Adrenocorticotrophic hormone stimulates phosphotyrosine phosphatase SHP2 in bovine adrenocortical cells: phosphorylation and activation by cAMP-dependent protein kinase

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During activation of adrenocortical cells by adrenocorticotrophic hormone (ACTH), tyrosine dephosphorylation of paxillin is stimulated and this correlates with protrusion of filopodial structures and a decreased number of focal adhesions. These effects are inhibited by Na₃VO₄, a phosphotyrosine phosphatase inhibitor [Vilgrain, Chinn, Gaillard, Chambaz and Feige (1998) *Biochem. J.* 332, 533–540]. However, the tyrosine phosphatases involved in these processes remain to be identified. In this study, we provide evidence that the Src homology domain (SH)2-containing phosphotyrosine phosphatase (SHP)2, but not SHP1, is expressed in adrenocortical cells and is phosphorylated upon ACTH challenge. ACTH (10^{−8} M) treatment of ³²P-labelled adrenocortical cells resulted in an increase in phosphorylated SHP2. By probing SHP2-containing immunoprecipitates with an antibody to phosphoserine we found that SHP2 was phosphorylated on serine in ACTH-treated cells in a dose- and time-dependent manner. Furthermore, using an *in vitro* kinase assay,

we showed that SHP2 was a target for cAMP-dependent protein kinase (PKA). Serine was identified as the only target amino acid phosphorylated in SHP2. Phosphorylation of SHP2 by PKA resulted in a dramatic stimulation of phosphatase activity measured either with insulin receptor substrate-1 or with the synthetic peptide [³²P]poly(Glu/Tyr) as substrate. In an in-gel assay of SHP2-containing immunoprecipitates, phosphorylated *in vitro* by PKA or isolated from adrenocortical cells treated with 10 nM ACTH, a pronounced activation of SHP2 activity was shown. These observations clearly support the idea that a PKA-mediated signal transduction pathway contributes to SHP2 regulation in adrenocortical cells and point to SHP2 as a possible mediator of the effects of ACTH.

Key words: ACTH, phosphotyrosine phosphatases, SH2-containing tyrosine phosphatase, insulin receptor substrate 1.

INTRODUCTION

Protein tyrosine phosphorylation has emerged as a key mechanism for signal transduction involved in the control of cellular processes, such as cell proliferation, differentiation and cytoskeletal remodelling [1]. The balance between the activities of protein tyrosine kinases and phosphotyrosine phosphatases (PTPases) determines the level of tyrosine phosphorylation. During the past few years a large number of PTPases have been isolated, cloned and sequenced [2], and have been found to fall into two groups, cytosolic and transmembrane enzymes [2]. Known mammalian cytosolic PTPases possess Src homology (SH)2 domains, which allow them to associate with molecules phosphorylated on tyrosine residues; these are SHP1, also termed SH-PTP1, and SHP2. Although their amino acid sequences are homologous, their tissue distribution differs. SHP1 is expressed predominantly in haematopoietic cells [3], whereas SHP2 is more widely distributed. SHP1 negatively regulates signalling by several cytokine and growth factor receptors, apparently by promoting dephosphorylation of the intracellular domain of the receptor and/or the receptor-associated Janus tyrosine kinase [4].

SHP2 (also known as SH-PTP3 [5], SH-PTP2 [6], PTP2C [7], PTP1D [8] and Syp [9]) is a cytosolic protein tyrosine phosphatase that is ubiquitously expressed and is thought to represent the mammalian homologue of the *Drosophila* protein CSW [10].

SHP2 has two SH2 domains, and ligation of SHP2 SH2 domains has been reported to contribute to activation of its phosphatase activity [11,12]. SHP2 has also been suggested to act as an adaptor in promoting the association of the insulin receptor with insulin receptor substrate (IRS)-1 [13]. SHP2 itself is tyrosine phosphorylated in response to cell stimulation by a number of different growth factors, including platelet-derived growth factor and epidermal growth factor, which also lead to its activation [8,9]. In addition to its activation downstream of growth factor receptors, several pieces of evidence suggest that SHP2 might play an important role in cell attachment and motility. Abrogation of the catalytic activity of SHP2 with a dominant negative mutant abolishes insulin-like growth factor-1-induced focal adhesion kinase (FAK) dephosphorylation and chemotaxis. Interestingly, cells expressing the dominant negative mutant showed a larger number of focal adhesion contacts [14]. Upon integrin engagement, it has been shown that a fraction of SHP-2 moves to focal contacts [15]. More recently, disruption of the mouse *Shp-2* gene was shown to lead to a lack of normal gastrulation and subsequent death in the early stages of embryogenesis [16], suggesting that SHP-2 might be involved in mediating the correct organization and migration of mesodermal cells during gastrulation.

Bovine adrenocortical fasciculata cells in primary culture respond to several hormonal effectors by increased steroidogenic activity. Adrenocorticotrophic hormone (ACTH) has a major

Abbreviations used: ACTH, adrenocorticotrophic hormone; DTT, dithiothreitol; FAK, focal adhesion kinase; IRS, insulin receptor substrate; PKA, cAMP-dependent protein kinase; PTPases, phosphotyrosine phosphatases; SH, Src homology domain; SHP, SH2-containing PTPases.

