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Src-Tyrosine Kinases Are Major Agents in Mitochondrial Tyrosine Phosphorylation

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Abstract Mitochondrial tyrosine phosphorylation is emerging as an important mechanism in regulating mitochondrial function. This article, aimed at identifying which kinases are the major agents in mitochondrial tyrosine phosphorylation, shows that this role should be attributed to Src family members. Indeed, various members of this family, for example, Fgr, Fyn, Lyn, c-Src, are constitutively present in the internal structure of mitochondria as well as Csk, a key enzyme in the regulation of the activity of this family. By means of different approaches, biochemical fractioning, Western blotting and immunogold analysis ''in situ'' of phosphotyrosine signaling, evidence is reported on the existence of a signal transduction pathway from plasma membrane to mitochondria, resulting in increasing Src-dependent mitochondrial tyrosine phosphorylation. The activation of Src kinases at mitochondrial level is associated with the proliferative status where several mitochondrial proteins are specifically tyrosine-phosphorylated. J. Cell. Biochem. 104: 840–849, 2008.

Key words: mitochondria; phosphorylation; tyrosine kinases; Src tyrosine kinase family

Protein phosphorylation/dephosphorylation is one of the major regulatory systems of signal transduction contributing to the control of almost all aspects of cell life. About 30% of cell proteins have a bound phosphate and abnormal levels of phosphorylation are crucial factors in several pathologies [Hunter, 1995; Cohen, 2002]. Furthermore, the importance of phosphorylation/dephosphorylation in the regulation of mitochondrial processes has recently been recognized and several protein kinases and phosphatases have been identified in mitochondria [Salvi et al., 2002, 2004, 2005; Thomson, 2002; Pagliarini et al., 2005; Pagliarini and Dixon, 2006]. Phosphorylation in mitochondria involves not only serine/threonine but also tyrosine and histidine residues. In particular, mitochondrial tyrosine phosphorylation is emerging as a central mechanism in regulating mitochondrial functions [Salvi et al., 2005; Pagliarini and Dixon, 2006], and the possible involvement of tyrosine phosphorylation in mitochondrial signaling is discussed in depth in a recent review [Salvi et al., 2005]. Cytochrome *c* oxidase (COX), the terminal respiratory complex in the respiratory chain, has been identified as a substrate for the tyrosine kinase c-Src [Miyazaki et al., 2003]. C-Src phosphorylates COX subunit II in vitro and in osteoclasts; it activates COX and, in turn, modulates the efficiency of the mitochondrial electron transport chain [Miyazaki et al., 2003]. C-Src belongs to the Src family of protein tyrosine kinases (SFKs), which have been implicated as critical regulators of a large number of intracellular signaling pathways from various cell membrane receptors to several

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cytoplasmic targets, most notably those controlling cell growth, proliferation and differentiation, survival and adhesion, and many of the members of this family have been identified as cellular oncogenes [Parsons and Parsons, 2004; Yeatman, 2004]. There are 11 members of the Src-family kinases in humans: Blk, Brk, Fgr, Frk, Fyn, Hck, Lck, Lyn, Src, Srm, and Yes [Manning et al., 2002]. Their main location is the cytoplasmic surface of the plasma membrane and cytoskeletal structures but evidence is also reported for other subcellular locations [Brown and Cooper, 1996; Parsons and Parsons, 2004; Yeatman, 2004]. The association of the activity of SFKs with mitochondria has recently been investigated, and SFKs in the internal structures of mitochondria were identified as Lyn tyrosine kinase [Salvi et al., 2002] and c-Src tyrosine kinase [Miyazaki et al., 2003]. The association of c-Src with mitochondria also seems to be dependent on the presence of two anchoring proteins: AKAP121 and Dok-4. AKAP121 is a well-known anchoring protein of protein kinase A (PKA) to outer mitochondrial membranes, which acts as multifunctional protein complexes and may bind several kinases and phosphatases. It has been demonstrated that, besides PKA, AKAP121 binds protein tyrosine phosphatase D1 (PTPD1) in HEK293 cells. PTPD1 activates c-Src and binds it by anchoring the activated enzyme to the outer mitochondrial membrane [Cardone et al., 2004; Livigni et al., 2006]. However, new evidence reveals that endogenous AKAP121 is also located in the inner mitochondrial membranes [Sardanelli et al., 2006] where c-Src and Lyn kinases have also been detected (see above).

The second anchoring protein, Dok-4, is an adapter protein, structurally characterized by an NH2-terminal tandem of conserved pleckstrin homology (PH) and the phosphotyrosine-binding (PTB) domain, linked to a single COOH-terminal region. The overexpression of Dok-4 increases the association of c-Src to the mitochondrial fraction in endothelial cells, whereas its reduction by RNA interference decreases its association. The submitochondrial location of Dok-4 has not been investigated [Itoh et al., 2005].

