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Research Article

Tyrosine phosphatase activity in mitochondria: presence of Shp-2 phosphatase in mitochondria

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Abstract. Tyrosine phosphorylation by unidentified enzymes has been observed in mitochondria, with recent evidence indicating that non-receptorial tyrosine kinases belonging to the Src family, which represent key players in several transduction pathways, are constitutively present in mitochondria. The extent of protein phosphorylation reflects a coordination balance between the activities of specific kinases and phophatases. The present study demonstrates that purified rat brain mitochondria possess endogenous tyrosine phosphatase activity. Mitochondrial phosphatases were found to be capable of dephosphorylating different exogenous substrates, including paranitrophenylphosphate, ^{32}P-poly(Glu-Tyr)$_{4:1}$ and ^{32}P-angiotensin. These activities are strongly inhibited by peroxovanadate, a well-known inhibitor of tyrosine phosphatases, but not by inhibitors of alkali or Ser/Thr phosphatases, and mainly take place in the intermembrane space and outer mitochondrial membrane. Using a combination of approaches, we identified the tyrosine phosphatase Shp-2 in mitochondria. Shp-2 plays a crucial role in a number of intracellular signalling cascades and is probably involved in several human diseases. It thus represents the first tyrosine phosphatase shown to be present in mitochondria.

Key words. Mitochondria; tyrosine phosphatase; Shp-2.

Protein tyrosine phosphorylation is a post-translational modification that plays a crucial role in many cell regulatory processes, including mitosis, differentiation, cell cycle control, survival and apoptosis, as well as development, growth and neurotransmitter signalling [1, 2]. It is therefore not surprising that functional perturbation via deregulation of protein tyrosine kinase and/or phosphatase activities is linked to several human diseases, including cancer and diabetes [3, 4].

Many of the major signal transduction pathways are mediated by tyrosine phosphorylation (via kinases) and/or dephosphorylation (via phosphatases) of tyrosine residues found in various intracellular proteins. Signal transduction involving tyrosine phosphorylation is generally initiated at cell membrane receptors by tyrosine kinase activity. Besides receptor tyrosine kinases, a group of intracellular kinases, exemplified by the Src family (e.g. Src, Fyn, Lyn, Fgr), may serve as control switches in a variety of signal transduction pathways governing essential cell processes [5, 6]. The inner surface of the plasma membrane has been considered the main site of this activity [1–3]. However, Src family activity has also been detected in association with the nuclear envelope and endoplasmic reticulum membranes [7].

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A previous paper [8] reported that treatment of highly purified rat brain mitochondria (RBM) with the oxidizing agent peroxovanadate, which acts as a phosphotyrosine phosphatase inhibitor, increases the levels of mitochondrial protein tyrosine phosphorylation, and three major substrate proteins have been detected [8]. These proteins are membrane bound and located on the inner surface of the outer membrane and/or the external surface of the inner membrane [8]. Immunoblotting and immunoprecipitation assays of mitochondrial lysates revealed 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 [8]. These tyrosine kinases were detected mainly on the surface of the inner membrane, facing the intermembrane space [8]. These biochemical data were accompanied by the results of immunogold microscopy performed on isolated mitochondria, which revealed the presence of Lyn [8]. 'In situ' immunogold analyses of mitochondria in whole cerebral cortex tissue showed that the Fyn, Src, Lyn and Csk tyrosine kinases are constitutive components of mitochondria (M. Salvi, A. Stringaro, A. M. Brunati, E. Agostinelli, G. Arancia and A. Toninello, unpublished data).

Baron and co-workers very convincingly identified the first mitochondrial protein tyrosine-phosphorylated by c-Src, cytochrome c oxidase (Cox) [9]. Moreover, they showed that c-Src directly modulates the efficiency of the mitochondrial electron transport chain at the level of complex IV [9]. Cox activity is in fact correlated with c-Src kinase activity: it decreases when c-Src is down-regulated by the overexpression of Csk or a dominant-negative mutant of c-Src, and increases when c-Src activity is up-regulated by the expression of a dominant-negative mutant of Csk, or in c-Src-deficient cells [9].

The level of tyrosine phosphorylation of a given protein is determined by the balance between the competing activities of protein tyrosine kinases and protein tyrosine phosphatases. The coordination of these activities renders this post-translational modification reversible and is modulated according to the different needs of the cell. The family of protein tyrosine phosphatases is divided into two major groups: receptor-like phosphatases, which contain one transmembrane domain and one or two phosphatase domains at the C terminus, and non-receptor tyrosine phosphatases, which lack a transmembrane domain and are either cytosolic or peripherally membrane associated [for reviews, see refs 10–14].

To our knowledge, tyrosine phosphatase activity in mitochondria has not been described in the literature. The present study therefore aimed at verifying the presence of tyrosine phosphatase activity in mitochondria in assays carried out using three exogenous substrates having different and quite generic motifs, in order to detect a broad spectrum of enzymes. We then investigated the locations of detected activities in various compartments of isolated mitochondria and identified a specific tyrosine phosphatase, Shp-2, in mitochondria.

Materials and methods

Materials

[γ^{32}P]ATP was purchased from Amersham, protease inhibitor cocktail from Roche, anti-Shp-2 polyclonal antibody from Santa Cruz Biotechnology, anti-flavoprotein of succinate ubiquinone reductase (complex II) monoclonal antibody from Molecular Probes, anti-Bcl-2 antibody from Santa Cruz Biotechnology, anti-PMCA from Upstate, anti-Golgi 58K antibody from Sigma Aldrich, and anti-calreticulin from Upstate. Calpeptin was obtained from Calbiochem (Darmstadt). Peroxovanadate was prepared by mixing hydrogen peroxide (3 mmol/l) and sodium orthovanadate (2 mmol/l). Excess H_2O_2 was removed with catalase (1000 units/ml). Other reagents were purchased from Sigma.

Preparation of mitochondria

Rat brain mitochondria were purified by the Ficoll gradient method, according to Nicholls [15], with some modifications [8]. Briefly, cerebral cortex was homogenized in isolation medium (320 mM sucrose, 5 mM Hepes, 0.5 mM EDTA, pH 7.4) plus 0.3% bovine serum albumin (BSA) and subjected to centrifugation (900 g) for 5 min. The supernatant was centrifuged at 17,000 g for 10 min to precipitate crude mitochondrial pellets. The pellets were resuspended in isolation medium plus 1 mM ATP and layered on top of a discontinuous gradient of Ficoll diluted in isolation medium, composed of 2-ml layers of 16% (w/v), 14% and 12% Ficoll, and a 3-ml layer of 7% Ficoll. Following centrifugation for 30 min at 75,000 g, mitochondrial pellets were suspended in isolation medium and centrifuged again for 10 min at 800 g. The resulting pellets were suspended in isolation medium without EDTA, and their protein content was measured by the biuret method with BSA as a standard [16].