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physiological role in this context and its mechanism of action involves cAMP-dependent protein phosphorylation processes [17]. Thus, the activation of cAMP-dependent protein kinase (PKA) is an essential mediator of ACTH effects. Beyond its effect on steroidogenesis, ACTH inhibits adrenocortical cell proliferation in primary culture. This was observed in the Y1 mouse adrenocortical tumour cell line [18], as well as in normal adrenocortical cells isolated from a variety of species, including rat, cow and human (for a review see [19]). ACTH arrests dividing adrenal cells by interfering with progression through the G1 phase of the cell cycle [20], and inhibits the initiation of DNA synthesis in G1-arrested cells following the addition of serum or growth factors [21]. Moreover, in bovine adrenocortical cells, it has been shown that ACTH blocks the effects of fibroblast growth factor-2 on mitogen-activated protein kinase activation [22]. ACTH also has a powerful capacity to induce changes in cell shape in adrenocortical cells; particularly, it increases dendricity and protrusion in filopodial structures, suggesting a potential role for ACTH in cell-matrix interactions through reorganization of the actin filament cytoskeleton and focal adhesions [23,24].

In adrenocortical cells, it has been shown that the integrin-induced tyrosine phosphorylation of several focal adhesion proteins and the ability to spread on fibronectin were defective in ACTH-treated cells [24]. Indeed, ACTH-treated cells exhibited a decreased number of focal adhesions, which was accompanied by a decreased rate of paxillin-tyrosine phosphorylation. Forskolin, a direct activator of adenylate cyclase, was found to reproduce the effect of ACTH, suggesting an essential role for cAMP in this process. These effects, as well as the changes in cell shape, induced by ACTH were impaired by Na_3VO_4 , a potent inhibitor of phosphotyrosine phosphatases [24]. This model predicts a requirement for phosphotyrosine phosphatase in the regulation of signals downstream of integrins and triggered by ACTH and cAMP. However, a role for a specific cytoplasmic PTPase remains to be defined.

In the present study, the first evidence that SHP2 is expressed in adrenocortical cells and is phosphorylated upon ACTH challenge is presented. Importantly, we show that SHP2 is a target for PKA, which results in a dramatic stimulation of phosphatase activity. These observations clearly support the idea that a PKA-mediated signal-transduction pathway contributes to SHP2 regulation in adrenocortical cells, and point to SHP2 as a possible mediator of the effects of ACTH.

MATERIALS AND METHODS

Reagents

Benzamidine, leupeptin, pepstatin A, poly(Glu/Tyr) and Triton X-100 were purchased from Sigma (St Louis, MO, U.S.A.). ACTH was obtained from Ciba-Geigy (Rueil Malmaison, France). $^{32}\text{P}\text{IP}_1$ (28 Ci/mmol; 1 Ci = 37 GBq) was from Amersham (Les Ulis, France) and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (4000 Ci/mmol) from DuPont-NEN (Les Ulis, France). Protein G-Sepharose was from Pharmacia (Les Ulis, France). Monoclonal antibodies against human SHP1 and SHP2 were from Transduction Laboratories (Lexington, KY, U.S.A.). The Src kinase was obtained from Oncogene Science. The monoclonal antibody to phosphotyrosine (clone 4G10) was from Upstate Biotechnology (New York, NY, U.S.A.) and the monoclonal antibody to phosphoserine was from Sigma. Purified SHP2 was a gift from Dr E. Fisher (University of Washington, Seattle, WA, U.S.A.); the catalytic subunit of PKA was purified from bovine heart as described in [25]. Horseradish peroxidase-conjugated affinity-purified rabbit anti-mouse IgG was from Bio-Rad (Hercules, CA, U.S.A.). For immunoprecipitation of IRS-1, a polyclonal

antibody against a fusion protein, glutathione-S-transferase-rat IRS-1 (residues 201–861), was prepared (INSERM Unit 145, Nice, France). Enhanced chemiluminescence detection reagents were purchased from DuPont-NEN. Nitrocellulose was obtained from Schleicher and Schuell (Ecqueville, France). The micro-bicinchoninic acid protein assay reagent kit was from Pierce (Oud Beijerland, The Netherlands).

Buffers

Buffer A was: 20 mM Tris/acetate (pH 7.0), 0.27 M sucrose, 1% (v/v) Triton X-100, 1 mM dithiothreitol (DTT), 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4 , 1 mM benzamidine, 4 $\mu\text{g}/\text{ml}$ leupeptin and 1 $\mu\text{g}/\text{ml}$ pepstatin A. Buffer B was: 10 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X-100 and 0.5% (v/v) Nonidet P-40.

Cell culture, extraction and immunoprecipitation

Bovine adrenocortical fasciculata reticularis cells were prepared from adrenal glands obtained from the local slaughter house and grown at 37 °C in Ham's F-12 medium (Gibco, Grand Island, NY, U.S.A.) supplemented with 10% (v/v) horse serum, 2.5% (v/v) fetal-calf serum and 1 mM glutamine, as described previously [26]. For cell extracts, the cells were sonicated for 10 s in 500 μl of buffer A. Lysates were cleared by centrifugation at 12000 g for 10 min, then protein content was measured using the micro-bicinchoninic acid protein assay and samples were stored at –80 °C before use.

For immunoprecipitation, 500 μg of protein of different adrenocortical cell extracts were incubated with 2 μg of the monoclonal antibody to SHP2 in 500 μl of buffer B for 1 h at 4 °C. Immunoprecipitates were separated on protein G-Sepharose and fractions were collected for 45 min at 4 °C. The fractions were centrifuged on a microfuge for 5 min at 4 °C and the immunoprecipitates were washed five times with buffer A.