Considering the emerging role of tyrosine phosphorylation in mitochondria, the aim of this study is to demonstrate the constitutive presence in the internal structure of rat brain mitochondria (RBM) of SFKs, the identification of these kinases, and to investigate the possible presence of the Csk kinase in mitochondria, which should modulate SFK activity by phosphorylation.

Here we show that SFKs are the major players in mitochondrial tyrosine phosphorylation and, by means of different approaches, we report evidence of the existence of a signal transduction from plasma membrane to mitochondria, resulting in increased SFK-dependent mitochondrial tyrosine phosphorylation.

MATERIALS AND METHODS

Materials

Anti-phosphotyrosine monoclonal antibodies were purchased from ICN Biotechnology; anti-polyclonal antibodies against c-Src, Fyn, Fgr, Lyn, and Csk from Santa Cruz Biotechnology; anti-flavoprotein of succinate ubiquinone reductase (Complex II) monoclonal antibody from Molecular Probes; amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo [3,4*d*] pyrimidine (PP2) from Boehringer; and SU-6656 from Calbiochem. The synthetic peptide cdc-2 (KVEKIGEGTYGVVYK) was kindly provided by Dr. O. Marin (University of Padova, Italy). Other reagents were purchased from Sigma.

Cell Culture

The N2A cell line was cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, at 37°C in a 5% CO_2 incubator.

Mitochondrial Isolation and Purification

Rat brain was homogenized in isolation medium (250 mM sucrose, 1 mM EDTA, 5 mM Hepes, pH 7.4) and subjected to centrifugation (900*g*) for 5 min. The supernatant was centrifuged at 12,000*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

12,000*g*. The resulting pellets were suspended in isolation medium without EDTA, and their protein content was measured by the biuret method, with bovine serum albumin as a standard.

The high purity of the RBM obtained using the procedure applied here is exemplified in previous articles demonstrating the negligible degree of activity of acetylcholinesterase [Nicholls, 1978] and lactate dehydrogenase [Nicholls, 1978; Ciman et al., 1992] in such preparations—it is also demonstrated that the mitochondria are free of plasma membrane and cytosol contamination. In addition, electron microscopy has demonstrated the complete absence of contaminating membrane fragments in the preparations [Ciman et al., 1992]. In order to verify that our own preparations were free of microsomes, a subcellular fraction with high tyrosine kinase activity, frequently present in isolated mitochondria, was assayed for two microsomal marker enzymes, NADPH-cytochrome *c* reductase and glucose-6-phosphatase, and they turned out to be almost undetectable (Table I).

NADPH-cytochrome *c* reductase activity was assayed as described by Sottocasa et al. [1967]. Glucose-6-phosphatase activity was measured according to Swanson [1950].

Phosphorylation Assays

Tyrosine phosphorylation was assayed by incubating 50 μg of brain homogenate or brain mitochondria at 30°C for 10 min in 30 μl of reaction medium containing: 50 mM Tris, pH 7.5, 10 mM $MnCl_2$ and 20 μM [γ-^{33}P]ATP (3×10^6 cpm/nmol) and 1 mg/ml random polymer poly(Glu-Tyr)$_{4:1}$ (Sigma–Aldrich) which served as exogenous substrate. After incubation, samples were analyzed by SDS–PAGE and revealed by a Cyclone Storage Phosphor Imager (Packard).

Assay of the tyrosine kinase activity of Optiprep™ fractions was analyzed by adding 10 μl of each fraction in a final 30 μl of incubation mixture containing 50 mM Tris/HCl, pH 7.5, 10 mM $MnCl_2$, 20 μM [γ-^{33}P]ATP (3×10^6 cpm/nmol), 200 μM sodium orthovanadate and 200 μM cdc2 peptide, which served as specific substrates for SFKs. After 10 min incubation at 30°C, the samples were analyzed by SDS–PAGE and revealed by a Cyclone Storage Phosphor Imager.

Immunogold Analyses

Rat brain cortical tissue samples were processed as previously described [Salvi et al., 2004]. The ultrathin sections were incubated overnight at 4°C with anti-tyrosine kinase polyclonal antibodies and subsequently labeled with anti-rabbit IgG 10-nM gold conjugate (diluted 1:10; Sigma) as described in [Salvi et al., 2004]. Control samples were obtained by omission of the primary antibody. For blocking/competition, we used the specific blocking peptide (Santa Cruz Biotechnology, Inc.) with a fivefold excess compared with the anti-kinase antibody diluted in the specific blocking buffer (PBS containing 0.1% (w/v) BSA). Incubation was carried out overnight at 4°C.

