demonstrated for the first time that the 39-kDa subunit (band 2 in fig. 7A) was tyrosine-phosphorylated. This subunit has been suggested to form a biosynthetic module with the acyl-carrier protein SDAP of complex I [44].

Our results were not conclusive concerning tyrosine phosphorylation of the other complexes, as the second-dimension SDS-PAGE did not resolve associated proteins (fig. 7B, arrows). However the respective subunits of the respiratory complexes, identified by LC-MS/MS, have already been found to be phosphoproteins [8, 9, 14].

In conclusion, we demonstrate clearly the presence of tyrosine-phosphorylated proteins in mitochondria and their sensitivity to H_2O_2, in both *in vitro* and physiologic conditions, along with their variation according to energetic-state conditions. We have shown that the Src family kinases are in part involved in state 3 respiration. We have also detected the presence of tyrosine phosphatase PTP 1B, exclusively in brain mitochondria. We have identified the 39-kDa subunit of complex I as being tyrosine-phosphorylated. We have experimental evidence that the α chain of FoF1-ATP synthase and flavoprotein of complex II, subunit II of complex III, and subunit II of complex IV are likely tyrosine-phosphorylated.

Therefore, our study identifies some mitochondrial proteins and associated kinases which deserve attention in future studies on the role of tyrosine phosphorylation in cellular energy metabolism.

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