^{32}P -Labelling of adrenocortical cells

Adrenocortical cells growing in 100-mm dishes were washed twice in phosphate-free medium and then incubated for 4 h in the same medium supplemented with ^{32}P i (0.5 mCi/ml). Thereafter, ACTH (10^{-8} M) was added and the cells were incubated for a further 5–60 min. Proteins were solubilized in buffer A and immunoprecipitated by antibody to SHP2.

Phosphorylation *in vitro* of SHP2 by PKA

Immunoprecipitates of SHP2 from adrenocortical cell extracts or recombinant SHP2 were incubated with the catalytic subunit of PKA at 30 °C in 50 μl of 25 mM Tris/HCl (pH 7.5), 10 μM ATP/ $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (2 μCi), 1 mM DTT and 5 mM MgCl_2 for 20 min (or as stated). The samples were then subjected to SDS/PAGE and autoradiographed.

Determination of PTPases activity

Enzyme activities were determined in adrenocortical cell extracts using an in-gel assay as described in [27], where ^{32}P -poly(Glu/Tyr) is incorporated into the gel prior to migration.

Preparation of poly(Glu/Tyr) was as follows: 3 μl of c-Src in 50 mM Hepes (pH 7.0)/0.1 mM EDTA containing 0.1 $\mu\text{g}/\text{ml}$ BSA, 0.015% (v/v) Brij 35, 0.2% (w/v) 2-mercaptoethanol, 12 μg of poly(Glu/Tyr) and 20 ml of a solution containing 10 μM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ /6 mM MgCl_2 was incubated overnight at 30 °C in a final volume of 60 ml. At the end of the incubation, the

reaction was stopped by the addition of 20% (w/v) trichloroacetic acid and 50 μ l of BSA (20 μ g/ml), as a carrier protein. After centrifugation for 10 min at 4 °C, the pellet was washed three times with 20% trichloroacetic acid. The radioactivity in the pellet was estimated and the pellet was dissolved in 0.2 M Tris/HCl (pH 7.5) to give 15000–25000 c.p.m./5 μ l. The 32 P-labelled poly(Glu/Tyr) was incorporated, at approx. 10^5 c.p.m./ μ l, into a 12% acrylamide/0.1% bis-acrylamide/SDS gel mixture before polymerization.

The phosphotyrosine phosphatase assay was as follows: 1 μ g of protein from the various adrenocortical cell extracts was loaded on to the gel containing 32 P-labelled poly(Glu/Tyr). After SDS/PAGE, all incubations were performed at room temperature with shaking. The gels were first incubated in 50 mM Tris/HCl (pH 8.0)/20% (v/v) propan-2-ol either for 1.5 h or overnight to remove the SDS, then washed twice, for 30 min each, in 50 mM Tris/HCl (pH 8.0)/0.3% (v/v) 2-mercaptoethanol and then incubated for 90 min in 50 mM Tris/HCl (pH 8.0), 0.3% (v/v) 2-mercaptoethanol, 6 M guanidine hydrochloride, 1 mM EDTA. For renaturation, the gels were incubated three times, for 1 h each, in 50 mM Tris/HCl (pH 8.0), 0.3% 2-mercaptoethanol, 0.04% (v/v) Tween 40, 1 mM EDTA and finally overnight in 50 mM Tris/HCl (pH 8.0), 0.3% 2-mercaptoethanol, 0.04% Tween 40, 1 mM EDTA, 4 mM DTT. The dried gels were autoradiographed and quantification was performed using a PhosphorImager and an Image-Quant program.

Phospho IRS-1 was prepared as follows: the full-length IRS-1 cDNA subcloned into the pBluescript KS vector was obtained from Dr M. F. White and Dr C. R. Kahn (Joslin Diabetes Center, Boston, U.S.A.). The IRS-1 cDNA was subcloned into a pCEP-4 expression vector (Invitrogen, San Diego, CA, U.S.A.) as described previously [28]. Cell culture, transfection of 293 EBNA cells and phosphorylation *in vivo* of IRS-1 have been described previously [28].

IRS-1 was dephosphorylated by purified SHP2. IRS-1 phosphorylated *in vivo* was incubated with buffer or purified SHP2 (0.5 μ g/sample) in 25 mM imidazole (pH 7.0), 4 mM EDTA, 1 mM DTT and 100 μ g/ml BSA for 15 min at 30 °C. After separation by SDS/PAGE, proteins were immunoblotted with antibodies to phosphotyrosine as described previously [28]. The associated radioactivity was determined using phosphorimager quantification. SDS/PAGE and phospho amino acid analyses were performed according to Hunter and Sefton [29].

Western blotting

Protein (40 μ g) from the various adrenocortical cell extracts was analysed by SDS/PAGE (12% acrylamide). Proteins were then transferred from the gel to nitrocellulose for 1 h and the residual binding sites were blocked by incubating the filters for 1 h in PBS containing 0.05% (v/v) Tween 20 and 5% (w/v) non-fat milk. The blots were subsequently incubated for 1 h with antibodies to SHP2, phosphotyrosine (1 μ g/ml) or phosphoserine. After being washed, the blots were incubated for 1 h with horseradish-peroxidase-conjugated rabbit anti-mouse IgG diluted in PBS containing 0.05% (v/v) Tween 20. Immunoreactive proteins were visualized by chemiluminescence (ECL[®]; Amersham, Little Chalfont, U.K.).