As summarized in table 1, the absence of other contaminating subcellular compartments in our mitochondrial preparations had been demonstrated in previous studies, which detected negligible traces of acetylcholinesterase, lactate dehydrogenase [17], NADPH-cytochrome c reductase and glucose-6 phosphatase [8] activity. Furthermore, electron microscopy demonstrated the complete absence of contaminating membrane fragments in the preparations [13], and Western blot analysis of the RBM to detect other extramitochondrial markers (β-actin, PCNA, Bip, PDGF-R, caveolin) yielded completely negative results [8].

To separate mitochondrial membranes from the soluble fraction, 10 mg/ml of mitochondria were sonicated in an MSE Sonicator and subjected to eight freeze/thaw cycles.

Table 1. Absence of contaminants in brain mitochondrial preparations.

Method	Extra mitochondrial markers	Observations
Electron microscopy	contamining membrane fragments and other organelles [19]	absent
Western blot analysis	β-actin, PCNA, Bip, PDGF-R, caveolin [8]	not detectable
Enzymatic activity	acetylcholinesterase [19], lactate dehydrogenase [19], NADPH-cytochrome c reductase [8], glucose-6-phosphatase [8]	negligible

Mitochondrial suspensions were then centrifuged at 100,000 g to obtain membrane pellets and supernatant fractions.

Determination of mitochondria integrity
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 [18].

Marker enzymes
Monoamine oxidase activity was assayed as described by Tabor et al. [19]. Adenylate kinase (ADK), Cox, and malate dehydrogenase (MDH) activities were determined according to Dorbani et al. [20].

Digitonin treatment
Submitochondrial fractions were obtained by the digitonin extraction method (0.2 or 0.6 mg digitonin/mg of mitochondrial protein), as described by Dorbani et al. [20].

Assays for ^{32}P-Tyr phosphatase activity
Phosphatase assays using ^{32}P-poly(Glu-Tyr)$_{4:1}$ and ^{32}P-angiotensin were carried out as described elsewhere [21, 22], by incubating RBM (50 µg) at 30 °C for 15 min in a 30-µl reaction mixture containing 150 pmol of the ^{32}P-labelled substrate. The released ^{32}P was quantified as described previously [21, 22].
The phosphatase assay using paranitrophenylphosphate (p-NPP) as a substrate is based on the formation of paranitrophenol from p-NPP at 410 nm as described in Clari et al. [21].

Anti-Shp-2 immunoprecipitation
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 pre-cleared by incubation with protein A-Sepharose for 45 min at 4 °C. Samples (1 mg mitochondrial protein) were then incubated overnight at 4 °C with anti-Shp-2 antibody bound to protein A-Sepharose. Immune complexes were washed three times by centrifugation and resuspensed in 50 mM Tris/HCl, pH 7.5.

Immune complex phosphatase assays
Tyrosine phosphatase assays of immune complexes, obtained as described above, were performed in 30-µl incubation mixtures containing 50 mM Tris/HCl, pH 7.5, plus the exogenous substrate. After incubation for 10 min at 30 °C, released ^{32}P was quantified as described above.

Proteinase K treatment
Purified RBM were treated with 50 ng/ml proteinase K in isolation medium without EDTA (see preparation of mitochondria) in the absence or presence of 0.5% Triton X-100 at room temperature for 30 min. The reaction was stopped by the addition of protease inhibitor cocktail, and then analysed by Western blotting using antibodies to Shp-2, Bcl-2 and the flavoprotein of succinate ubiquinone reductase (complex II).

Subcellular fractionation
Rat brain (250 mg) was homogenized in 1 ml of isolation medium (250 mM sucrose, 5 mM Hepes, pH 7.4) and subjected to centrifugation for 10 min at 900 g (nuclei fraction, pellet I). The supernatant was then centrifuged for 1 h at 100,000 g to separate cytosol from the post-nuclear particulate fraction (pellet II). The two pellets were resuspended in 1 ml of isolation medium.
Subcellular fractionation of the post-nuclear particulate fraction was performed using OptiPrep (Accurate Chemical and Scientific Corporation). A discontinuous gradient was prepared with 30, 25, 20, 15 and 10% OptiPrep solution. A 200-µl sample of the post-nuclear particulate fraction was overlaid on the discontinuous gradient and centrifuged at 100,000 g for 1 h at 4 °C. The gradient was removed in 15 equal fractions of 200 µl collected from the top of the gradient; 50 µl of each fraction was analysed by Western blotting.

Immunogold analyses
Rat brain cortical tissue samples were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) by the perfusion method, dehydrated in an ethanol gradient and embedded in Lowicryl HM20 resin (Electron Microscopy Sciences. The resin was polymerized under UV light for 2 h at 4 °C and for 22 h at room temperature. Ultrathin sections were then prepared using an LBK ultramicrotome (Ultratome Nova), collected on gold grids, and treated for 5 min with PBS containing 0.15% (w/v) glycine. After washing by quickly floating the grids on

PBS drops containing 0.4 % (w/v) gelatin and 0.1 % (w/v) BSA, the sections were incubated overnight at 4 °C with a polyclonal antibody against Shp-2. Then, after washing by floating the grids in PBS drops containing 0.4 % (w/v) BSA for 1 h at room temperature, the sections were pre-incubated with PBS plus 0.4% (w/v) gelatin and 0.1% (w/v) BSA for 10 min, washed in PBS containing 0.1% (w/v) BSA, and then labelled with anti-rabbit IgG conjugated to 10-nm gold particles (diluted 1:10; Sigma) for 20 min at room temperature. Control sections were prepared by omitting the primary antibody and pre-treating with a specific blocking peptide (Santa Cruz Biotechnology), applied in a fivefold excess compared to the anti-phosphatase antibody diluted in the specific blocking buffer [PBS containing 0.1 % (w/v) BSA]. Incubation was carried out overnight at 4 °C.

Ultrathin sections were stained with uranyl acetate and lead citrate for examination under a transmission electron microscope (Philips 208; Fei Company).

Results

Tyrosine phosphatase activities in RBM

The presence of protein tyrosine phosphatase (PTP) activity was measured in highly purified RBM using the exogenous phosphatase substrates ^{32}P-poly(Glu-Tyr)$_{4:1,}$ ^{32}P-angiotensin and p-NPP. p-NPP does not contain a phosphotyrosine but is commonly used as a generic substrate to test for PTP activity. ^{32}P-poly(Glu-Tyr)$_{4:1}$ is a mixture of synthetic peptides of differing molecular weights, consisting of phosphotyrosines surrounded by glutamic acid residues, with a constant 4:1 ratio of Glu to Tyr. ^{32}P-angiotensin is a natural peptide in which phosphotyrosine is surrounded by neutral residues (DRVYIHPF).