For ultrathin cryosections, cells were fixed with 4% paraformaldehyde plus 0.5% glutaraldehyde in PBS, pH 7.4, for 2 h at 4°C, washed, and embedded in 2% agarose low melting point (LMP), solidified on ice. Agarose blocks were infused with 2.3 M sucrose in PBS overnight at 4°C, frozen in liquid N_2, and cryosectioned following the method described by Tokuyasu (1973). Ultrathin cryosections obtained on a Leica Ultracut UCT device (Leica Microsystem, Vienna, Austria), were collected by means of sucrose and methylcellulose and incubated with the antiphosphotyrosine antibody, then revealed with a goat anti-mouse 10-nM gold conjugate (1:10 diluted, Sigma–Aldrich, Milan, Italy). Lastly, ultrathin cryosections were stained with solutions of 2% methylcellulose and 0.4% uranyl acetate. Samples were

TABLE I. Activity of NADPH-Cytochrome *c* Reductase and Glucose-6-Phosphatase in Rat Brain Mitochondria and Microsomes

Enzyme	Microsomes (μmol/min/mg prot)	Mitochondria (μmol/min/mg prot)
NADPH-cytochrome *c* reductase	16.2 ± 1.3	0.03 ± 0.004
Glucose-6-phosphatase	11.3 ± 0.5	0.02 ± 0.002

Activity expressed as μmol/min per mg protein. Values are the means ± SD from three independent experiments.

examined under a transmission electron microscope (Philips 208, Fei Company).

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 analyzed by Western blotting with antibodies to specific tyrosine kinase, 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. Two hundred microliters of the post-nuclear particulate fraction was overlaid on the discontinuous gradient and centrifuged at 100,000*g* for 3 h at 4°C. The gradient was removed in 15 equal fractions of 200 μl, collected from the top of the gradient. Fifty microliters of each fraction were analyzed by Western blotting.

Phosphatase λ Treatment

RBM (50 μg) were incubated with 500 U λPPase in phosphatase buffer (50 mM Tris/HCl pH 7.5, 0.1 mM EDTA, 5 mM dithiothreitol, 0.01% BRIJ 35, 2 mM $MnCl_2$) for 45 min at 30°C in the presence of 0.5% Triton X-100. Phosphatase activity was stopped by adding phosphatase inhibitor cocktails 1 and 2 (Sigma) and 1 mM sodium orthovanadate. Samples were then subjected to Western blot analysis.

Cell Viability Assay

Cell viability was detected by means of 3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltetrazolium bromide (MTT) reagent, a tetrazolium salt which is metabolized by mitochondrial dehydrogenases and which produces a purple precipitate in viable cells. Cells were grown in a 96-well plate in various conditions. Then, 1 h before the end of incubation, 10 μl of MTT solution (5 mg/ml in PBS) was added to each well. Incubation was stopped by the addition of 20 μl of lysis solution, consisting of 20% (w/v) SDS, 50% (v/v) *N*,*N*-dimethylformamide, 2% (v/v) acetic acid and 25 mM HCl, at pH 4.7. Plates were read for attenuance (D) at $\lambda = 590$ nm on a Titertek Multiskan Plus plate reader (Flow Laboratories).

Statistical Analysis

The values of each parameter in each group were expressed as arithmetic averages and standard deviations (means ± SD). Where applicable, Student's *t*-test was performed. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

SFKs Are Major Players in Mitochondrial Tyrosine Phosphorylation

When highly purified rat brain mitochondria (RBM) were incubated in the presence of ATP, together with the strong tyrosine phosphatase inhibitor peroxovanadate, endogenous and exogenous substrates become tyrosine phosphorylated [Salvi et al., 2002]. To discriminate the role of various tyrosine kinase families in mitochondria, we tested the kinase activity of brain homogenates (Fig. 1A) and highly purified RBM (Fig. 1B) on a generic poly(Glu-Tyr)4:1 substrate, in the presence or absence of various specific kinase inhibitors. We used two, structurally non-related, SFK inhibitors, PP2 and SU6656; an inhibitor of Syk tyrosine kinase, piceatannol; an inhibitor of JAK kinases, AG490; two inhibitors of receptorial tyrosine kinases, AG1478 for EGF and AG1296 for PDGF respectively; and a more generic inhibitor for receptorial and non receptorial tyrosine kinases, genistein. Figure 1A shows that the tyrosine kinase activity of brain homogenate is inhibited by all these compounds to different extents. Instead, tyrosine kinase activity in RBM is almost completely inhibited by PP2 and SU6656 and partially by genistein, indicating the primary role of SFKs in mitochondria (Fig. 1B). The other inhibitors were almost without effect, except for slight

