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Characterization and location of Src-dependent tyrosine phosphorylation in rat brain mitochondria

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Abstract

Analysis of protein phosphorylation in highly purified rat brain mitochondria revealed the presence of several alkali-stable phosphoproteins whose phosphorylation markedly increases upon treatment with peroxovanadate and Mn^{2+}, a property indicating tyrosine phosphorylation. These include three prominent bands, with apparent sizes of 50, 60, and 75 kDa, which are detectable by anti-phosphotyrosine. Tyrosine phosphorylation disappears when mitochondria are treated with PP2, an inhibitor of the Src kinase family, suggesting the presence of members of this family in rat brain mitochondria. Immunoblotting and immunoprecipitation assays of mitochondrial lysates confirmed the presence of Fyn, Src and Lyn kinases, as well as Csk, a protein kinase which negatively controls the activity of the Src kinase family. Results show that tyrosine-phosphorylated proteins are membrane-bound and that they are located on the inner surface of the outer membrane and/or the external surface of the inner membrane. Instead, Src tyrosine kinases are mainly located in the intermembrane space – in particular, as revealed by immunogold experiments for Lyn kinase, in the cristal lumen. Rat brain mitochondria were also found to possess a marked level of tyrosine phosphatase activity, strongly inhibited by peroxovanadate.

Keywords: Mitochondria; Tyrosine kinase; Src kinase family

1. Introduction

Protein phosphorylation is implicated in a large number of cell processes, particularly signal transduction pathways and enzymatic regulation [1]. Signal transduction involving protein phosphorylation is generally triggered at cell surface receptors with tyrosine kinase activity and, as a consequence, the plasma membrane has been considered the main subcellular site of such activities [2]. However, tyrosine phosphorylation has also been detected in the nuclear envelope and endoplasmic reticulum membranes [3]. Besides receptor tyrosine kinases, a group of intracellular type, exemplified by the Src family (Src, Fyn, Lyn, Fgr, etc.), may serve as control switches in a variety of signal transduction pathways governing essential cell processes [4]. Receptor tyrosine kinases and receptors lacking intrinsic tyrosine kinase activity interact with members of the Src family [5–9]. Tyrosine phosphorylation by these kinases

Abbreviations: RBM, rat brain mitochondria; $\Delta\Psi$, membrane potential; PTK, protein-tyrosine kinase; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine

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is a reversible process which depends on the activity of tyrosine phosphatases. Depending on the cellular context, tyrosine phosphatases can either antagonize the action of tyrosine kinases or cooperate with them. The activation of the Src family kinases by tyrosine phosphatases is an example of positive cooperation between these systems [8]. Inhibition of cellular tyrosine phosphatase activities may result in increased levels of tyrosine-phosphorylated proteins, implying the existence of a dynamic relationship between tyrosine phosphorylation and dephosphorylation pathways. Conditions of oxidative stress may modify the activity of major signalling enzymes, including tyrosine kinases and phosphatases [10].

Mitochondria and individual mitochondrial proteins are known to participate in processes involving signal transduction pathways, including programmed cell death [11], neoplastic proliferation and oxidative stress [11–13]. Protein phosphorylation has been recognized to cover very important functions in signal transduction; however, whereas Ser/Thr phosphorylation in mitochondria has been studied in depth [14–16], there are few studies on the presence of a mitochondrial tyrosine kinase [17–19]. In particular, in brain mitochondria no report deals with this subject.

The main aim of the present study was to identify the occurrence of tyrosine kinase activity and of corresponding substrates associated with brain mitochondria, to gain information on the family(ies) of tyrosine kinases mainly involved and to reveal tyrosine phosphatase activity which modulates tyrosine phosphorylation. Another aim was to gain information on the location of these enzymes and their substrates in mitochondrial subcompartments.

2. Materials and methods

2.1. Materials

[γ-^{32}P]ATP was purchased from Amersham. 4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2), genistein, piceatannol and radicicol were obtained from Calbiochem and protease inhibitor cocktail from Boehringer. Peroxovanadate was prepared by mixing hydrogen peroxide (3 mmol/l) and sodium orthovanadate (2 mmol/l).

Anti-phosphotyrosine monoclonal antibodies were purchased from ICN Biotechnology, anti-cytochrome *c* monoclonal antibodies from PharMingen, anti-β-actin polyclonal antibodies from Sigma, anti-PCNA, anti-PDGF receptor, anti-Bip, anti-HSP-60, anti-Lyn, anti-Fyn, anti-Src and anti-Csk polyclonal antibodies from Santa Cruz Biotechnology, anti-caveolin polyclonal antibodies from Lab. Transduction. Other reagents were purchased from Sigma.

2.2. Preparation of mitochondria

Rat brain mitochondria (RBM) were purified by the Ficoll gradient method, according to Nicholls, with some modifications. Briefly, rat brain (cerebral cortex) was homogenized in isolation medium (320 mM sucrose, 5 mM HEPES, 0.5 mM EDTA, pH 7.4; 0.3% BSA was added during homogenization and the first step of purification) and subjected to centrifugation ($900\times g$) for 5 min. The supernatant was then centrifuged at $17\,000\times g$ for 10 min, to precipitate crude mitochondrial pellets. These were resuspended in isolation medium plus 1 mM ATP and layered on top of a discontinuous gradient, composed of 2 ml of isolation medium containing 16% (w/v) Ficoll, 2 ml of isolation medium containing 14% (w/v) Ficoll, 3 ml of isolation medium containing 12% (w/v) Ficoll, and 3 ml of isolation medium containing 7% (w/v) Ficoll. The gradient was centrifuged for 30 min at $75\,000\times g$. Mitochondrial pellets were suspended in isolation medium and centrifuged for 10 min at $17\,000\times g$. Again the pellets were suspended in isolation medium without EDTA. Protein content was measured by the biuret method with bovine serum albumin as a standard [22]. To separate mitochondrial membranes from the soluble fraction, 10 mg/ml of mitochondria were exposed to ultrasonic energy (MSE Sonicator) and subjected to eight freeze/thaw cycles. Mitochondrial suspensions were then centrifuged at $100\,000\times g$ to obtain membrane pellets and supernatant fractions.

2.3. Marker enzymes

NADPH-cytochrome *c* reductase activity was assayed as described by Sottocasa et al. [23]. Glucose-6-phosphatase activity was measured according to Swanson [24]. The inorganic phosphate liberated during incubation was determined as described by

Baginski et al. [25]. Monoamine oxidase activity was assayed as described by Tabor et al. [26]. Adenylate kinase, cytochrome *c* oxidase, and malate dehydrogenase activities were determined according to Dorbani et al. [27].

2.4. Western blot analysis to assess the mitochondrial purity

Brain homogenate in mitochondrial isolation medium [21] and mitochondrial suspension were lysed with 20 mM Tris–HCl, pH 7.5, 10% glycerol, 1% Nonidet P-40, 1 mM EDTA, 50 mM NaCl. Both in the amount of 30 μg protein were subjected to SDS–PAGE and analysed by Western blotting.