Results of phosphatase assays revealed dephosphorylation of all three substrates by mitochondrial lysate (fig. 1). Assays carried out in varying conditions indicated that pNPP-ase exhibits maximum activity at pH 5 in the presence of 5 mM Mg^{2+} (fig. 1 A). PTP activity detected using ^{32}P-poly(Glu-Tyr)$_{4:1}$ showed two peaks of activity with different sensitivities to pH and Mg^{2+}, one at pH 7 in the presence of 5 mM Mg^{2+}, and the other at pH 8 in the absence of Mg^{2+} (fig. 1 B). PTP activity measured using ^{32}P-angiotensin was most active at pH 7.5, in the presence of 1 mM Mg^{2+} (fig. 1 C). To ascertain the presence of true tyrosine phosphatase activity instead of an action of broad-specificity alkaline and Ser/Thr phosphatases, we used various inhibitors: okadaic acid, a general inhibitor of Ser/Thr phosphatases; a cocktail I phosphatase inhibitor set containing bromotetramisole oxalate, a specific inhibitor of alkaline phosphatases; and two inhibitors of Ser/Thr phosphatases, cantharidin and microcystin. All these compounds had no effect on observed tyrosine phosphatase activities (table 2). PTPs, unlike alkaline or Ser/Thr phosphatases, catalyse phosphate hydrolysis via a covalent phosphocysteine intermediate, which is therefore critical for its activity. Peroxovanadate inhibits tyrosine phosphatases by irreversibly oxidizing catalytic cysteine [23]. In our assay, peroxovanadate strongly inhibited all these activities, indicating the presence of true PTPs in mitochondrial lysate (table 2).

Distribution of tyrosine phosphatase activities between soluble and membrane fractions

We next attempted to determine the sub-mitochondrial location of PTP and pNPP-ase activities by measuring them in two different mitochondrial fractions: the soluble fraction, represented by the intermembrane space and matrix, and the membrane fraction, represented by the outer and inner membranes. The activities of (ADK) and (MDH) were measured as markers for the membrane and soluble fractions, respectively (see inset, fig. 2).

Results showed that pNPP-ase and PTP activities were detected in both soluble and membrane fractions, but to different extents (figs. 2 A–D).

Localisation of tyrosine phosphatase activities

To examine the location of these activities in more detail, intact mitochondria were extracted with two different concentrations of digitonin, as described previously [8]. Digitonin interacts with cholesterol molecules of the outer mitochondrial membrane, forming a complex in a 1:1 ratio and resulting in dose-dependent, progressive molecular disordering of the membrane until its disruption. This leads to selective release of proteins from the intermembrane space/outer membrane compartments, which can be traced using marker enzymes [15]. The inner membrane is not affected by this treatment, as it lacks cholesterol.

Figure 3 shows the effects of a low concentration of digitonin (i.e. 0.2 mg/mg mitochondrial protein) (gray columns); after treatment, mitochondria were separated from the supernatant and PTP and pNPP-ase activities were assayed in the supernatant and pellet. pNPP-ase (fig. 3 A) and PTP activities for ^{32}P-poly(Glu-Tyr)$_{4:1}$ (pH 7, 5 mM Mg^{2+}) and ^{32}P-poly(Glu-Tyr)$_{4:1}$ (pH 8, –Mg^{2+}) were found to be equally distributed between the supernatant and pellet (fig. 3 B). In contrast, the activity detected using ^{32}P-angiotensin as substrate was much more evident in the supernatant than in the pellet (fig. 3 C).

The observation that phosphatase activity in the supernatant after low-concentration digitonin treatment was accompanied by release of the intermembrane marker ADK strongly suggests that the phosphatase(s) reside in this sub-compartment. In contrast, the outer membrane marker monoamine oxidase was not released (fig. 3 D). This result most probably reflects a leakage, which induces efflux from the intermembrane