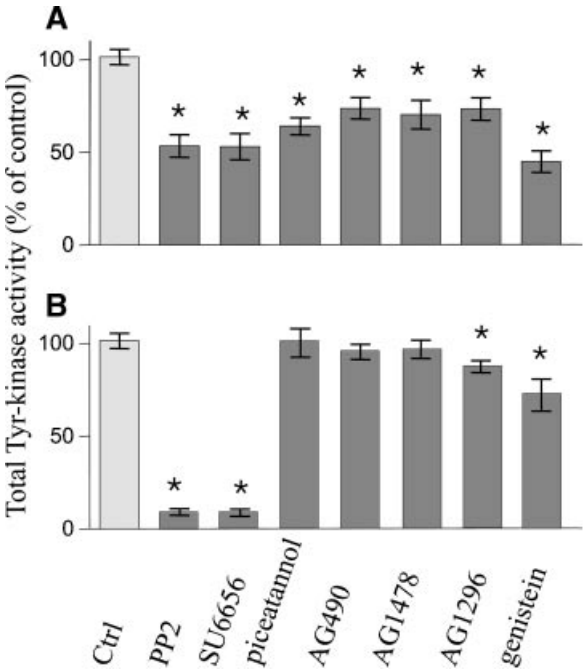

Fig. 1. Tyrosine phosphorylation in brain homogenate (**A**) and isolated RBM (**B**) in presence of various inhibitors. Tyrosine kinase activity of 50 μg of brain homogenate (A) and of RBM (B), was assayed as described in Materials and Methods Section by aspecific peptide substrate poly(Glu-Tyr)4:1, in absence (Ctrl) or presence of: 1 μM PP2, 1 μM SU6656, 5 μM piceatannol, 10 μM AG490, 10 μM AG1478, 10 μM AG 1296, or 50 μM genistein. Data represent means of experiments run in triplicate with SE (*$P < 0.05$).

inhibition (statistically significant; $P < 0.05$) observed in the presence of AG1296 (Fig. 1B), the inhibitor of PDGF, indicative of its possible association with mitochondria.

Different Members of SFKs Are Constitutively Present in Rat Brain Mitochondria

The primary role of SFKs in tyrosine kinase activity associated with mitochondria led us to identify specific members of SFKs and possibly their regulatory kinase Csk in the internal structures of mitochondria. Evidence for an intramitochondrial location have only been reported for two members of SFKs: Lyn kinase [Salvi et al., 2002] and c-Src kinase [Miyazaki et al., 2003]. Here, we used two techniques: direct visualization by immunogold in "in situ" mitochondria of whole rat cerebral cortex, and Western blot detection in mitochondria before and after proteinase K treatment.

Post-embedding immunolabeling experiments revealed the reactivity of a panel of antibodies directed against several protein tyrosine kinases: Fgr (A), Fyn (B), Csk (C), Lyn (D), and c-Src (E). In ultrathin sections of rat cortex, fractions of all these tyrosine kinases appear to be associated with mitochondria (Fig. 2, panels A–D). In particular, in order to investigate the submitochondrial location, as illustrated in the magnification of Figure $2A_2$, which shows a typical situation, the gold particles are clearly associated with dark areas representing internal cristae (intermembrane space and inner membrane) and much less peripherically associated with the outer membrane. The four antibodies supply differing labeling intensities due to the different affinity of the antibody against the various epitopes. However, a different abundance of each kinase cannot be ruled out.

Sections labeled with the gold-conjugated secondary antibody appeared to be completely

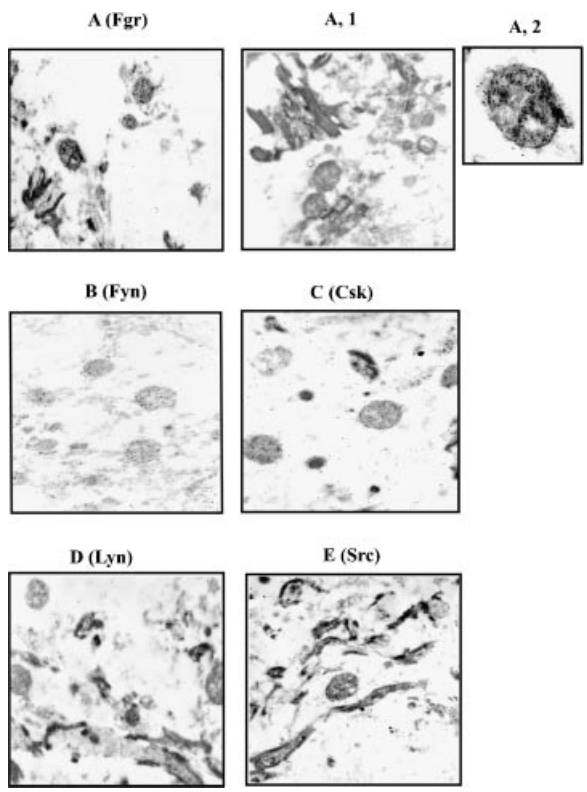

Fig. 2. Detection of intramitochondrial location of Lyn, Fgr, Fyn, Src, and Csk kinases by immunogold on whole cerebral cortex tissue. Thin sections of rat cerebral cortex were incubated with Fgr (**A**), Fyn (**B**), Csk (**C**), Lyn (**D**), and Src (**E**) antibodies, followed by gold-conjugated secondary antibody. **Panel A_1**: negative controls in which gold-labeled rabbit secondary antibody was applied in absence of primary antibody. **Panel A_2**: magnification of a single mitochondrion.

negative (Fig. $2A_1$), showing the lack of aspecificity of the secondary antibody.