2.5. Determination of integrity of mitochondria

Membrane potential ($\Delta\Psi$) was measured by monitoring the distribution of the lipophilic cation, tetraphenylphosphonium, across the mitochondrial membrane, using a selective electrode prepared in our laboratory according to published procedures [28]. Membrane potential measured with this electrode was corrected as proposed by Jensen [29]. Care was taken to keep the tetraphenylphosphonium concentration below inhibitory levels, as previously indicated [30]. The mitochondrial matrix volume was calculated from the distribution of [^{14}C]sucrose and $^{3}H_2O$, according to Palmieri and Klingenberg [31].

A centrifugal filtration method was used to measure mitochondrial uptake of [^{14}C]ADP [30]. Swelling was estimated by changes in absorbance at 540 nm on a Perkin-Elmer Lambda 5 spectrophotometer equipped with thermostatic control.

2.6. Digitonin treatment

Submitochondrial fractions were obtained by the digitonin method (0.2 or 0.6 mg digitonin/mg of mitochondrial protein), as described by Dorbani et al. [27].

2.7. Phosphorylation assays

Endogenous phosphorylation of mitochondria was measured by incubating 50 μg of mitochondria at 30°C for 10 min in 30 μl of reaction medium containing 200 mM sucrose, 10 mM HEPES (pH 7.4), 5 mM $MnCl_2$, and 20 μM [γ-^{32}P]ATP (3×10^6 cpm/nmol). Enolase phosphorylation assays were performed as described previously [32].

Reactions were stopped by the addition of 2% SDS and 1% 2-mercaptoethanol (final concentrations) followed by 5 min treatment at 100°C, as described [33]. Solubilized mitochondria were analysed by SDS–PAGE, essentially according to Laemmli [34].

After electrophoresis, gels were stained with Coomassie brilliant blue according to Laemmli [34], submitted to 2 M NaOH treatment at 55°C as described [33] and again fixed. Dried gels were then autoradiographed at −80°C with intensifying screens. Otherwise, after electrophoresis, gels were transferred to nitrocellulose membranes and immunostained with anti-phosphotyrosine antibody. A blocked anti-phosphotyrosine antibody has also been prepared that was incubated overnight with an excess of phosphotyrosine.

Quantitation of band intensity in Western analysis was accomplished using Image Station 440, Kodak.

2.8. Phosphoamino acid analysis

To determine phosphoamino acid levels in mitochondria, the phosphorylation reaction carried out as described above was stopped by adding an appropriate volume of HCl (final concentration 6 N), and acid hydrolysed at 110°C for 2 h in vacuum-sealed tubes. The HCl was removed by evaporation and the residue was dissolved in a marker mixture containing phosphoserine, phosphothreonine and phosphotyrosine (1 mg/ml each) and analysed by bidirectional paper electrophoresis [33] as follows. 50 μl aliquots of hydrolysate were spotted on Whatman 3 MM paper (100 μM) paper and subjected to electrophoresis in the first direction at pH 1.9 (acetic acid/formic acid/H_2O, 150:25:825) for 2 h at 2.4 kV and in the second direction at pH 3.5 (acetic acid/pyridine/H_2O, 50:5:945) for 1 h at 2.4 kV. Markers were detected by staining with ninhydrin, and ^{32}P-labelled phosphoamino acids were detected by autoradiography.

2.9. Assay for ^{32}P-Tyr phosphatase activity using poly(Glu-^{32}P-Tyr)4:1 as substrate

The phosphatase assay was carried out by incubating RBM (50 μg) at 30°C for 15 min in a medium (30 μl) containing 15 pmol ^{32}P-poly(Glu-Tyr) (corresponding to 0.024 μCi/μg) previously phosphorylated as described [33]. The reaction was stopped by the addition of 2% SDS and 1% 2-mercaptoethanol (final concentrations) followed by 5 min treatment at 100°C as described [33].

2.10. Anti-Lyn, anti-Fyn, anti-Src and anti-Csk immunoprecipitation (IP)

RBM were extracted for 1 h at 4°C with 20 mM Tris–HCl, pH 7.5, 10% glycerol, 1% Nonidet P-40, 1 mM EDTA, 50 mM NaCl and a protease inhibitor cocktail. After centrifugation, supernatants were precleared by incubation with protein A-Sepharose for 45 min at 4°C. Samples (2 mg mitochondrial protein) were then incubated overnight at 4°C with the appropriate antibody bound to protein A-Sepharose. Immune complexes were washed three times by centrifugation and resuspended in 50 mM Tris–HCl, pH 7.5, containing a protease inhibitor cocktail.

2.11. Immune complex kinase assays

Tyrosine kinase assays of immune complexes, obtained as described above, were performed in basal medium (see Section 2.7) containing poly(Glu-Tyr)4:1, which served as exogenous substrate. After incubation for 10 min at 30°C, samples were analysed by SDS–PAGE followed by autoradiography.

2.12. Immunogold labelling

RBM were fixed for 2 h in 4% paraformaldehyde and 0.25% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), post-fixed for 1 h in 1% osmium tetroxide in the same buffer, dehydrated in ethanol, and embedded in London resin white. Ultrathin sections picked up on gold grids were deosmicated with sodium metaperiodate, washed with 0.01 M PBS (pH 7.2), incubated for 20 min on 1% BSA in PBS, and treated with rabbit primary polyclonal antibody against tyrosine kinase Lyn. After washing with PBS, sections were incubated with colloidal gold (15 nm) conjugated with goat anti-rabbit IgG. Sections were then stained with uranyl acetate followed by lead citrate and examined under the electron microscope. A control experiment was performed by eliminating the incubation of sections with the primary antibody.

3. Results

Some cellular signal transduction events involving mitochondria are associated with phosphorylation/dephosphorylation processes, involving tyrosine residues in particular [7–10]. In order to verify whether this post-translational modification takes place in the mitochondrion, our main concern was to obtain mitochondrial preparations free of contaminating membranes, as tyrosine kinase is particularly active in the plasma membrane and microsomes. The high purity of the RBM obtained using the procedure applied here is exemplified in previous papers demonstrating the negligible degree of activity of acetylcholinesterase [20] and lactate dehydrogenase [20,21] in such preparations – an indication that the mitochondria are free of plasma membrane and cytosol contamination. Furthermore, electron microscopy demonstrated the complete absence of contaminating membrane fragments in the preparations [21]. In order to verify that our own preparations were free of microsomes, a subcellular fraction with high tyrosine kinase activity frequently present in isolated mitochon-

Table 1
Activity of NADPH-cytochrome *c* reductase and glucose-6-phosphatase in rat brain mitochondria and microsomes

Enzyme	Microsomes (nmol/min/mg protein)	Mitochondria (nmol/min/mg protein)
NADPH-cytochrome *c* reductase	10.02 ± 0.6	0.02 ± 0.003
Glucose-6-phosphatase	8.1 ± 0.4	0.02 ± 0.002

Activity expressed as nmol/mg protein.
Values are the means ± S.D. from three independent experiments.

dria, we assayed for two microsomal marker enzymes, i.e. NADPH-cytochrome *c* reductase and glucose-6-phosphatase, and found them to be nearly undetectable (Table 1). Besides the above reported enzymatic activity determinations and ultrastructural observations, in order to have a complete evidence of the purity of mitochondrial preparations, were also performed Western blot analyses with other extramitochondrial markers. These markers are: β-actin (cytoplasm), PCNA (nucleus), PDGFR (cell membrane), and Bip (endoplasmic reticulum). We also tested for caveolin, a protein particularly found in caveolae, specialized regions of the plasma membrane. The results reported in Fig. 1 show the Western blot analyses performed in RBM suspension (lane A) and in a brain homogenate (lane B). As observable in the figure, the immunoreactions are completely negative for all the extramitochondrial markers in the RBM preparations (lane A). Positive reactions, instead, are observed for comparison, for all markers, in the homogenate (lane B). The positive reaction in both lanes refers to cytochrome *c* used as mitochondrial marker.