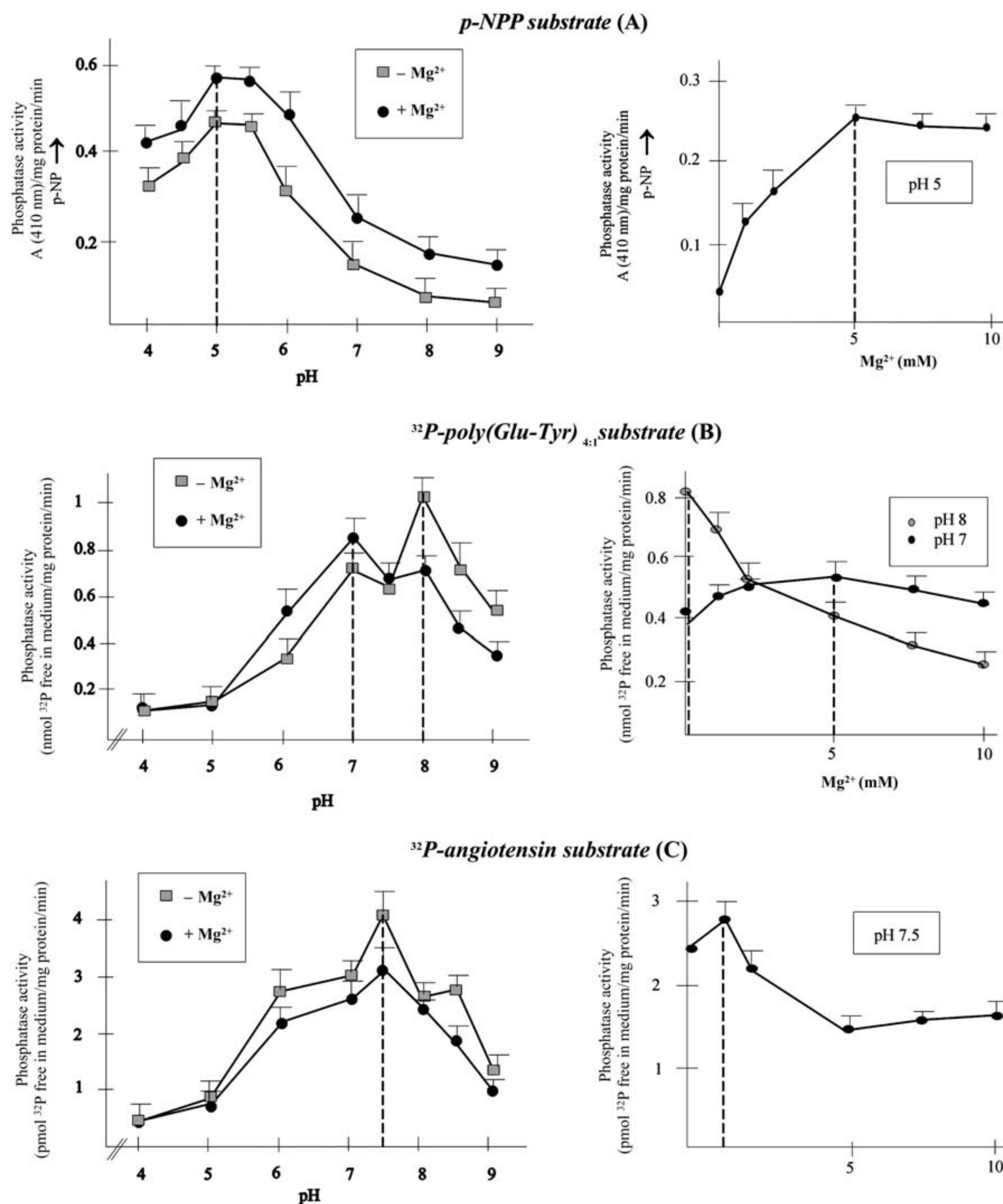

Figure 1. Tyrosine phosphatase activity in RBM; pH and Mg^{2+} dependence. pNPP-ase activity against paranitrophenylphosphate (*A*) and PTP activity against ^{32}P-poly(Glu-Tyr)$_{4:1}$ (*B*) and ^{32}P-angiotensin (*C*) was measured at various pHs (4–9) in the absence or presence of 5 mM Mg^{2+} (left) and at the pH indicated, in the presence of indicated concentrations of Mg^{2+} (right). Reported values are means + SD from three separate experiments for each pH and Mg^{2+} concentration.

space of soluble enzymes without disrupting or greatly damaging the outer membrane, as previously demonstrated [8].

Extraction with a high concentration of digitonin (i.e. 0.6 mg/mg mitochondrial protein) disrupted the outer membrane, resulting in the release of both ADK and monoamine oxidase in the supernatant fraction, with very little activity in the pellet, which consisted of mitoplasts (i.e. outer membrane-deprived mitochondria) (fig. 3D). In these conditions, most of the phosphatase activity measured using all three substrates was found in the supernatant fraction (fig. 3A–C), indicating that these activi-

Table 2. Effect of inhibitors with different specificity on the tyrosine phosphatase activities observed.

Inhibitor	Activity (% of the control)			
	PNPP substrate	^{32}P-poly(Glu-Tyr)$_{4:1}$ substrate pH 7 + Mg^{2+}	^{32}P-poly(Glu-Tyr)$_{4:1}$ substrate pH 8 – Mg^{2+}	^{32}P-angiotensin substrate
Inhibitor cocktail I	99.7 ± 0.5	100 ± 0.5	98.9 ± 0.6	99.4 ± 0.5
Okadaic acid	100 ± 0.6	99.8 ± 0.6	99.9 ± 0.6	99 ± 0.6
Peroxovanadate	12 ± 2.5	10 ± 3.5	8 ± 3.5	12 ± 3

pNPP-ase activity against p-NPP and PTP activity against ^{32}P-poly(Glu-Tyr)$_{4:1}$ and ^{32}P-angiotensin were measured as described in Materials and methods in the presence or absence of 1/100 inhibitor cocktail I (Sigma), 5 µM okadaic acid and 0.5 mM peroxovanadate.