The specificity of kinase immunolabeling was further confirmed by blocking assays using specific kinase peptides. The competitive binding exerted by these peptides results in complete inhibition of kinase immunolabeling (results not reported), demonstrating the specificity of the primary antibodies in these conditions.

The intramitochondrial location of SFKs was also then demonstrated by assessing the sensitivity of the mitochondria-associated kinases to proteinase K. Intact RBM were incubated with proteinase K in the absence or presence of Triton X-100, and mitochondrial proteins were Western blotted for specific kinases, Bcl-2, and the flavoprotein of Complex II. As shown in Figure 3, the flavoprotein of Complex II, which is located inside mitochondria at the level of the inner membrane, is fully protected from proteinase K in the absence of detergent, whereas Bcl-2, which is peripherically associated with the outer mitochondrial membrane, is completely degraded, regardless of whether Triton X-100 is present or not. Fgr, Fyn, and Csk are not degraded by proteinase K in the absence of Triton X-100, suggesting that they are mainly located inside mitochondria (Fig. 3). To our knowledge, the intramitochondrial location of Fgr and Fyn and the regulatory kinase Csk is demonstrated here for the first time, whereas the intramitochondrial location of Lyn and c-Src, already clearly shown [Salvi et al., 2002; Miyazaki et al., 2003], is reconfirmed here.

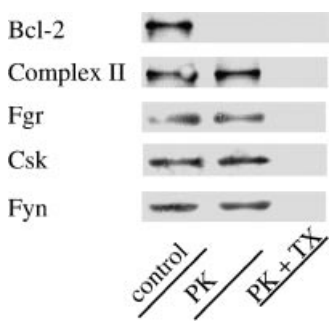

Fig. 3. Detection of intramitochondrial location of Fgr, Fyn, Csk kinases by proteinase K sensitivity. Intact RBM were treated with 50 ng/ml proteinase K in absence or presence of 0.5% Triton X-100 at RT for 30 min. Reaction was analyzed by Western blotting with antibodies anti-Fgr, -Fyn, Csk, -Bcl-2, and anti-Flavoprotein of Complex II.

Triggering of Mitochondrial Tyrosine Phosphorylation in Neuroblastoma N2A Cells

As mentioned above, SFKs play key roles in regulating the signal transduction of fundamental cellular processes, including cell growth, differentiation, cell shape, migration and survival.

Since tyrosine phosphorylation of rat brain mitochondrial proteins is increased by in vitro addition of ATP and H_2O_2 and since Src kinase inhibitor PP2 decreases tyrosine phosphorylation and respiratory rates at state 3 [Augereau et al., 2005], we investigated the phosphotyrosine signal and activation state of SFKs in various conditions on a cellular model: neuroblastoma cells N2A.

We monitored mitochondrial tyrosine phosphorylation in neuroblastoma cells N2A in the presence of serum (10% FBS) (+FBS) and in cells starved overnight (−FBS) (Fig. 4A,B, respectively). The absence of serum in N2A cells is a sufficient condition to shift cells from a proliferation state towards a differentiative one [Shea et al., 1991; Wang et al., 2004]. Several techniques were employed: measurement of SFK activity in differing subcellular fractions, immuno-detection of tyrosine phosphorylated proteins on mitochondrial fractions western blotted on PVDF membranes, and direct visualization of phosphotyrosine labeling of mitochondria "in situ" by electron microscopy (immunogold staining).

For cell fractioning, $N2A_{+FBS}$ and $_{-FBS}$ were lysed and the post-nuclear particulate fraction were ultracentrifuged on Optiprep™ discontinuous gradients (Fig. 4). These fractions were Western blotted using organelle-specific antibodies, including anti-plasma membrane Ca^{2+} ATP-ase (PMCA) (plasma membrane), anti-Golgi 58K (Golgi complex), anti-calnexin (endoplasmic reticulum) and anti-flavoprotein of Complex II (mitochondria). The plasma membrane was present in the lighter fractions 1 and 2. The Golgi complex was distributed between fractions 4 and 7. In fractions 9–11, endoplasmic reticulum was present, whereas mitochondria were found to be distributed between fractions 13–15. All these fractions were assayed for tyrosine kinase activity against the synthetic peptide cdc-2, specific for SFKs. In $N2A_{+FBS}$, SFK-dependent activity is found to different extents in all fractions; in particular, as well as in plasma membrane and