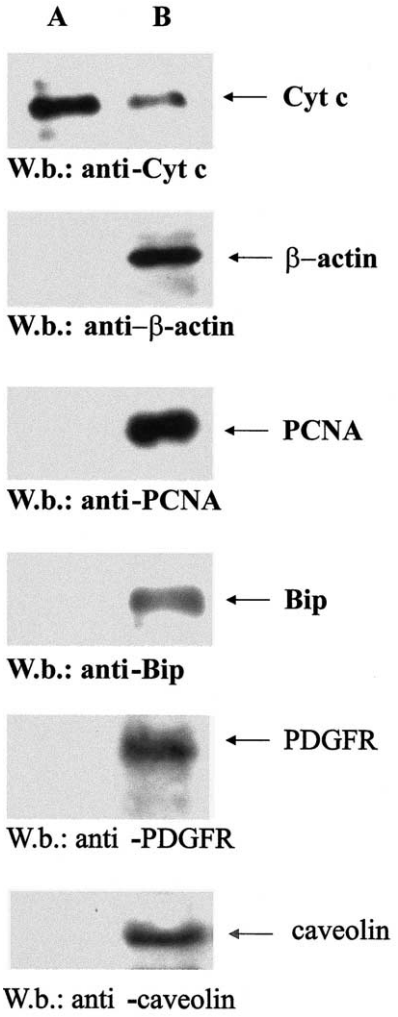

Fig. 1. Western blot analysis of mitochondrial and brain homogenate lysates, with anti-cytochrome (Cyt) *c*, anti-β-actin, anti-PCNA, anti-PDGF receptor (PDGFR), anti-Bip and anti-caveolin. Mitochondrial lysates (30 μg) were subjected to immunoblot analysis with anti-Cyt *c*, anti-β-actin, anti-PCNA, anti-Bip, anti-PDGFR and anti-caveolin (lane A). Brain homogenate lysates (30 μg) were subjected to immunoblot analysis with anti-Cyt *c*, anti-β-actin, anti-PCNA, anti-PDGF receptor and anti-caveolin (lane B).

Fig. 2 shows the patterns of phosphorylated mitochondrial proteins retained after NaOH treatment. Patterns change according to phosphorylation conditions. In the absence of peroxovanadate, only two proteins, of 50 and 60 kDa, with a very low phosphorylation level, are observable (lanes a, b). The addition of 5 μM oligomycin, an inhibitor of F_1F_0-ATPase, increases their endogenous phosphorylation. The addition of 2 mM peroxovanadate to the incubation medium stimulates the phosphorylation of several proteins (lane c), including that of three proteins of 50, 60 and 75 kDa, the levels of which are further increased by treatment with oligomycin (lane d). The effect of oligomycin is very probably due to the increased supply of non-hydrolysed ATP available in the presence of this inhibitor. A protein loading standard of HSP 60 by Western blot is included in this figure for quantitative purpose.

Phosphorylation targets were identified by phosphoamino acid analysis of mitochondria, ^{32}P-labelled in a reaction mixture containing oligomycin in the absence or presence of peroxovanadate (Fig. 3). In its absence, phosphorylation mainly involves Ser and to a lesser extent Thr residues, whereas tyrosine phosphorylation is almost totally absent (Fig. 3A). The addition of peroxovanadate markedly increases the presence of ^{32}P-Tyr, to a level comparable with the phosphorylation of Thr residues (Fig. 3B).

To ascertain whether tyrosine phosphorylation specifically involves the proteins of 50, 60 and 75 kDa, Western blot analysis was performed using a monoclonal anti-phosphotyrosine antibody, and the presence of P-Tyr in each of the three proteins was confirmed (Fig. 4A, lanes a, b). The other two per-

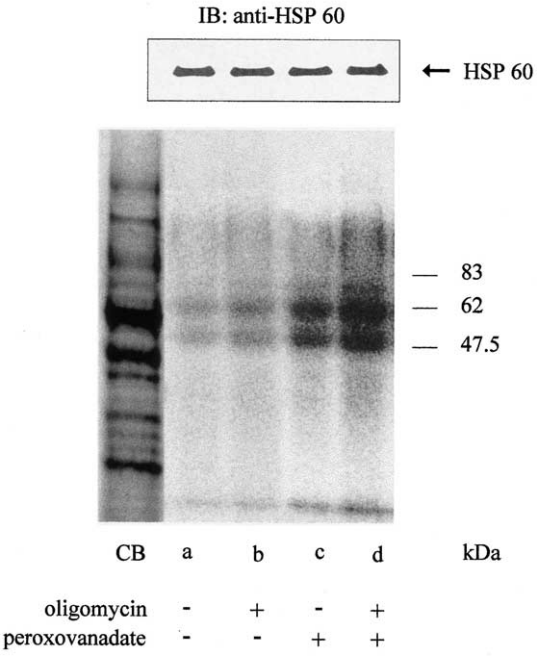

Fig. 2. Effects of oligomycin and peroxovanadate on alkali-stable protein phosphorylation of rat brain mitochondria. RBM (50 μg) were incubated alone at 30°C for 10 min in the reaction medium described in Section 2 (lane a) or in the presence of 5 μM oligomycin (lane b), 2 mM peroxovanadate (lane c) or 5 μM oligomycin plus 2 mM peroxovanadate (lane d). Samples were analysed by 0.1% SDS–10% PAGE, and gels were transferred to PVDF membrane, immunostained with anti-HSP 60 as a protein loading standard. Then the membranes were subjected to NaOH treatment as described. Lane CB, NaOH-treated Coomassie brilliant blue-stained gel of mitochondrial proteins. Molecular size markers are shown on the right. Autoradiograms were exposed for 20 h.

oxovanadate-insensitive bands were clearly non-specific, as shown by a previously blocked anti-phosphotyrosine antibody (Fig. 4A, lanes c, d).

As peroxovanadate is a well-known inhibitor of tyrosine phosphatases [35], to check whether the observed increase in phosphorylation was ascribable to its action, the occurrence of tyrosine phosphatase activity was verified in RBM using ^{32}P-labelled poly(Glu-Tyr)4:1 as a substrate, as described in Section 2 (Fig. 4B). A ~75% reduction in the phosphorylation of ^{32}P-poly(Glu-Tyr)4:1 was observed in the presence of 50 μg RBM; with peroxovanadate this activity was almost completely inhibited.

In order to identify the protein-tyrosine kinases (PTKs) responsible for phosphorylation, we tested the capability of a series of tyrosine kinase inhibitors of differing specificities to inhibit phosphorylation in the 50, 60 and 75 kDa proteins, detected after treatment with peroxovanadate. Fig. 5 shows their inhibitory effects on tyrosine phosphorylation of the 75 kDa protein; similar results were obtained with 50 and 60 kDa protein (data not shown).