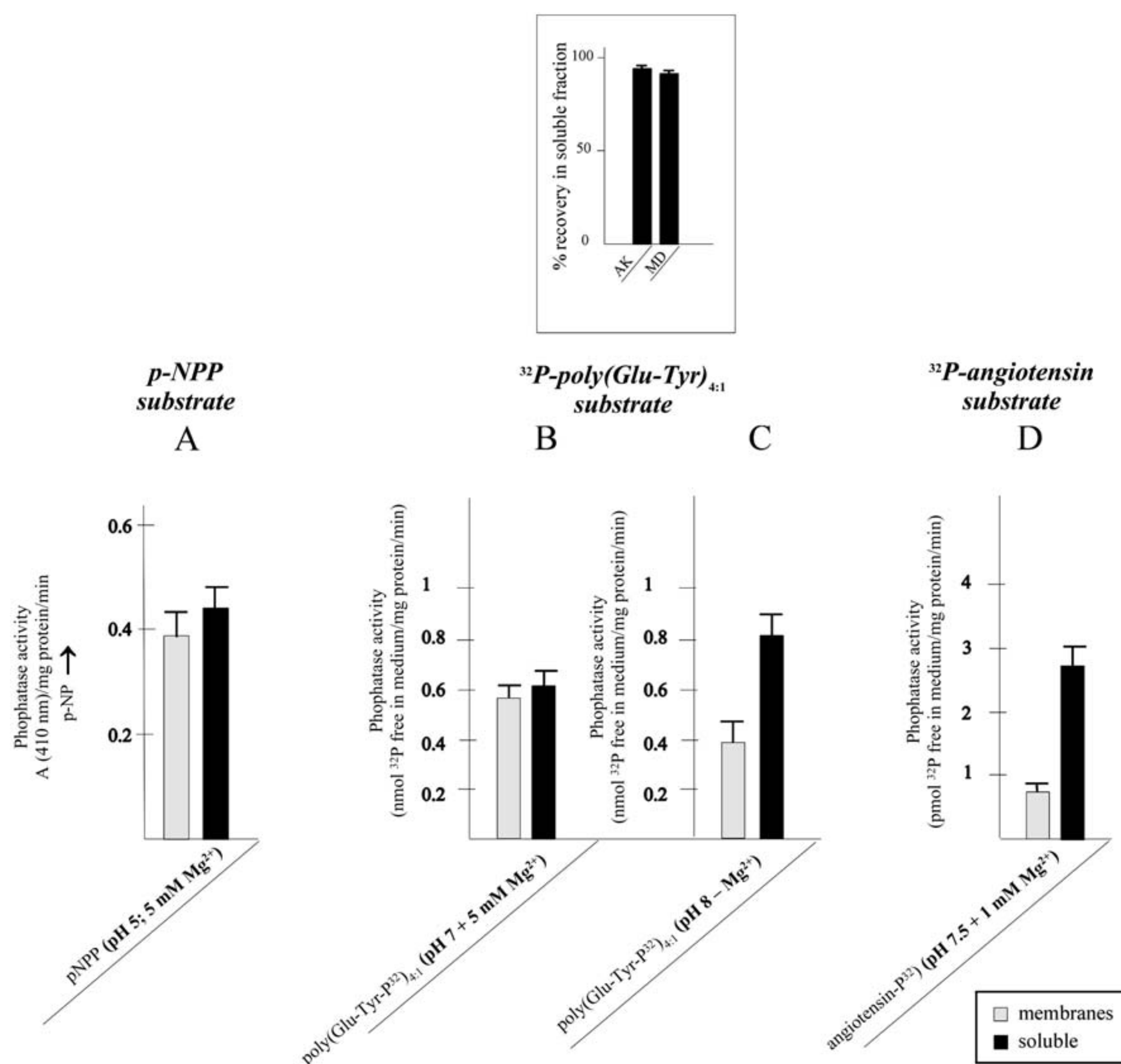

Figure 2. Tyrosine phosphatase activity in soluble and membrane fractions. Mitochondria were separated into soluble and membrane fractions, as described in Materials and methods. Inset: ativities of ADK and MDH recovered in the soluble fraction. pNPP-ase activity against p-NPP (*A*) and PTP activity against ^{32}P-poly(Glu-Tyr)$_{4:1}$ (*B*, *C*) and ^{32}P-angiotensin (*D*) were measured at indicated pH and Mg^{2+} concentrations, shown as described in Materials and methods. Each column represents a mean + SD of four independent experiments.

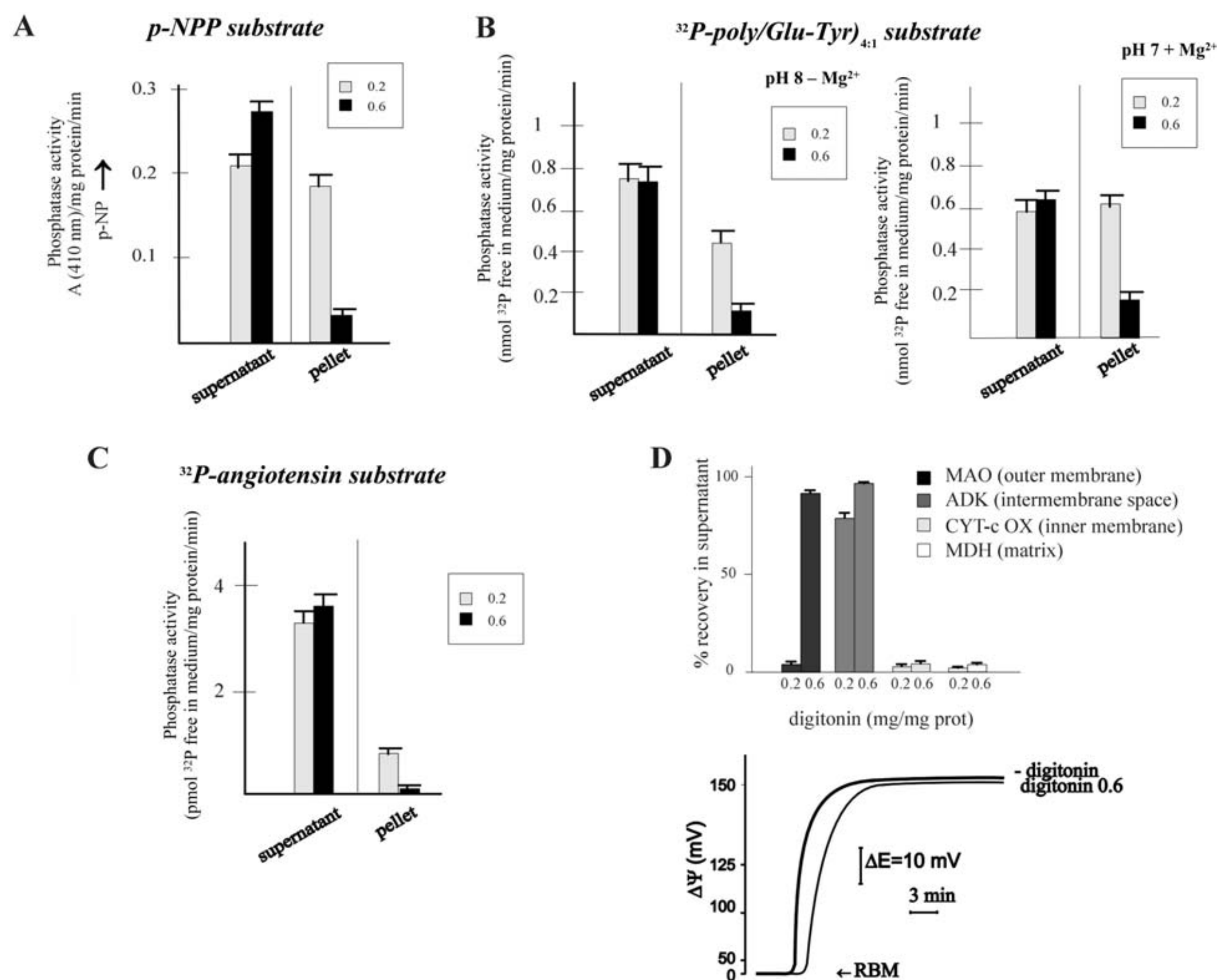

Figure 3. (*A–C*) Tyrosine phosphatase activity in various fractions of digitonin-treated mitochondria. Mitochondria were fractionated by the digitonin method and resulting soluble and pellet fractions were assayed for phosphatase activity, as described in Materials and methods. Each column represents the mean + SD of four independent experiments. (*D*) Results of marker enzyme assays of supernatant and mitochondrial $\Delta\Psi$ values after digitonin treatment.

ties are mainly located in the outer membrane (see black columns).

The integrity of the inner membrane after treatment with high digitonin concentrations was demonstrated by (i) the detection of Cox and MDH activities, markers for the inner membrane and matrix, respectively, only in the mitoplasts, and (ii) the high $\Delta\Psi$ value exhibited by these organelles (fig. 3 D).