Data analysis

Values represent the means $\pm$ S.D. of determinations from three different wells or dishes in the same experiment. Each experiment was performed at least three times under identical or similar conditions with comparable results.

RESULTS

PTPases in adrenocortical cells

We have shown previously that ACTH-induced change in cytoskeletal shape was impaired by Na_3VO_4 and was correlated with the tyrosine dephosphorylation of paxillin [24]. These data suggested that one or several PTPases could be responsible for the tyrosine dephosphorylation in response to ACTH stimulation. To investigate this hypothesis, we examined the PTPases present in bovine adrenocortical cells, based on their electrophoretic mobility and immunoreactivity. Adrenocortical cell lysates were Western blotted with monoclonal antibodies to PTP1B, SHP1 and SHP2, with predicted molecular masses of 50, 68 and 70 kDa respectively. As shown in Figure 1(A), only one species was detected with each antibody. PTP1B was detected as a 50 kDa protein and SHP2 as a 70–72 kDa protein. In contrast, SHP1 was not detected in adrenocortical cell lysates, when compared with the positive control (5 μ l of Jurkat cells). Although a proteolytically degraded form of these PTPases could be recognized by the antibodies, no degradation products were seen in Figure 1(A), indicating that rapidly solubilizing cells from the culture dish with ice-cold buffer containing protease inhibitors prevented proteolysis. Thus we conclude that PTP1B and SHP2 are expressed in adrenocortical cells in primary culture.

Phosphorylation of SHP2 in adrenocortical cells

SHP2 was recently found to interact with FAK and paxillin in focal contacts [31], and could represent a target for an ACTH effect in adrenocortical cells. Therefore we tested whether ACTH could modulate SHP2. First the amount of SHP2 protein in control and ACTH-treated cells was examined by Western blotting. As shown in Figure 1(B), 60 min treatment with ACTH did not significantly modify the amount of SHP2 protein when compared with untreated cells.

Because ACTH signal transduction involves serine/threonine protein phosphorylation, we investigated whether SHP2 could be phosphorylated in adrenocortical cells upon ACTH challenge. 32 P-Labelled adrenocortical cells were stimulated by ACTH (10 nM) for 5–60 min and SHP2 was immunoprecipitated. SHP2-containing immunoprecipitates were analysed by autoradiography after SDS/PAGE. As shown in Figure 1(C), a phosphorylated form of SHP2 was detected within 5 min of ACTH stimulation and this increased up to 60 min. For the time course of ACTH-induced phosphorylation of SHP2, adrenocortical cells were treated with ACTH (10 nM) for 0–60 min. The cells were lysed, SHP2 was immunoprecipitated with α -SHP2 and the immunocomplexes were analysed by Western blotting with an antibody specific for phosphoserine. The phosphorylation of SHP2 was detected within 5 min and increased up to 60 min (Figure 1D).

To determine the concentration dependence of ACTH-induced SHP2 phosphorylation, cells were treated with increasing concentrations of ACTH for 30 min, lysed and processed as above (Figure 1D). SHP2 phosphorylation was induced by the lowest concentration of ACTH tested (1 nM) and the maximum effect was achieved with 10 nM ACTH. Thus ACTH induced a sensitive time- and dose-dependent serine phosphorylation of SHP2. The levels of SHP2, as determined by Western blotting, were not affected under these conditions (Figure 1D, lower panel).

Phosphorylation of SHP2 by PKA *in vitro*

Because ACTH acts on a G-protein-coupled receptor and activates adenylate cyclase, we investigated whether the cAMP-signalling pathway could modulate SHP2. cAMP specifically

Figure 1 Phosphorylation of SHP2 in adrenocortical cells

Adrenocortical cells grown on fibronectin-coated plates were untreated or treated with 10 nM ACTH for 15 min and then rinsed in PBS on ice. Proteins were extracted in 100 μ l of lysis buffer. **(A)** A sample of 40 μ g of adrenocortical cell extract (adrenal) was immunoblotted (Blot) with monoclonal antibodies to PTP1B, SHP1 or SHP2. The right lane of each gel shows the positive control (5 μ l of total cell lysate from human Jurkat cells). Proteins were visualized by ECL[®]. The positions of molecular-mass standards (in kDa) are shown on the extreme left. The results are representative of three experiments. **(B)** Adrenocortical cells grown on fibronectin-coated plates were untreated (none) or treated with 10 nM ACTH for 60 min, rinsed in PBS on ice and extracted into 100 μ l of buffer A. A sample of cell extract (40 μ g) was immunoblotted with the monoclonal antibody to SHP2. The right lane of the gel shows the positive control (5 μ l of total cell lysate from human Jurkat cells). **(C)** Adrenocortical cells growing in 100-mm dishes were washed twice in phosphate-free RPMI 1640 medium and then incubated for 4 h in the same medium supplemented with [³²P]_i (0.5 mCi/ml). Thereafter, ACTH (10⁻⁸ M) was added and the cells were incubated for 5, 15, 30 or 60 min. Proteins were solubilized in buffer A and cell extracts were immunoprecipitated with an antibody to SHP2. The immunoprecipitates were analysed by SDS/PAGE (12% gel) and the dried gel was exposed to a PhosphorImager. **(D)** Adrenocortical cells were treated either with 10⁻⁸ M ACTH for 0, 5, 15, 30 or 60 min (left panel) or with 10⁻⁹ M, 10⁻⁸ M or 10⁻⁷ M ACTH for 60 min (right panel). Proteins were solubilized in buffer A and immunoprecipitated (IP) with an antibody to SHP2. The immunoprecipitates were analysed by Western blotting with an antibody to phosphoserine (upper panels) or to SHP2 (lower panels).