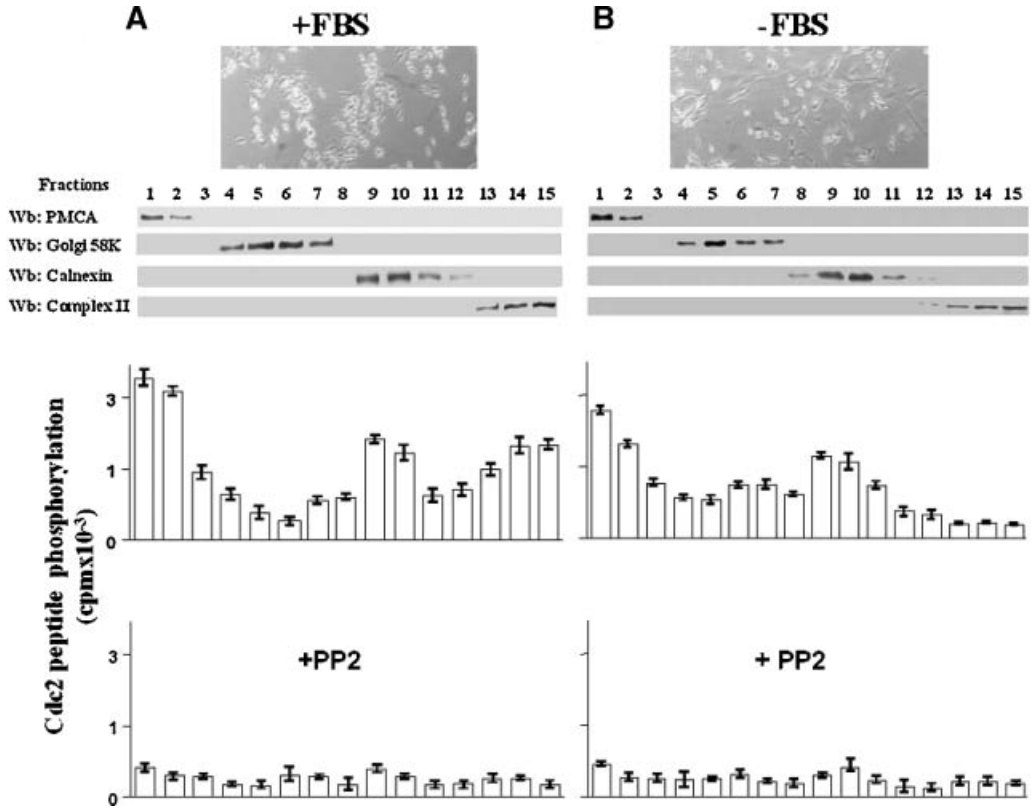

Fig. 4. Tyrosine kinase activity in subcellular fractionation of homogenized N2A cells. Neuroblastoma cells N2A were growth in the presence of serum (10% FBS) (+FBS) (**A**) or deprived of serum overnight (−FBS) (**B**). Particulate fraction from $N2A_{+FBS}$ (A) and ($N2A_{-FBS}$) (B), fractionated by centrifugation on discontinuous Optiprep™ gradients as described in Materials and Methods Section, and aliquots of resulting fractions were analyzed by Western blotting with antibodies anti-PMCA (plasma membrane), anti-Golgi 58K (Golgi complex), anti-calnexin (endoplasmic reticulum), anti-flavoprotein of Complex II (mitochondria). Tyrosine kinase activity of equal amounts of each fraction was also assayed (see Materials and Methods Section) with 200 μM cdc2 peptide, in presence or absence of 1 μM PP2. Data on kinase activity are means of four experiments with SE.

endoplasmic reticulum, a peak of activity also occurred in the mitochondrial fractions. This activity, like the others, was almost completely inhibited in all fractions in the presence of PP2 (see Fig. 4A) or SU-6656 (data not shown), two specific and structurally non-related SFK kinase inhibitors (for PP2, see Hanke et al., 1996; for SU6656, see Blake et al., 2000). The SFK activity of $N2A_{-FBS}$ was slightly reduced in plasma membrane and endoplasmic reticulum, except in mitochondrial fraction, in which it was almost completely abolished (Fig. 4B).

The observation that, in $N2A_{-FBS}$ cells, SFK activity still occurs, although to a reduced extent, at the level of plasma membrane, Golgi and endoplasmic reticulum (Fig. 4B) may be explained by the fact that SFKs have been implicated not only in proliferative pathways but also in differentiating ones [reviewed in Parsons and Parsons, 2004] and the absence of serum is a condition for the induction of differentiation in N2A cells [Shea et al., 1991; Wang et al., 2004].