Shp-2: a tyrosine phosphatase constitutively present in mitochondria

The detection of PTP activity mainly in the soluble fraction indicated the presence of non-receptor PTPs. The level of tyrosine phosphorylation of band 3, triggered by members of Src kinases, has recently been reported to be modulated by the action of the tyrosine phosphatase Shp-2, which recognizes the phosphotyrosine on band 3 first as a docking site and then as a target substrate [24]. This study revealed the relationship between members of the Src family and Shp-2 in modulating phosphorylation levels. Shp-2 is a cytoplasmatic SH_2 domain-containing tyrosine phosphatase which is involved in signalling pathways mediated by a variety of growth factors and cytokines; it is highly expressed in brain tissue [for recent reviews, see refs 25–27]. The possible involvement of Shp-2 in the modulation of mitochondrial tyrosine phosphorylation was therefore investigated. Western blot analysis of the mitochondrial lysates with anti-Shp-2 revealed one band at 72 kDa, which corresponds to the apparent size of Shp-2 (fig. 4 A, a); a primary antibody pre-treated with a Shp-2 blocking peptide failed to reveal this band, showing the specificity of the antibody-protein interaction (fig. 4 A, b). To verify whether Shp-2 is located inside or peripherally associated with the outer membrane of mitochondria, we

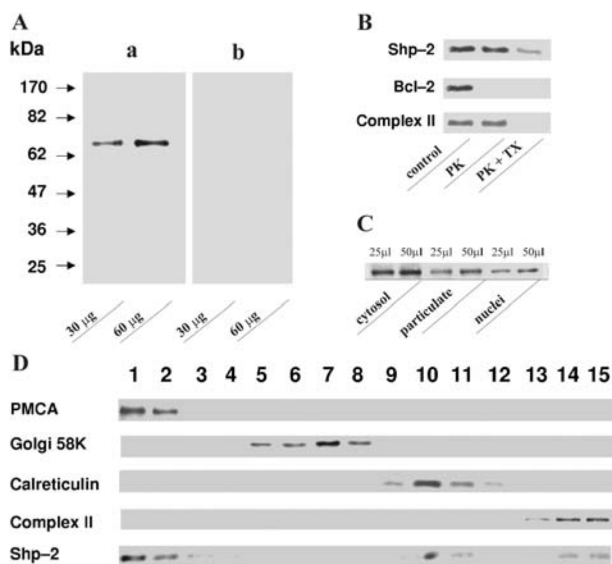

Figure 4. Shp-2 location in purified mitochondria and subcellular fractions. (*A*) Detection of Shp-2 by Western blotting. Aliquots (30 and 60 µg) of mitochondria were subjected to SDS-PAGE, transferred to nitrocellulose and incubated with anti-Shp-2 antibody (a) or an anti-Shp-2 antibody previously incubated with specific blocking peptide (b). (*B*) Intact RBM were treated with 50 ng/ml proteinase K (PK) in the absence or presence of 0.5% Triton X-100 (TX) at room temperature for 30 min. The reaction was analysed by Western blotting using the antibodies anti-Shp-2, -Bcl-2, -flavoprotein of complex II. (*C*) Sub-cellular fractionation of homogenised brain lysate. Aliquots (25 and 50 µl) of cytosol, the post-nuclear particulate fraction and nuclei were analysed by Western botting using the antibody anti-Shp-2. (*D*) The post-nuclear particulate fraction was centrifuged at discontinuous Optiprep gradients, as described in Materials and methods, and aliquots of resulting fractions were analysed by Western blotting using the antibodies anti-PMCA (plasmatic membrane), anti-Golgi 58K (Golgi complex), anti-calreticulin (endoplasmatic reticulum), anti-flavoprotein of complex II (mitochondria) and anti-Shp-2.

assessed the sensitivity of the mitochondria-associated Shp-2 to proteinase K. Intact brain mitochondria were incubated with proteinase K in the absence or presence of Triton X-100, and mitochondrial proteins were Western blotted for Shp-2, Bcl-2 and flavoprotein of complex II. As shown in figure 4B, flavoprotein of complex II, which is located inside mitochondria at the level of the inner membranes, was fully protected from proteinase K in the absence of detergent, whereas Bcl-2, which is peripherally associated with the external mitochondrial membrane, was completely degradated, regardless of whether Triton X-100 was present or not. The major part of Shp-2 was not degraded by proteinase K in the absence of Triton X-100, suggesting that Shp-2 is mainly located inside mitochondria (fig. 4B).

To determine the quantity of mitochondrial Shp-2 relative to other cell compartments, brain lysate was subfractionated into cytosol, nuclei and a post-nuclear particulate fraction and Western blotted using anti-Shp-2 antibody. As expected, Shp-2 was present mainly in the cytosol, but also present in nuclear and post-nuclear particulate fractions (fig. 4C). The post-nuclear particulate fraction was further separated by ultracentrifugation on Optiprep discontinuous gradients (fig. 4D). These fractions were Western blotted using anti-Shp-2 and organelle-specific antibodies, including anti-plasma membrane Ca^{2+} ATP-ase (PMCA) (plasmatic membrane), anti-Golgi 58K (Golgi complex), anti-calreticulin (endoplasmatic reticulum) and anti-flavoprotein of complex II (mitochondria). The plasma membrane was present in the lighter fractions 1–2. The Golgi complex was distributed between fraction 5–8. In fractions 9–12, endoplasmatic reticulum was present, whereas mitochondria were found to be distributed between fractions 13–15. Shp-2 was distributed in almost all fractions, demonstrating its association with various intracellular membranes, including mitochondria (fig. 4D). Mitochondrial Shp-2 represents about 20% of the total amount of the enzyme present in the post-nuclear particulate fraction.

Next, we immunoprecipitated Shp-2 from mitochondrial lysate and tested the ability of the immunoprecipitate complex to dephosphorylate exogenous substrates. In the particular conditions of pH and Mg^{2+} used, immunoprecipitated Shp-2 was able to dephosphorylate only ^{32}P-poly(Glu-Tyr)$_{4:1}$; no dephosphorylation was observed using p-NPP or ^{32}P-angiotensin as substrates (fig. 5A). Dephosphorylation of ^{32}P-poly(Glu-Tyr)$_{4:1}$ was fully inhibited by calpeptin (fig. 5A), a selective inhibitor of Shp-2 [24, 28].

To confirm the involvement of Shp-2 in the dephosphorylation of the three substrates by mitochondrial lysate (figs 1–3) and to assess the contribution of Shp-2 to mitochondrial tyrosine phosphatases, we tested these activities in the presence and absence of the selective Shp-2 inhibitor calpeptin. Results showed that calpeptin is without effect on the dephosphorylation of p-NPP, ^{32}P-angiotensin and ^{32}P-poly(Glu-Tyr)$_{4:1}$ at pH 8 in the absence of Mg^{2+}. Instead, it inhibits the activity of the lysate on ^{32}P-poly(Glu-Tyr)$_{4:1}$ at pH 7 in the presence of 5 mM Mg^{2+}, by about 20%, matching the activity of immunoprecipitated Shp-2 (see fig. 5B).

Figure 6 shows the results of immunogold electron microscopy performed on ultrathin sections of rat cortex with an anti-Shp-2 polyclonal antibody. Although the mild fixation and Lowicryl-embedding procedure were not optimal in terms of morphological preservation, they were found to be essential to retain immunological reactivity.