Observations made using these inhibitors allowed us to make some important considerations. First, phosphorylation of 50, 60 and 75 kDa bands is almost completely prevented by 1 μM PP2, a molecule which exerts a strong inhibitory effect principally on tyrosine kinase of the Src family [36], indicating that these proteins may be phosphorylated by this family of kinases. Second, the concentration range over which the other inhibitors act supports this hypothesis. Genistein, a broad range PTK inhibitor [37], has an appreciable effect at a very high concentration (50 μM). This inhibition cannot be considered specific when we recall that 5 μM are sufficient for complete inhibition of phosphorylation of the EGF receptor [37]. Instead, piceatannol has no effect over the 10–50 μM concentration range, thus excluding the involvement of PTKs of the Syk family [38]. Lastly, the inhibitory effect of radicicol, although very low, also suggests the involvement of Src family members [39]. Based on these observations, further experiments focused on verifying the presence of Src-PTKs in mitochondria. To start with, we performed quantitative analysis of Src kinases recovered in the mitochondrial fraction with respect to the total cellular amount. This was estimated by densitometric scanning of Western blots of kinases on brain homogenate and on mitochondrial fractions obtained from the same volume of homogenate. The presence of Src kinase associated with the mitochondrial fraction ranged from 4% (Lyn kinase) to 8% (Fyn kinase) (data not shown). The ratio of Src kinase in mitochondria was then analysed with respect to the other specific fractions. The Western blots of Fig. 6A show that the mitochondria contain Src-PTKs Fyn, Lyn and Src, as well as Csk, a protein kinase which negatively controls the activity of the Src family. The activity of each enzyme (Fig. 6B) was verified by the results of in vitro phosphorylation assays carried out using kinases immunoprecipitated from RBM and the exogenous substrate poly(Glu-Tyr)4:1. It is important to point out that, in each case, measured activity was closely related to the amounts of immu-

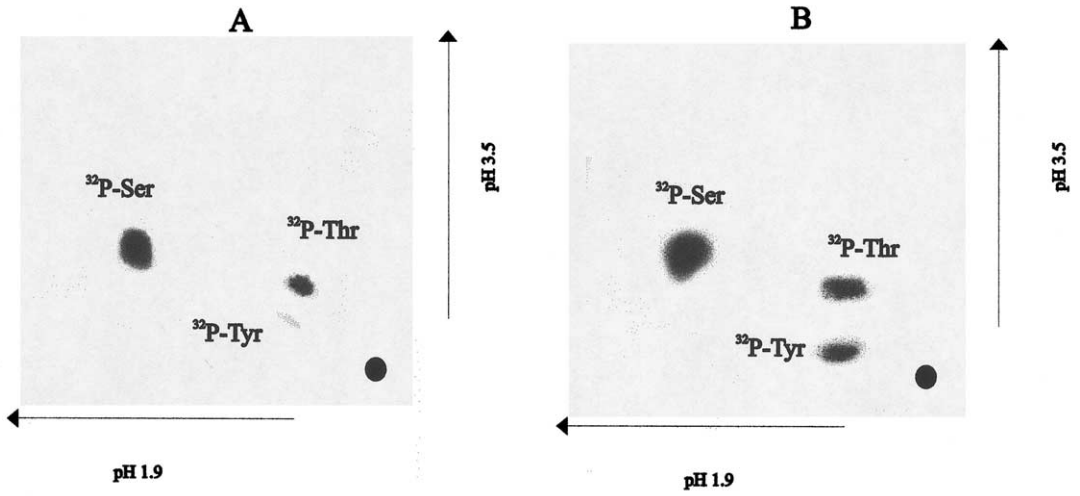

Fig. 3. Electrophoretic analysis of ^{32}P-phosphoamino acids in acid hydrolysate of ^{32}P-labelled rat brain mitochondria. RBM (200 μg) were incubated in the reaction medium as described, with 5 μM oligomycin at 30°C for 10 min in the absence or presence of 2 mM peroxovanadate, and then subjected to acid hydrolysis and phosphoamino acid analysis as described. Arrows in autoradiogram: first and second directions of electrophoretic migration from origin. Panels A and B are the patterns obtained for rat brain mitochondria incubated in the absence and presence of peroxovanadate, respectively. Autoradiograms were exposed for 24 h. This result is representative of three independent experiments.

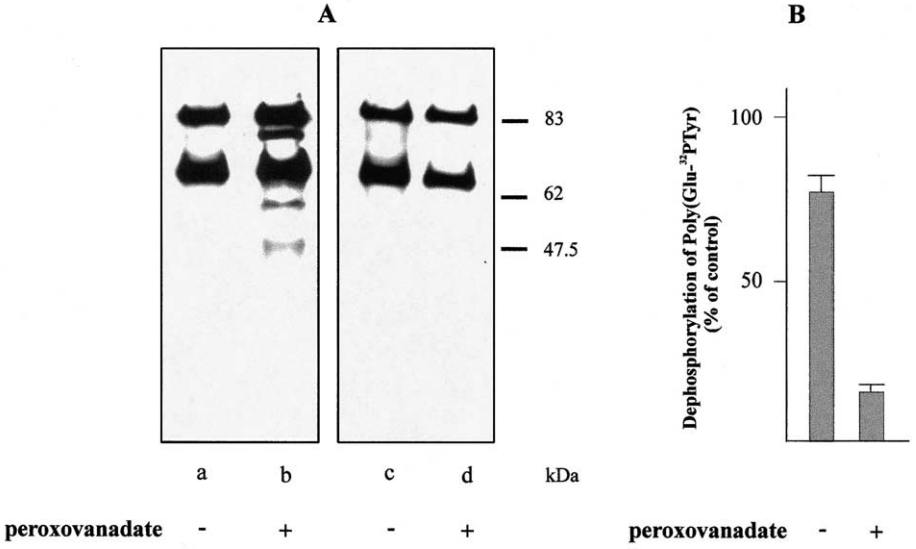

Fig. 4. Effects of peroxovanadate on tyrosine kinase (A) and phosphatase (B) activities. (A) RBM (50 μg) were incubated at 30°C for 10 min in the reaction medium as described, with 5 μM oligomycin, in the absence (lanes a, c) or presence of 2 mM peroxovanadate (lanes b, d). Samples were analysed by 0.1% SDS–10% PAGE, and gels were transferred to nitrocellulose membranes and immunostained with anti-phosphotyrosine antibody. Lanes c and d: a previously blocked anti-phosphotyrosine antibody was used: antibody was incubated overnight with an excess of phosphotyrosine to block all specific sites. Molecular size markers are shown on the right. (B) Tyrosine phosphatase activity in rat brain mitochondria (50 μg), measured with poly(Glu-^{32}P-Tyr)4:1 (0.024 μCi/1 μg), previously phosphorylated as described in Section 2, at 30°C for 15 min (c), in the absence (a) or presence (b) of peroxovanadate. Samples were analysed by 0.1% SDS–10% PAGE, and radioactivity was quantified using an Instant Imager (Packard). Tyrosine phosphatase activity is expressed as percentage of the control value obtained in the absence of RBM. Each column represents means ± S.D. of four independent experiments.