The strongly reduced tyrosine kinase activity in mitochondrial fractions from $N2A_{-FBS}$ cells should be confirmed by a decrease in the detection of mitochondrial tyrosine phosphorylated proteins. Cell lysates and mitochondrial fractions from $N2A_{+FBS}$ and $N2A_{-FBS}$ were Western blotted on PVDF membrane and stained with antiphosphotyrosine antibody. Phosphotyrosine antibody detected several bands in cell lysates from $N2A_{+FBS}$ cells (Fig. 5, left panel, lane 1), almost all sensitive to phosphatase λ treatment (Fig. 5, left panel, lane 2). In cell lysates from $N2A_{-FBS}$ cells (Fig. 5, left panel, lane 3), several phosphotyrosine proteins were also detected, but the pattern was completely different, indicating that the absence of serum is responsible for

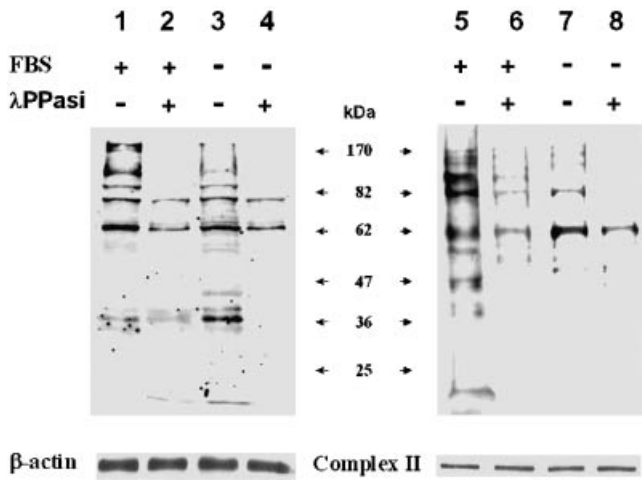

Fig. 5. Serum induces an increases in mitochondrial tyrosine phosphorylation. Detection of tyrosine phosphorylated proteins in cell lysates (left panel) and mitochondrial fractions (right panel) by Western blotting. Cell lysates (left panel) and mitochondrial fractions (right panel) from $N2A_{+FBS}$ (lanes 1–2 and 5–6) and $N2A_{-FBS}$ (lanes 3–4 and 7–8) were Western blotted on PVDF membrane and stained with antiphosphotyrosine antibody. Aspecific detection was revealed by phosphatase λ treatment (lanes 2, 4 and 6, 8). β-Actin and Complex II Western blot has been reported for loading control.

the activation of different signaling pathways, confirming the results shown in Fig. 4; this staining is also highly sensitive to phosphatase λ treatment (Fig. 5, left panel, lane 4). Several phosphotyrosine proteins (sensitive to phosphatase λ treatment) were aalso detected in the mitochondrial fraction from $N2A_{+FBS}$ cells (see Fig. 5, right panel, lane 5; and lane 6 for the phosphatase λ treatment). Instead, the amount and intensity of staining was strongly reduced in the mitochondrial fraction from $N2A_{-FBS}$ (Fig. 5, right panel, lane 7): only two bands were clearly detected, one of them aspecifically detected (Fig. 5, right panel, lane 8).

Immunogold analysis on cryosections, performed with monoclonal anti-phosphotyrosine and a gold-conjugated secondary antibody, revealed mitochondrial tyrosine phosphorylated proteins "in situ". $N2A_{+FBS}$ cells showed labeling at the levels of plasma membrane, cytosol and subcellular compartments (Fig. 6C). In particular, mitochondria were also labeled (Fig. 6D). $N2A_{-FBS}$ cells stained with antiphosphotyrosine antibody also showed a considerable reduction in labeling in mitochondria (Fig. 6A,B).

To confirm that the observed increase in mitochondrial tyrosine phosphorylation is SFKs-dependent, we repeated the previous experiments in the presence of the SFKs inhibitor PP2. Mitochondrial fractions from $N2A_{+FBS}$ without (Fig. 6G, lane 1) or with PP2 overnight treatment (Fig. 6G, lane 2) were Western blotted on PVDF membrane and stained with antiphosphotyrosine antibody. The Phosphotyrosine proteins detected in the mitochondrial fraction from $N2A_{+FBS}$ cells without PP2 treatment (see Fig. 6G, lane 1) were strongly reduced in the mitochondrial fraction from $N2A_{+FBS}$ followed by PP2 treatment (Fig. 6G, lane 2). It is to underline that the PP2 overnight treatment doesn't reduce significantly the cell viability (Fig. 6H). These results were confirmed by immunogold analysis on cryosections of $N2A_{+FBS}$ followed by PP2 treatment where mitochondrial labeling was strongly reduced (Fig. 6E,F). Similar results were obtained by incubating the cells with SU-6656 (data not shown).