targets the high-affinity cAMP binding sites in the regulatory R-I subunit of PKA [30], which results in the sustained activation of the serine/threonine type I PKA. To determine whether the serine phosphorylation of SHP2 in adrenocortical cells was

Figure 2 Phosphorylation *in vitro* of the recombinant SHP2 with PKA catalytic subunit

(A) Recombinant SHP2 (500 ng) was incubated with the PKA catalytic subunit (16 ng) (PK-A) in the presence of [³²P]ATP/MgCl₂ for the times shown, as described in the Material and Methods section. In the lane marked SHP2 no PKA was added, and in the lane marked PK-A, SHP2 was omitted. Samples were separated by SDS/PAGE (15% gel), and phosphorylated SHP2 was detected by autoradiography. Arrows on the extreme left indicate the positions of the phosphorylated form of recombinant SHP2 protein and the PKA catalytic subunit. Phosphorylated SHP2 was quantified by PhosphorImager and the results are shown in the lower panel. Error bars indicate S. D. The results are representative of three experiments. **(B)** Purified recombinant SHP2 (500 ng) was phosphorylated with increasing amounts of PKA catalytic subunit (PK-A). Arrows on the extreme right indicate the phosphorylated forms of SHP2 protein and the PKA catalytic subunit. Phosphorylated SHP2 was quantified by PhosphorImager and the results are shown in the lower panel. Error bars indicate S. D. The results are representative of three experiments. **(C)** Increasing amounts of recombinant SHP2 (0.1, 0.5, 1, 2.5 or 5 μ g) were incubated with [³²P]ATP/MgCl₂ and 30 ng of the PKA catalytic subunit (PK-A) for 15 min at 30 °C. At the end of the incubation period, [³²P]-labelled recombinant SHP2 was separated by SDS/PAGE and revealed by autoradiography. Quantification of [³²P]SHP2 after PhosphorImager analysis is shown in the lower panel. Error bars indicate S.D. The results are representative of three experiments. **(D)** Shows the identification of phospho amino acids after phosphorylation of SHP2 by PKA. Following the phosphorylation reaction in the presence of [³²P]ATP/MgCl₂, the radioactive band corresponding to SHP2, obtained as described in **(A)**, was excised from fixed gels, rehydrated, digested with trypsin and washed. The dried peptide precipitates were then hydrolysed with 6 M HCl and the hydrolysates were analysed by electrophoresis on cellulose thin-layer plates in pyridine/acetic acid (1:10, v/v) at pH 3.5, together with phosphoserine (P-Ser) and phosphothreonine (P-Thr) as standards. The unlabelled standards were then stained with ninhydrin (arrows), and the plate was subjected to autoradiography (dark spots). P_i, location of P_i after the electrophoretic analysis.

mediated by PKA, we performed an experiment *in vitro* using purified PKA and recombinant SHP2. Recombinant SHP2 (500 ng) was incubated with the PKA catalytic subunit (16 ng) in the presence of [³²P]ATP/MgCl₂ for increasing periods of time.

Figure 3 Activation of SHP2 by PKA to dephosphorylate IRS or poly(Glu/Tyr)

(A) Dephosphorylation of IRS-1 by SHP2 phosphorylated by PKA. Recombinant SHP2 was phosphorylated with 30 ng of the PKA catalytic subunit (PK-A) and unlabelled ATP *in vitro*, incubated with phosphorylated IRS-1, as described in the Materials and methods section. Samples were subjected to SDS/PAGE (12% gels) and Western blotted with antibody to phosphotyrosine. Proteins were detected by ECL[®]. In the lane IRS1 + PK-A, no SHP2 was added; in the lane IRS1 + SHP2, SHP2 was not phosphorylated by the PKA catalytic subunit; in the lane IRS1 + SHP2 + PK-A, SHP2 was phosphorylated. The phosphotyrosine content of IRS-1 was quantified by PhosphorImager. Error bars indicate S.D. The results are representative of three experiments. (B) Activation of bovine adrenocortical SHP2 by phosphorylation by PKA. Adrenocortical control cell protein (500 μ g) was subjected to immunoprecipitation with the monoclonal antibody to SHP2. The immunoprecipitate was divided into two portions; one portion was incubated with 650 ng of PKA/10 μ M ATP in the presence of 10 mM MgCl₂ (+ PK-A/ATP-Mg) for 60 min at 30 °C and the other portion was treated similarly but without 10 mM MgCl₂ (Ctrl). After incubation, the samples were processed for in-gel assay as described in the Material and methods section. The gel was dried and PTPase activity was detected by autoradiography and quantified by PhosphorImager. Error bars indicate S.D. The results are representative of three experiments.

Following the phosphorylation reaction, the mixtures were subjected to SDS/PAGE and analysed by autoradiography. As shown in Figure 2(A), [³²P]_i incorporation into SHP2 was detected within 5 min of incubation and the protein phosphorylation rate at 30 °C was linear for 10 min. Half-maximal incorporation was apparent after approx. 15 min. When PKA was omitted (Figure 2A, right panel, lane SHP2), no endogenous phosphorylation in SHP2 was detected. When SHP2 was omitted from the reaction, only one band migrating around 38–40 kDa, which represents the PKA autophosphorylation form, was detected (Figure 2A, right panel, lane PKA). Key catalytic parameters of SHP2 phosphorylation by PKA were further examined. Figure 2(B) shows that incubation of SHP2 with increasing amounts of PKA (from 8–32 ng) for 30 min at 30 °C led to a dose-dependent phosphorylation of SHP2. As shown in Figure 2(C), incubation of PKA with increasing concentrations of SHP2 (from 0.25–5 μ g) resulted in a dose-dependent phosphorylation of the tyrosine phosphatase. The corresponding Lineweaver–Burk plot gave an apparent K_m value of 15.5 μ M.