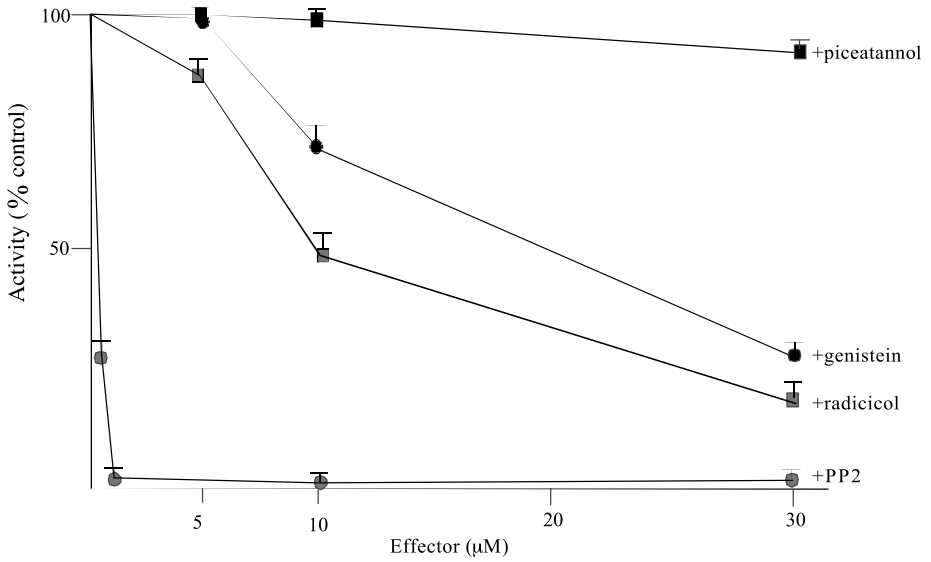

Fig. 5. Effect of different tyrosine kinase inhibitors on the phosphorylation of a 75 kDa mitochondrial protein. RBM (50 μg) were incubated alone or in the presence of different concentrations of the indicated inhibitors for 10 min under the conditions described in Section 2. The samples were submitted to SDS–PAGE, and gels were transferred to nitrocellulose membranes, immunostained with anti-phosphotyrosine antibody and quantified as described in Section 2. Activity is expressed as the percentage of the control values obtained in the absence of effectors. Reported values represent means ± S.D. from four separate experiments.

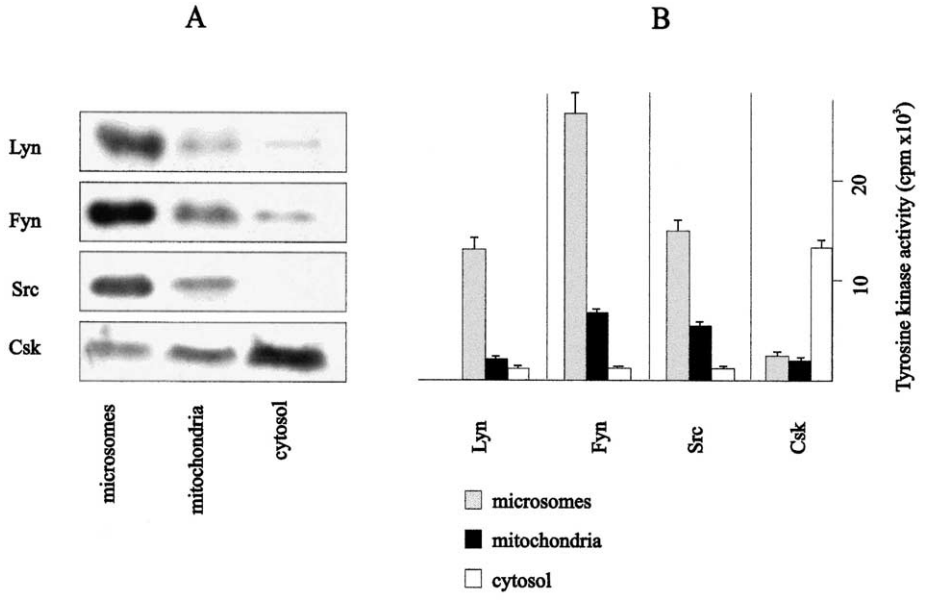

Fig. 6. Subcellular distribution of Lyn, Fyn, Src and Csk in rat brain. (A) Detection of Lyn, Fyn, Src and Csk in differing subcellular fractions using specific antibodies. 50 μg aliquots of mitochondria, microsomes and cytosol were subjected to SDS–PAGE, transferred to nitrocellulose membranes, and incubated with anti-Lyn, -Fyn, -Src, and -Csk antibodies. (B) Determination of activity of Lyn, Fyn, Src and Csk immunoprecipitated from differing subcellular fractions. Protein extracts (2 mg) from mitochondria, microsomes and cytosol were immunoprecipitated by anti-Lyn, -Fyn, -Src, or -Csk antibodies, and tyrosine kinase activity of immunocomplexes was tested in vitro as described. Values are means ± S.D. of four independent experiments.