CONCLUSIONS

Data shown in this work demonstrate that members of the SFK family are major players in mitochondrial tyrosine phosphorylation in rat brain, and apart from c-Src and Lyn, already shown [Miyazaki et al., 2003; Salvi et al., 2002], fractions of Fgr, Fyn and the regulatory kinase Csk are also located in the internal structure of mitochondria, although all these enzymes lack a classical mitochondrial import sequence.

The registration of a shift in the activation of mitochondrial SFK tyrosine phosphorylation in N2A cells in presence of serum, compared with that of cells in serum deprivation, demonstrates that SFK activity could be under the control of

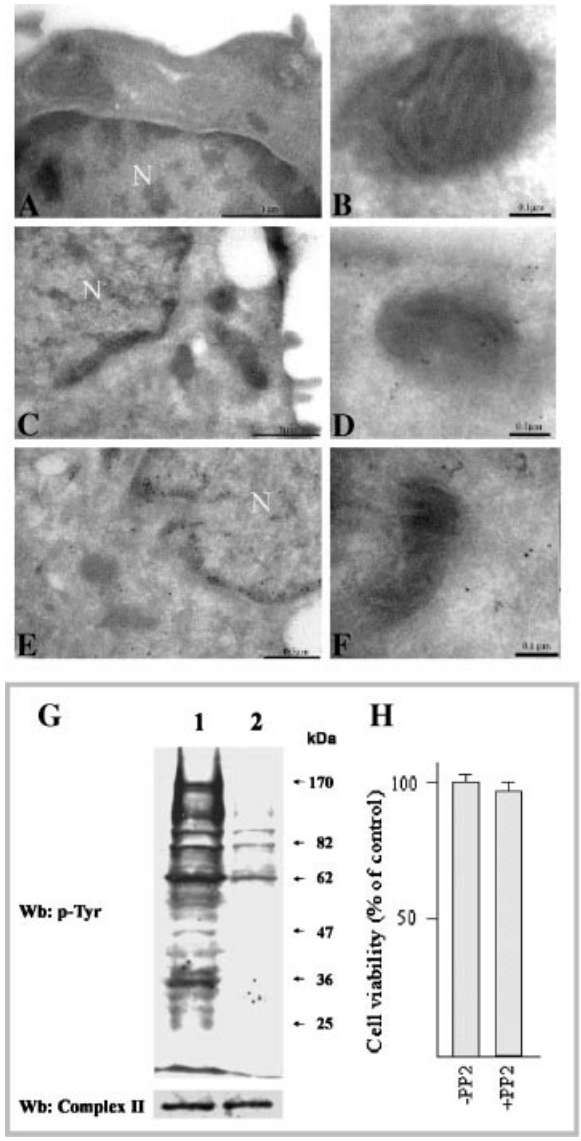

Fig. 6. Immunogold detection on cryosections of phosphotyrosine signal in ''in situ'' mitochondria of whole N2A cells and effect of PP2. The upper panels show thin sections of N2A cells incubated in absence of serum (**A**,**B**), in presence of serum (**C**,**D**) or in presence of serum plus 10 μM PP2 (**E**,**F**), treated with anti-PTyr antibody, followed by gold-conjugated secondary antibody. Panels B,D,F: magnification. In the lower panels it has been reported the detection of tyrosine phosphorylated proteins in mitochondrial fractions by Western blotting (**panel G**) and the effect of PP2 on cell viability (**panel H**). Mitochondrial fractions from $N2A_{+FBS}$ without (lane 1) or with 10 μM PP2 overnight treatment (lane 2) were Western blotted on PVDF membrane and stained with antiphosphotyrosine antibody. Complex II Western blot has been reported for loading control. Panel H reports cell viability measurement by MTT assay as described in Materials and Methods Section.

plasma membrane receptors, and suggests the role of SFK activity in modulating mitochondrial functions in response to activated cell membrane receptors. Further work will be necessary to establish if the increase in mitochondrial tyrosine phosphorylation Src-dependent is due to a translocation of kinases from cytosol to mitochondria or to an activation of a pool of kinases resident in mitochondrial compartments. Moreover the activation of Src at mitochondrial level is strictly correlated with the proliferative status where several mitochondrial proteins are specifically tyrosine phsophoryated. Since the proliferative status should be supported by an increasing in the production of energy metabolism, in particular at mitochondrial level, we expect that Src tyrosine kinases act as positive regulators of mitochondrial functions. This statement is well supported by functional studies on the role of Src tyrosine kinase in mitochondria demonstrating that these enzymes stimulate the activity of the electron transport chain [Miyazaki et al., 2003] and a down regulation of tyrosine phosphorylation by overexpressing PTPM1, a tyrosine phosphtases localized in mitochondria, reduce the production of ATP [Pagliarini et al., 2005].

COX is the only mitochondrial substrate of Src kinases identified up to date and for this reason future research should be addressed to discover all the mitochondrial targets of these enzymes, a possibility which could really open new scenarios on the regulation of energy metabolism at mitochondrial level.

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