To determine the target amino acids of PKA in the phosphorylated SHP2, a maximally ³²P-labelled SHP2, obtained as described above, was excised from fixed gels, rehydrated and digested with trypsin. Dried peptide precipitates were subjected to HCl hydrolysis and the resulting amino acid mixture was analysed by TLC on cellulose plates in pyridine/acetic acid (1:10, v/v) at pH 3.5. As shown in Figure 2(D), autoradiography disclosed that the only detectable labelled amino acid corresponded to phosphoserine; no phosphothreonine was detected.

Stimulation of recombinant SHP2 by PKA to dephosphorylate IRS-1 *in vitro*

Tyrosine phosphorylation of IRS-1 by the activated receptors for insulin, insulin-like growth factor-1, and various cytokines creates binding sites for signalling proteins with SH2 domains. IRS-1 possesses as many as 18 potential tyrosine phosphorylation sites and has been shown to be a substrate for SHP2 [28]. It was used as substrate to examine the activity of recombinant SHP2 after phosphorylation *in vitro* by PKA. A specific phosphotyrosine monoclonal antibody was used for the detection and quantification of dephosphorylation. Figure 3(A) shows that SHP2 decreased the level of phosphotyrosine in IRS-1 by 50%. Importantly, when SHP2 was phosphorylated by the PKA catalytic subunit in the presence of unlabelled ATP, the phosphotyrosine level in IRS-1 became almost undetectable. Notably, the presence of the PKA catalytic subunit in the assay mixture did not modify tyrosine phosphorylation of IRS-1.

Activation of immunopurified SHP2 from adrenocortical cells by PKA

Because these results strongly suggested that PKA was a key activator of SHP2, we tested whether SHP2 immunoprecipitated from adrenocortical cells presented a modified PTPase activity after incubation with a PKA/ATP/MgCl₂ mixture. Equivalent amounts of SH2-containing immunoprecipitates were incubated with or without the PKA catalytic subunit in 1 mM ATP/10 mM MgCl₂ for 60 min. The PTPase activity of SHP2-containing immunoprecipitates was then estimated using an in-gel assay with [³²P]poly(Glu/Tyr) as substrate. As shown in Figure 3(B), in immunoprecipitates where PKA catalytic subunit had been omitted, the band corresponding to active SHP2 was barely detectable. In contrast, the addition of exogenous PKA resulted in a dramatic increase in SHP2 activity. The PKA catalytic

Figure 4 Stimulation of SHP2 activity in adrenocortical cells in primary culture by ACTH

(A) Upper panel: proteins (5 μ g) from control cells (CTL) and cells treated with 10 nM ACTH (ACTH) were electrophoresed on a 12% acrylamide gel containing [32 P]poly(Glu/Tyr). The gel was then treated as described in the Materials and methods section to renature PTPase activity, dried and autoradiographed. Arrows on the right indicate the position of renatured PTPases. Lower panel (histogram): PTPase activity was detected on autoradiographs and quantification was performed using the Image-Quant program. Error bars indicate S.D. The results are representative of four independent experiments; the same amount of protein was loaded onto each gel. (B) Adrenocortical cells grown on fibronectin-coated plates were untreated or treated with 10 nM ACTH for the indicated times. The cell lysates were prepared as described in the Materials and methods section and 500 μ g of protein was immunoprecipitated with the monoclonal antibody to SHP2. The immunoprecipitates were then electrophoresed on a 12% acrylamide gel containing [32 P]poly(Glu/Tyr). The renatured PTPase activity in the immunoprecipitates, detected by autoradiography, was quantified with the Image-Quant program. Error bars indicate S.D. The results are representative of three experiments.

subunit alone was analysed under the same conditions and no PTPases activity was detected (results not shown).

Detection of PTPases activities using an in-gel assay: activation of SHP2 in adrenocortical cells by ACTH

In addition to detection of the activity of the individual PTPases, changes in activity because of covalent modification of PTPase, such as proteolysis or phosphorylation, was detected using the renaturation assay. In contrast, changes in activity due to non-covalent interactions were disrupted by denaturation, and the interacting components could be separated by SDS/PAGE. The pattern of PTPases activities in adrenocortical cells was also examined with cell lysates from untreated or ACTH-treated cells. The presence of two major bands of 50 and 70 kDa and three discrete bands with molecular masses of approx. 36, 78 and 130 kDa were detected (Figure 4A, left lane). Upon treatment with 10 nM ACTH for 15 min (Figure 4A, right lane), the major difference was in the intensity of the 72 kDa band. Quantification of each band was performed using the

Image-Quant program. This showed that only the 72 kDa species was increased 2-fold in ACTH-treated cells when compared with untreated cells (Figure 4A, lower panel). This experiment was performed four times under similar conditions with comparable results. We found that the activities measured using the in-gel assay were specific for PTPases since they were inhibited by Na_3VO_4 and activity was unchanged in the presence of okadaic acid, a serine/threonine phosphatase inhibitor (results not shown).