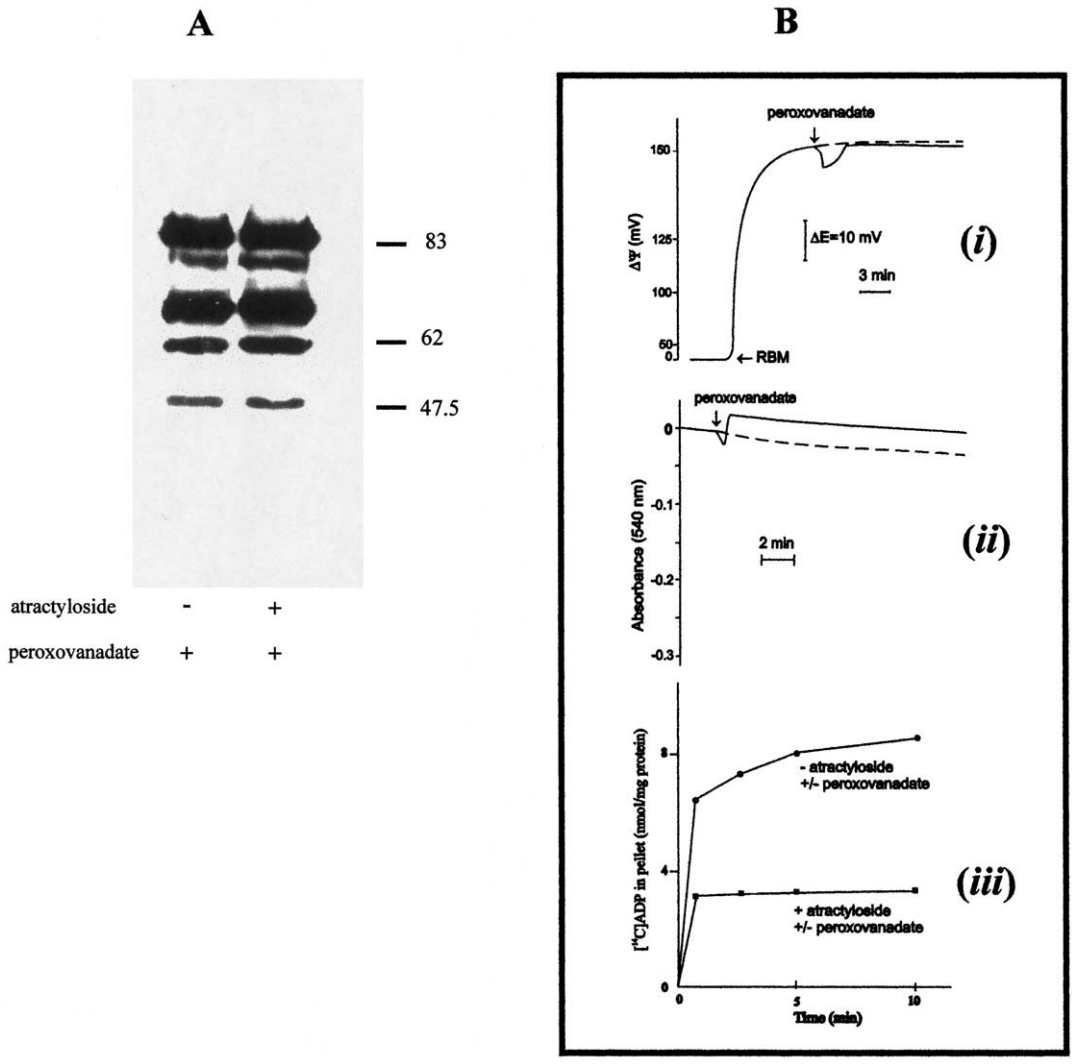

Fig. 7. Tyrosine phosphorylation in intact mitochondria in the presence of atractyloside (A) and determination of mitochondrial integrity in the presence of peroxovanadate (B). (A) RBM (50 μg) were incubated in tyrosine phosphorylation assay reaction medium as described, with 5 μM oligomycin and 2 mM peroxovanadate in the absence or presence of 10 μM atractyloside. Samples were analysed by 0.1% SDS–10% PAGE, and gels were transferred to nitrocellulose membranes and immunostained with anti-phosphotyrosine antibody. Molecular size markers are shown on the right. (B) RBM (50 μg) were incubated in medium as described, with 5 mM succinate, 1.25 μM rotenone and 1 mM phosphate in the absence or presence of 2 mM peroxovanadate. In (iii), the sample is supplemented with [^{14}C]ADP (500 μmol l^{-1}; 1.85 MBq $mmol^{-1}$). (i) Membrane potential, (ii) absorbance measurements and (iii) [^{14}C]ADP uptake.

nostained proteins evaluated as densitometric values. These assays identified Fyn and Src as the most abundant and having the highest total tyrosine kinase activity found in mitochondria; Lyn and Csk are less evident and exhibit lower activities. Comparisons of the ratios among the activities of these enzymes in mitochondria, microsomes and cytosol, taking 1 as the ratio for mitochondria, revealed differences in distribution, as demonstrated by ratios of 1:3.85:0.19 for Fyn, 1:5.88:0.47 for Lyn, and 1:2.86:0.2 for Src. They clearly demonstrate that the amounts of these three kinases, when evaluated at mitochondrial level, are more abundant than in cytoplasm and less abundant than in microsomes. They also indicate that, when compared with other kinases, Src is relatively more represented in mitochondria than in the other fractions. In fact, in these organelles, Src exhibits the highest ratio compared

with that of cytoplasm (5 times higher) and the top of the lower compared with that of microsomes (2.85 times lower).

We next attempted to identify the location of the tyrosine phosphorylation detected in our assays. Intact RBM were incubated in standard medium supplemented with oligomycin and peroxovanadate in the absence or presence of 10 μM atractyloside, an inhibitor of adenine nucleotide translocase. As shown in Fig. 7, the inhibitor does not change the phosphorylation levels of RBM proteins. Furthermore, 2 mM peroxovanadate does not alter the mitochondrial membrane potential, which remains constant (≅160 mV), nor do their osmotic properties change, i.e. no swelling is observed (Fig. 7, inset i and ii, respectively). These observations demonstrate that peroxovanadate does not affect the integrity of the membrane and that, as it does not have any effect on the translocase inhibition by atractyloside, ATP cannot enter the matrix as a result of membrane damage. The observed partial uptake in the presence of the inhibitor accounts for its entry into the inner membrane space (Fig. 7, inset iii). Therefore, the kinases and their substrates appear to be located outside the matrix space. Subsequent experiments dealt with identification of their specific location.

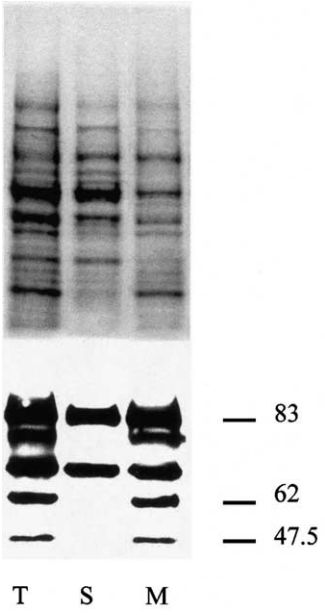

Fig. 8. Endogenous tyrosine-phosphorylated proteins. RBM (50 μg) were incubated in tyrosine phosphorylation assay reaction medium as described, with 5 μM oligomycin and 2 mM peroxovanadate. RBM were then broken (T) and mitochondrial membranes (M) were separated from the soluble fraction (S) as described. Samples were analysed by 0.1% SDS–10% PAGE, and gels were transferred to nitrocellulose membranes and immunostained with anti-phosphotyrosine antibody. Molecular size markers are shown on the right (bottom). Coomassie stained gel of broken mitochondria: mitochondrial membranes and soluble fraction (top).

To study subcompartmentalization of substrates, after tyrosine phosphorylation, mitochondria were subfractionated and membranes separated from soluble fractions (see Section 2). The results (Fig. 8) show that all three proteins are located in the membrane fraction (lane M), and that they do not appear in the soluble fraction (lane S). These observations highlight the involvement of the outer membrane and/or external surface of the inner membrane as the location of PTK substrates.

For further information on PTK location, enolase was used as an exogenous substrate (45 kDa) which cannot cross the outer membrane (permeable to molecules lower than 10 kDa). The results (Fig. 9) demonstrate that enolase is much less phosphorylated by a suspension of intact mitochondria than by one of mitochondria previously broken, as described in Section 2. This experiment clearly shows that PTKs are mainly located between the outer and inner membranes of mitochondria, and are not exposed on the external surface.

To identify a more detailed location, intact mitochondria were subjected to digitonin treatment. Digitonin interacts with cholesterol, forming a complex in a 1:1 ratio, resulting in slight molecular disordering of the membrane at low concentrations. Increasing amounts of digitonin have the characteristic selective effect of disrupting the membrane, revealed in release of marker enzymes from brain mitochondria [27]. This differential release takes place from the intermembrane space/outer membrane compartment, because the inner membrane is lacking in cholesterol.