Gold particles corresponding to Shp-2 labelling were observed mainly in mitochondria and myelin sheets (fig. 6A). Higher magnification revealed intense labelling on the electron-dense areas of mitochondria and, taking into account previous observations on isolated mitochondria [8], they are presumed to be associated with the cristae

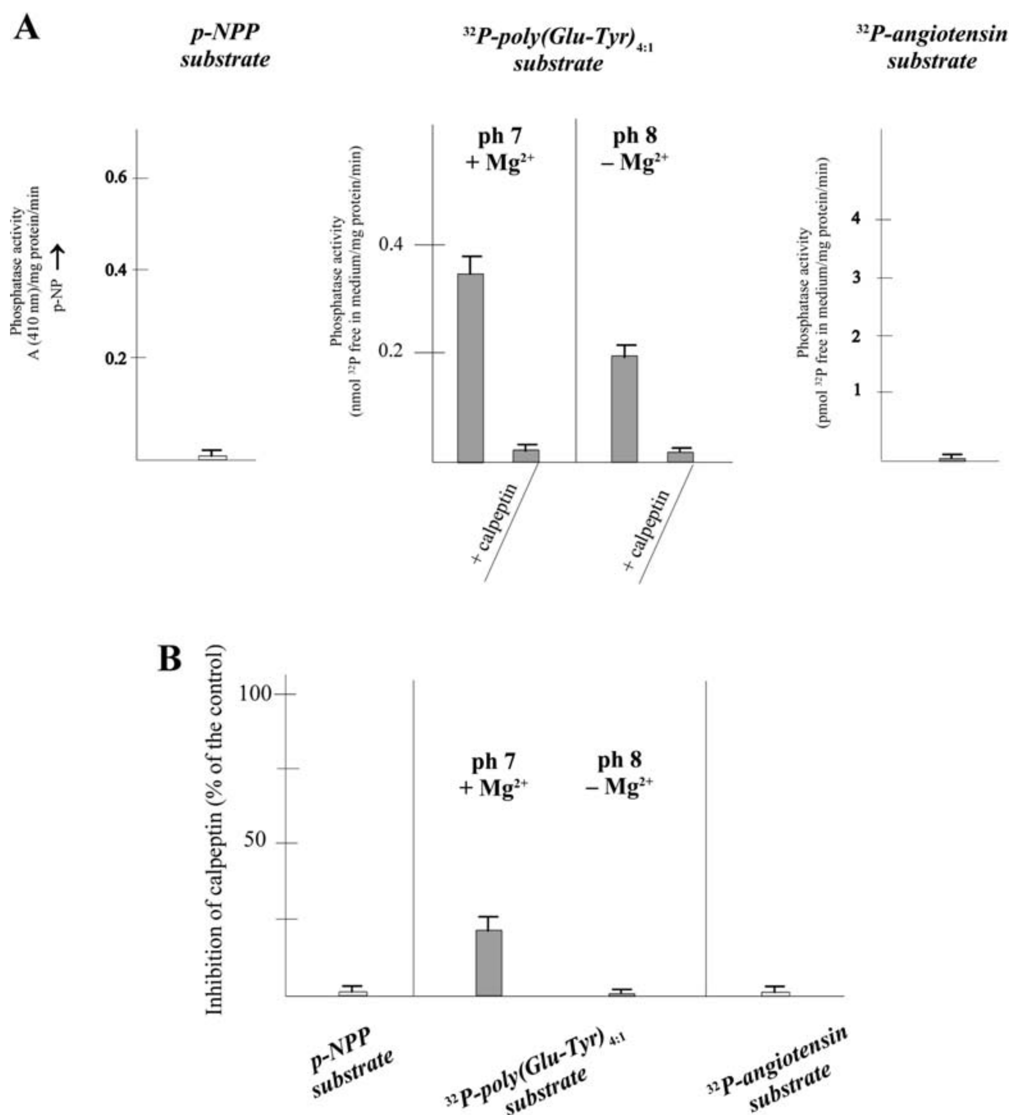

Figure 5. Dephosphorylation of three exogenous substrates by mitochondrial Shp-2. (*A*) Detection of Shp-2 activity in RBM. Protein extracts (1 mg) from mitochondria were immunoprecipitated with anti-Shp-2 antibody. Resulting immunocomplexes were then assayed for tyrosine phosphatase activity against three exogenous substrates, in the presence or absence of 0.5 mg/ml calpeptin. Reported values are means + SD from three separate experiments. (*B*) Effect of calpeptin on tyrosine phosphatase activity of mitochondrial lysate. Activities were measured as decribed in Materials and methods, in the presence or absence of 0.5 mg/ml calpeptin. Reported values are means + SD from three separate experiments.

and/or intracristal space; there was, however, no labelling of the outer mitochondrial membrane.
The cytoplasmic matrix appeared to be almost completely negative, since the weak fixation was not able to avoiding extracting of its soluble components during the subsequent steps of preparation.
Mitochondria were not labelled in the absence of primary antibody, thus confirming the specificity of the secondary antibody (fig. 6B). Pre-treatment of the sections with the primary antibody with an excess of Shp-2 blocking peptide prior to incubation also abolished labelling, thereby confirming the specificity of the primary antibody (fig. 6C). Higher magnification clearly showed the absence of mitochondria labelling in these conditions.
These results provide independent confirmation that Shp-2 is an intrinsic component of brain mitochondria.

Discussion

Over the past 20 years, several studies have provided evidence for the existence of unidentified tyrosine kinase activity in mitochondria [29–32]. Using different methodological approaches, we recently demonstrated that mitochondria harbor tyrosine kinases of the Src family as well as Csk [8]. Identification of these kinases in mitochondria was initially surprising, as these enzymes were generally thought to be primarily associated with the