Stimulation of SHP2 activity in adrenocortical cells in primary culture by ACTH

As the molecular mass of the 72 kDa species was in the range of that of SHP2, the activity of SHP2 in adrenocortical cells upon ACTH challenge was examined. SHP2 was immunoprecipitated from adrenocortical cells which had been treated with 10 nM ACTH for increasing periods of time, and SHP2-containing immunoprecipitates were then tested for PTPase activity using the in-gel assay. These immunoprecipitates were electrophoresed on a 12% acrylamide gel containing [32 P]poly(Glu/Tyr) and processed for renaturation of PTPase activity. As shown in Figure 4(B), one major band of activity with a molecular mass of 70 kDa was detected in each immunoprecipitate, which was in contrast to that obtained with the whole cell lysates. The activity of this species was increased by the addition of ACTH. The effect was detectable within 5 min of addition of the hormone and was sustained for up to 60 min. These results strongly suggest that ACTH treatment induces a rapid activation of SHP2 in adrenocortical cells.

DISCUSSION

In the present study, we present evidence that SHP-2, a ubiquitously expressed cytoplasmic PTPase with two SH2 domains at the N-terminus, is modulated in adrenocortical cells upon ACTH challenge through a cAMP/PKA signalling pathway. In endocrine cells, the role of PTPases in cAMP-induced secretory function remains to be defined. However, several lines of evidence indicated that cAMP could modulate PTPases, with the most compelling data arising from studies using Na_3VO_4 , a potent PTPase inhibitor. For example, in isolated corpora lutea from pseudopregnant rats, inhibition of PTPase activity with Na_3VO_4 appeared to inhibit luteotropic hormone-induced cAMP accumulation [32]. In contrast, in primary granulosa cell cultures, vanadate mimicked the effect of gonadotropic hormones or forskolin on progesterone production [33]. More recently, in rat granulosa cells, it was shown that expression of SHP-1 (but not SHP-2) was also hormonally regulated; SHP-1 mRNA and protein were high in granulosa cells of H rats, were decreased by oestrogen and follicle-stimulating hormone, and subsequently increased dramatically with luteinization [34]. In bovine adrenocortical cells, we reported previously that vanadate inhibited ACTH- and cAMP-induced disassembly of focal adhesions and tyrosine dephosphorylation of paxillin [24] without affecting steroid secretion. This model predicts the requirement for PTPase in the regulation of signals downstream of integrins and triggered by ACTH and cAMP. Using specific antibodies, we found that PTP1B and the SH2-containing phosphatase, SHP2, but not SHP1 were expressed in adrenocortical cells. PTP1B is a non-transmembrane PTPase which undergoes cell-cycle-dependent phosphorylation on serine residues by a PTP1B kinase with ill-defined identity [35]. The relevance of PTP1B in adrenocortical cells in relation to adrenal cell growth remains to be shown. The

SH2-containing phosphatase, SHP1, was not detectable in adrenocortical cell lysates. This was not surprising, since its expression is restricted to haematopoietic cells [3]. In contrast, SHP2, which is ubiquitously expressed, was found in adrenocortical cells.

SHP2 is expressed in many tissues and several studies have shown that SHP2 itself becomes tyrosine phosphorylated in growth-factor-stimulated cells [8,9]. Regulation of its activity by serine/threonine phosphorylation has been less well documented. However, it has been shown that p44 mitogen-activated protein kinase was able to phosphorylate SHP2 on threonine residues *in vivo* and *in vitro*, leading to a decrease in phosphatase activity measured with the epidermal growth factor receptor as a substrate [36]. Our present data strongly suggest that SHP2 can be phosphorylated on serine in ACTH-treated cells in a dose- and time-dependent manner. As ACTH acts through a Gs-coupled receptor, the cAMP-dependent pathway is implicated in most of the pleiotropic effects of the hormone. SHP2 was one of the first identified SH2-domain-containing PTPases where activity is modulated through a G-protein-coupled receptor via the PKA pathway. Stimulation of the G-protein-coupled somatostatin [37,38] and dopamine D2 [39] receptors has been shown to increase PTPase activity, but the identity of the PTPases involved in the antiproliferative effects of these hormones is unknown. A receptor-like PTPase, with expression restricted to bone and testis, was found to be regulated by parathyroid hormone and cAMP [40]. In monkey kidney cells, cAMP and PMA have been shown to activate a membrane-bound PTPase, which was extracted with detergent as a 150 kDa complex that contained a 55 kDa catalytic subunit [41].

ACTH acts on a G-protein-coupled receptor and activates adenylate cyclase; thus cAMP targets the high-affinity cAMP binding sites in the regulatory R-I subunit of PKA [32] and activates the serine/threonine type I PKA. Therefore PKA could be the intermediate which directly phosphorylates and activates SHP2. This hypothesis is supported by phosphorylation *in vitro* of recombinant SHP2 with the PKA catalytic subunit, which was concomitant with activation and stimulation of PTPase activity in SHP2-containing immunoprecipitates preincubated with PKA. When phosphorylated *in vitro*, SHP2 was found to be more active in dephosphorylating either ^{32}P -poly(Glu/Tyr) or IRS-1. It is now important to identify the physiological cellular targets of SHP2. *In vivo*, SHP2 binds directly, via its SH2 domains, to phosphorylated IRS-1 in response to insulin. This activates SHP2 and is likely to play a role in insulin action, since IRS-1 can be dephosphorylated by SHP2. Therefore it is tempting to suggest that SHP2 is part of a negative feedback loop leading to attenuation of the insulin signal by dephosphorylation of IRS-1. The biological significance of the phosphorylation of SHP2 by PKA remains to be established in this context. Whatever the molecular mechanism involved, the fact that PKA induces increased SHP2 activity towards IRS-1 is particularly intriguing, given the overall antagonistic action of cAMP and insulin [42]. Indeed, we would like to suggest that the increased dephosphorylation of IRS-1 seen with PKA activation might contribute, at least in part, to the biological antagonism between insulin and cAMP.