The results (Fig. 10) show the effects of a low concentration of digitonin (0.2 mg/mg mitochondrial protein). After treatment, mitochondria were separated from the supernatant and tyrosine kinase activity was assayed in both fractions by means of exogenous substrate enolase (see Section 2). As shown (Fig. 10, lane a), the supernatant fraction exhibits clear-cut phosphorylation activity, but the mitochondrial fraction does not (lane b). As the exter-

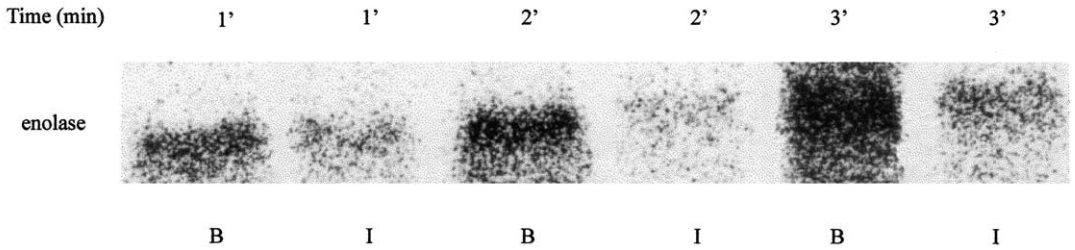

Fig. 9. Tyrosine phosphorylation of exogenous substrate (enolase) by intact and broken mitochondria. Intact (I) and broken (B) RBM (50 μg) were incubated in tyrosine phosphorylation assay reaction medium as described, with 5 μM oligomycin and 2 mM peroxovanadate plus enolase. Samples were analysed by 0.1% SDS–10% PAGE, and gels were subjected to NaOH treatment as described.

nal surface of mitochondria, demonstrated in the experiment with intact mitochondria (Fig. 9), does not exert any tyrosine kinase activity, the observed activity in the supernatant cannot be due to release of PTKs from the external surface of the outer membrane, but is almost certainly due to migration from the intermembrane space. This conclusion is strongly supported by the observation that enzyme release into the supernatant after digitonin treatment is accompanied by release of the intermembrane marker adenylate kinase, but not by that of the outer membrane marker monoamine oxidase, which is detected in the mitochondrial fraction (Fig. 10B). This result is most probably due to some leak pathway which induces efflux from the intermembrane space of soluble enzymes without disrupting or greatly damaging the outer membrane, as previously demonstrated [27]. The same results on phosphorylation activity, which confirm the location of PTKs in the intermembrane space, were achieved with a high concentration of digitonin (0.6 mg/mg mitochondrial protein) (see Fig. 10A). It should be stressed that, in this condition, besides adenylate kinase, monoamine oxidase is also detected in the supernatant fraction, thus explaining outer membrane disruption (Fig. 10B).

The integrity of the inner membrane after treatment with high digitonin concentrations is demonstrated by the detection of cytochrome *c* oxidase (inner membrane) and malate dehydrogenase activity (matrix) only in the fractions of mitoplasts (mitochondria deprived of their outer membrane by concentrated digitonin treatment) (Fig. 10B) and by the high $\Delta\Psi$ value exhibited by these organelles (result not reported). This fact also demonstrates that the

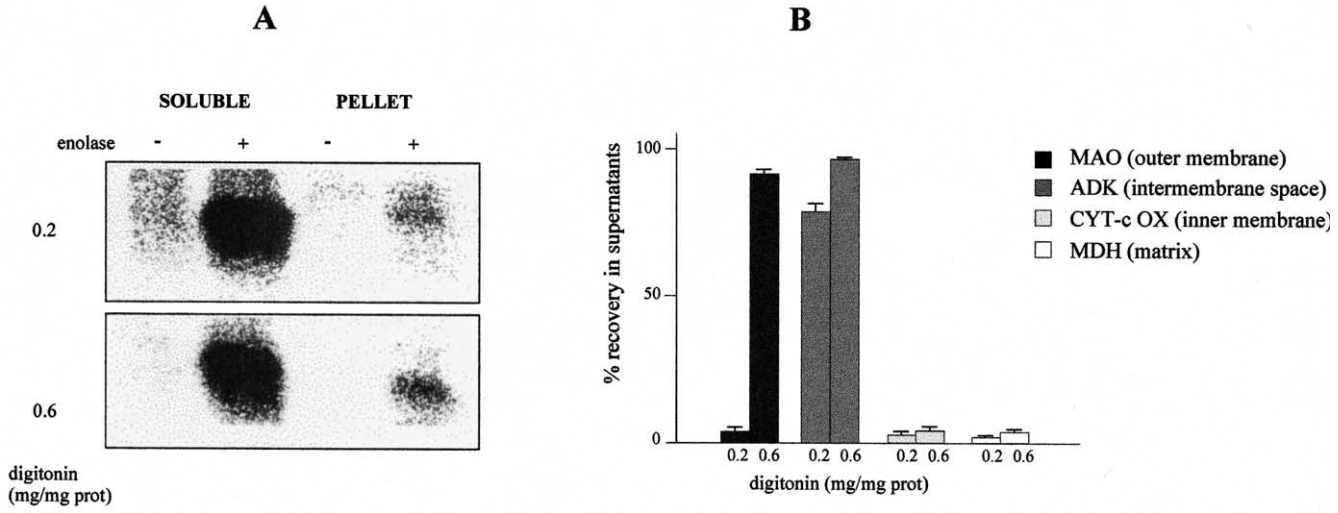

Fig. 10. Tyrosine kinase activity by different fractions of digitonin-treated mitochondria. Mitochondria were fractionated by the digitonin method as described. (A) The resulting two fractions (soluble and pellet) were incubated in tyrosine phosphorylation assay reaction medium as described, with 5 μM oligomycin and 2 mM peroxovanadate plus enolase. Samples were analysed by 0.1% SDS–10% PAGE, and gels were subjected to NaOH treatment as described. (B) Marker enzyme assay on supernatants after digitonin treatment.

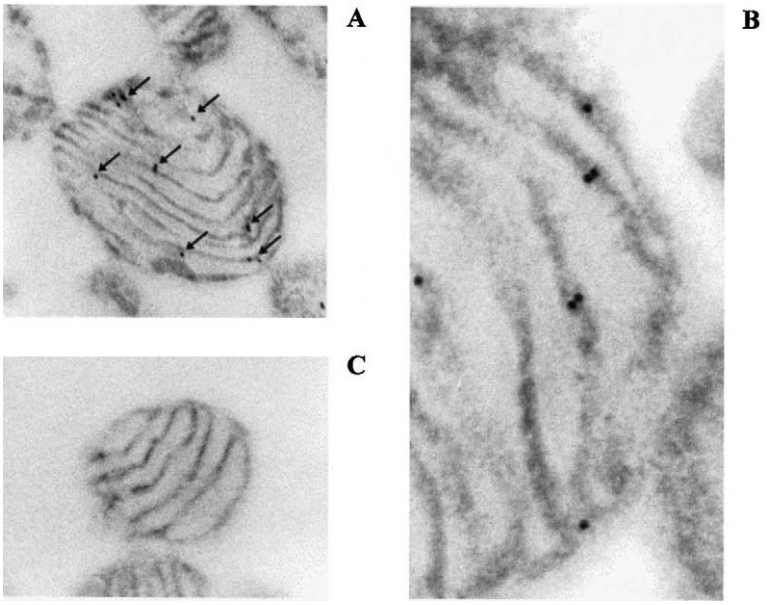

Fig. 11. Immunogold detection of Lyn kinase in RBM. (A,B) Immunogold labelling of Lyn kinase (arrows) of isolated RBM. (A) Magnification, ×20 000. (B) Magnification, ×68 000. (C) As negative control, gold-labelled secondary antibody was applied in the absence of Lyn antibody; no Lyn protein was detected.