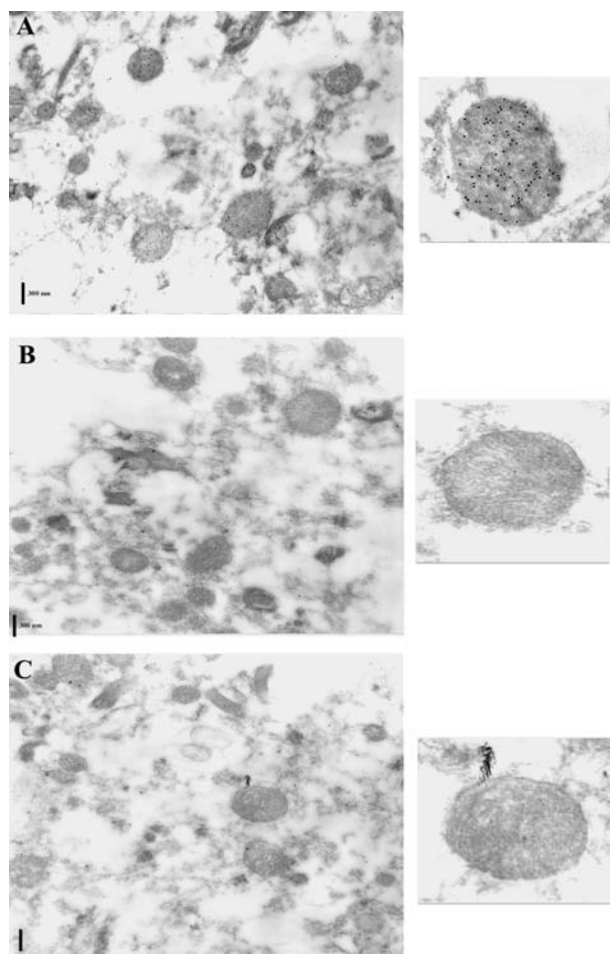

Figure 6. Immunogold detection of Shp-2 phosphatase in RBM. (*A*) Section of rat cerebral cortex incubated with Shp-2 antibody, followed by gold-conjugated secondary antibody. (*B*, *C*) Negative controls, in which gold-labelled secondary antibody was applied in the absence of Shp-2 antibody (*B*) and in which the section was pretreated with an excess of Shp-2 competitor peptide prior to incubation with primary and secondary antibodies (*C*).

plasma membrane, endoplasmic reticulum and nuclear envelope (for reviews, see refs 5–7).

The data reported in the present study provide first evidence of the presence of tyrosine phosphatase in mitochondria. Taking into account previous observations demonstrating the presence of tyrosine kinases [8], one may state that tyrosine phosphorylation in mitochondria is a reversible process which depends on the activities of kinases and phosphatases.

We showed that RBM possess different tyrosine phosphatases able to dephosphorylate three different exogenous substrates, i.e. p-NPP, ^{32}P-poly(Glu-Tyr)$_{4:1}$ and ^{32}P-angiotensin. Tyrosine phosphatases are generally accepted to exhibit some degree of substrate specificity, although their consensus sequences are not that rigid, a property which allows PTPs to recognize phosphopeptides that deviate from their 'ideal' substrate [10, 11]. These activities are strongly inhibited by peroxovanadate (table 2), a well-known inhibitor of tyrosine phosphatases which interact with the thiol that forms the covalent phosphocysteine intermediate, which is essential for catalysis of phosphate hydrolysis in all PTPs [23]. Inhibitors of Ser/Thr phosphatases, cantharidin, and okadaic acid and microcystin have no effect (table 2). Resistance to bromotetramisole oxalate and the pattern of activity versus pH clearly distinguish them from the alkaline phosphatases, which show optimal activity at pH 9 and sensitivity to tetramisole [33]. Indeed, Mg^{2+} may behave in different ways on PTP activity: as inhibitor [34], essential cofactor [35] or with no effect [36].

pNPP-ase activity (maximum at pH 5, 1 mM Mg^{2+}) mainly occurs in the intermembrane space and in the outer membrane (figs 2A, 3A). PTP activity detected using ^{32}P-poly(Glu-Tyr)$_{4:1}$ at pH 7 in the presence of 5 mM Mg^{2+} shows the same distribution (figs 2B, 3B). In contrast, activity detected using this substrate at pH 8 in the absence of Mg^{2+} (figs 2C, 3B), as well as that detected using ^{32}P-angiotensin, mainly occurs in the intermembrane space (figs 2D, 3C).

PTPs may be divided into two groups: soluble and transmembrane. All PTPs contain at least one highly conserved catalytic domain of approximately 240 amino acids with a unique motif [10, 11]. Our data indicate the presence of both receptorial and non-receptor tyrosine phosphatases in RBM. The protein phosphatase Shp-2, a non-receptor protein which possesses two SH2 domains followed by a tyrosine phosphatase domain and a C-terminal extension (for reviews, see refs 25–27), is at least partly responsible for the observed soluble PTP activities in mitochondria. The presence of Shp-2 in mitochondria was demonstrated by Western botting (fig. 4A), proteinase K treatment (fig. 4B), cell fractionation (fig. 4C, 4D), immunoprecipitation (fig. 5A) and, to avoid completely the possible artefactual consequence of the cell fractionation process, by 'in situ' immunogold analyses of mitochondria in whole rat cerebral tissue (fig. 6). Shp-2 is mainly located inside mitochondria and seems to be associated with cristae and/or the intercristal space. The observation that Shp-2 immunoprecipitated by RBM shows activity only against ^{32}P-poly(Glu-Tyr)$_{4:1}$ (fig. 5A) allows us to suggest the existence of other mitochondrial enzymes in RBM which are able to dephosphorylate p-NNP and ^{32}P-angiotensin. The partial inhibition by calpeptin of only ^{32}P-poly(Glu-Tyr)$_{4:1}$ in the activity of mitochondrial lysate (fig. 5B) confirms this statement. The specificity that we observed is confirmed by a recent report demonstrating the strong preference of Shp-2 for an acidic amino acid at position –2 [37].

Extensive studies have demonstrated the importance of protein tyrosine phosphorylation in neuronal survival, cell fate determination, axon guidance and connectivity. Many PTPs are expressed in the developing nervous sys-

tem and several maintain expression into adulthood. Since its discovery in the early 1990s, considerable knowledge has been gained regarding the structure and regulation of Shp-2, the biological consequences of changes in its concentration and its involvement in several transduction pathways. Transgenic mice overexpressing a dominant-negative form of Shp-2 are more susceptible than controls to ischaemia-induced brain damage and neuronal death [38]. A recent report demonstrated that the catalytic activity of Shp-2 is required to regulate the PI3K/Akt pathway and thus probably participates in anti-apoptotic signalling by suppressing caspase 3-mediated apoptosis [39]. Moreover, Shp-2 appears to play a pivotal role in several human diseases. For example, Shp-2-activating mutations are found in about 50% of patients with Noonan syndrome, an autosomal dominant disorder characterized by skeletal and cardiac defects [40]. In addition, somatic Shp-2 mutations have been found in about 20–25% of sporadic juvenile myelomonocytic leukaemia cases [41]. The inappropriate activation of Shp-2 may also be important in the pathogenesis of *Helicobacter pylori*, the major cause of gastric ulcer and carcinoma worldwide [42].

In conclusion, RBM contain a complete tyrosine phosphorylation system composed of endogenous tyrosine kinase and tyrosine phosphatases, mainly located at the level of the intermembrane space and the membranes facing it. The discovery of the new specific subcellular location of Shp-2 in the organelle that plays a critical role in controlling the life and death of cells may therefore open up new perspectives regarding cell regulatory processes.

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