PTKs identified in the supernatant are not of inner membrane origin.

To confirm these biochemical results, we used an ultramicroscopic technique for direct visual localization of Lyn kinase in mitochondria. RBM treated with antibodies gold labelled against Lyn kinase were labelled with 5–15 gold particles (Fig. 11A). All the particles were found in the intermembrane space. In this regard, this space can be functionally separated into two subcompartments: the cristal lumen, and the intermembrane space lying between the inner and outer membranes [13]. As shown even more clearly in Fig. 11B, all the labelled gold appears in both subcompartments, but mainly in the cristal lumen. In contrast, no labelling was found in the matrix. In the absence of the Lyn kinase antibody, no mitochondria showed labelling (Fig. 11C).

4. Discussion

The patterns of endogenously phosphorylated mitochondrial proteins detected in isolated mitochondria are very intriguing. Three major phosphoproteins of 50, 60, 75 kDa as well as others of various sizes were detected (see Fig. 2). The requirement of Mn^{2+}, an essential cofactor for in vitro tyrosine kinase activity, the stimulatory effects of peroxovanadate, a well-known inhibitor of tyrosine phosphatase activity [35], and the stability of phosphorylation to alkaline hydrolysis, all clearly demonstrate that RBM contains tyrosine kinase and that it has several endogenous substrates. Maximum phosphotyrosine labelling occurs in the presence of oligomycin, an inhibitor of F_1F_0-ATPase, which protects ATP from hydrolysis. The addition of peroxovanadate produces a substantial increase in phosphorylation on Tyr but does not affect that on Ser and Thr (Fig. 3B). The anti-phosphotyrosine antibody confirms that all three proteins, 50, 60 and 75 kDa, are phosphorylated on tyrosine residues. The necessary presence of peroxovanadate for tyrosine phosphorylation correlated with the inhibition of tyrosine phosphatase activity in rat brain mitochondria (Fig. 4B) clearly shows that the level of tyrosine phosphorylation reflects not only the activity of tyrosine kinases but also protein tyrosine phosphatase in coordination.

The ability of PP2 to alter peroxovanadate-dependent phosphorylation patterns had provided a good indication of the types of tyrosine kinases present in RBM, as PP2 is a highly potent inhibitor of non-receptor tyrosine kinases – mainly of tyrosine kinase belonging to the Src family [36]. Its effects, and those

of genistein, piceatannol and radicicol, other well-known inhibitors of tyrosine phosphorylation (see Fig. 5), had been a good indication that members of the Src family can phosphorylate individual phosphoproteins.

The results (Fig. 6) show that several members of this family (Src, Fyn, Lyn) accompanied by the Src-PTK activity regulator, Csk, are found in RBM, Fyn and Src being the most abundant and active. It is also noteworthy that, compared with other kinases, Src exhibits the highest ratio between mitochondria and microsomes. The activity of Src-PTKs in cells of differing types is negatively controlled through phosphorylation of their C-terminal tyrosine residues by Csk, which is mainly cytoplasmic, whereas Src-PTKs are predominantly plasma membrane-associated. The presence of Src-PTKs and their substrates, accompanied by Csk in brain mitochondria, indicates that these organelles give rise to regulated tyrosine phosphorylation/dephosphorylation closely associated with their functions. As discussed above, this hypothesis is strongly supported by the identification of tyrosine phosphatase activity (Fig. 4B).

Many efforts have been made to establish the exact location of tyrosine kinases and their substrates. The results reported here clearly show that the 50, 60 and 75 kDa proteins, substrates of mitochondrial PTKs, are membrane-bound (Fig. 8), particularly on the inner surface of the outer membrane and/or the outer surface of the inner membrane. Although the identity of these proteins cannot yet be established precisely, a 75 kDa protein has been identified, belonging to the heat shock family, present in the mitochondrial fraction of rat hepatoma cells, which undergoes a marked increase in tyrosine phosphorylation level [40]. Tyrosine phosphorylation of this protein is selective, as other members of this family do not exhibit phosphorylation. The 60 kDa protein Grb 10, belonging to a small family of adapter proteins and found in several tumours and cell lines, has been recognized as a mitochondrial protein, most probably located in the outer membrane [41]. Grb 10 has also been identified as a direct substrate for Src-PTK members [42] and may play a role in signalling regulation between plasma membrane receptors and the apoptosis-inducing machinery located on the mitochondrial outer membrane [41]. Experiments are in progress to verify whether the above-mentioned 75 kDa heat shock and Grb 10 proteins are also present in brain mitochondria. Moreover, a Src kinase-dependent channel, closely involved in the regulation of apoptosis is present in the plasma membrane [43], a similar channel has very recently been identified in the inner mitochondrial membrane (I. Szabò, pers. commun.).

Src-PTKs are generally extrinsic proteins, bound to plasma and intracellular membranes by hydrophobic interactions occurring between their myristoylated and/or palmitoylated tails and membrane proteins having selective affinity for acylated proteins [44]. The results reported in this paper show that most activity occurs in the intermembrane space and less in the mitoplast fraction, meaning that a few kinases are also inner membrane-bound. As mentioned above, acylation of these proteins is necessary for their membrane insertion [3,4], but some myristoylated Src molecules have also been found free in the cytosol [45], indicating that myristoylation does not guarantee membrane association. Indeed, non-myristoylated Src molecules are also found in the soluble fraction [46]. Although the results reported here do not allow us to state whether the Src-PTKs identified in the intermembrane space are acylated or not, a very important point must be emphasized. Some reviews have reported that Src-PTKs need to remain at the membrane in order to signal and that their detachment from it means loss of activity and of specific location. It is recognized that non-myristoylated and soluble myristoylated proteins still retain their specific properties [46] and the abrogation of myristoylation may lead to a highly specific alternative pattern of location [45]. The immunogold experiment (results shown in Fig. 11) clearly demonstrates the presence of Lyn kinase in brain mitochondria. In particular, its location appears to be associated with cristal structures. However, taking into account the results reported above, demonstrating that most Src kinases are present in the intermembrane space, Lyn kinase should also be present in the cristal lumen.

Src-PTKs regulate a plethora of events, including cell growth, division, differentiation, survival, and death of eukaryotic cells, and are particularly important in controlling communications between and within cells through signal transduction pathways [5–8]. Several members of the Src family, including

Src, Fyn, Lyn and Yes, are known to be highly expressed in the central nervous system and play important roles in both its development and function [47]. The possibility that, besides the recognized endogenous substrates, other exogenous peptides or proteins able to cross the inner membrane can be phosphorylated by Src-PTKs, allows speculation on the role played by these enzymes. A very recent paper reports that genistein, a specific natural tyrosine kinase inhibitor [37], can induce apoptosis of RPE-J cells [48], perhaps due to opening of the mitochondrial permeability transition pore which promotes cytochrome *c* release, a phenomenon closely connected with apoptosis [11–13]. Results obtained in our laboratory have also shown that genistein can induce permeability transition in isolated brain mitochondria, whereas peroxovanadate is a strong inhibitor of the phenomenon (manuscript in preparation). These observations, which must be confirmed using other specific inhibitors of tyrosine kinases and phosphatases, fit the hypothesis that tyrosine phosphorylation/dephosphorylation is involved in the modulation of neuronal pro-apoptotic signal transduction.

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