Using an in-gel assay, we analysed the pattern of PTPases expressed in bovine adrenocortical cells in primary culture. The activity measured provides an indication of the apparent subunit mass of the detected PTPases and the relative activities of the individual bands are easily assigned. Two major PTPases species of 50 and 70 kDa and three weaker activities with molecular masses of 130, 78 and 36 kDa were found in adrenocortical cell lysates. These activities might represent both cytoplasmic and

membrane-associated PTPases, since the cell lysates were prepared with a detergent-containing buffer. A similar pattern of PTPases activity has been described in several other cell types. In erythrocytes, the activity was ascribed to two species at about 70 and 55 kDa; in platelets, monocytes and lymphocytes and in mammalian tissues, seven or eight major species ranging in molecular mass from approx. 30–130 kDa have been described [27]. With the in-gel assay, differences in the intensities of the bands were observed within the same cell sample. This suggests that the affinity of [^{32}P]poly(Glu/Tyr) as substrate may differ for the different phosphatases detected. This seems to be the case for the 36, 78 and 130 kDa species. In contrast, the 50 and 70 kDa species had both a high intrinsic enzymic activity to the substrate [^{32}P]poly(Glu/Tyr) and were efficiently renatured in the gel. We were unable to detect high-molecular-mass PTPases > 130 kDa, such as transmembrane PTPase, which suggests that the conditions did not renature this type of PTPase.

Furthermore, we have shown that the activity of the 70 kDa species, detected by the in-gel assay in whole cell extracts and tentatively identified as SHP2, was increased upon ACTH exposure. This was confirmed by the finding that the activity of immunopurified SHP2 was rapidly stimulated in ACTH-treated cells. The rapid kinetics of the effect of ACTH on SHP2 activity correlated with post-translational modification rather than transcriptional control.

In adrenocortical cells, studies on the role of SHP2 in the biological effects of ACTH could provide an interesting model for investigating the involvement of PTPases in G-protein-activated signalling pathways. Our data showing the activation of SHP2 following ACTH stimulation suggest that SHP2 may function as an essential modulator of tyrosine phosphorylation status during adrenal metabolism. Using vanadate to inhibit PTPases, we have previously shown that paxillin, a component of focal adhesion, was tyrosine dephosphorylated after ACTH stimulation [24]. On the other hand, it was shown that the tyrosine dephosphorylation of paxillin in rat 3Y1 fibroblasts overexpressing phospholipase C γ 1 accompanies the morphological change of the transformed cells [43].

Thus paxillin could represent a potential target for SHP2 and its dephosphorylation could be part of the mechanism of ACTH-induced focal adhesion disassembly. This model was supported by time-course experiments in which SHP2 activation was concomitant with paxillin dephosphorylation [24]. These results allow us to make an intriguing proposal with regard to the dynamic interplay between a protein tyrosine kinase and a PTPase in the control of cell mobility and focal adhesion. SHP-2 might be involved in the turnover of focal adhesions by mediating the dephosphorylation of several proteins, including FAK and paxillin, which influence cell spreading, migration and cytoskeletal architecture. At present, we can only speculate on the role of this phosphatase in adrenocortical cell differentiation and migration. Indeed, the fetal adrenal cortex is a dynamic organ in which cells proliferate at the periphery, migrate centripetally and differentiate to form the specialized cortical compartments, and then undergo senescence [22].

To summarize, our present data lead to three key points. First, we have shown that SHP2 is expressed in adrenocortical cells in primary culture. Secondly, we found that SHP2 is a substrate for PKA and cAMP in adrenocortical cells; it may therefore mediate the effects of ACTH on cytoplasmic signalling pathways, leading to modification of adrenocortical cell shape. Thirdly, our data suggest a positive-feedback model in which, upon ACTH stimulation, basal levels of PKA activity catalyse the serine phosphorylation of SHP2, and SHP2 then transduces positive signals for downstream events, such as cell shape changes.

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REFERENCES

- Cantley, L., Auger, K., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R. and Soltoff, S. (1991) Oncogenes and signal transduction. *Cell* **64**, 281–302.
- Tonks, N. and Neel, B. G. (1996) From form to function: signaling by protein tyrosine phosphatases. *Cell* **87**, 365–368.
- Yi, T., Cleveland, J. L. and Ihle, J. N. (1992) Protein tyrosine phosphatase containing SH2 domains: characterization, preferential expression in hematopoietic cells, and localization to human chromosome 12p12-p13. *Mol. Cell. Biol.* **12**, 836–846.
- Jiao, H., Berrada, K., Yang, W. and Tabrizi, M. (1996) Direct association of the dephosphorylation of Jak2 kinase by the SH2-domain-containing protein tyrosine phosphatase SHP1. *Mol. Cell. Biol.* **16**, 6985–6992.
- Adachi, M., Sekiya, M., Miyachi, T., Matsuno, K., Hinoda, Y., Imai, K. and Yachi, A. (1992) Molecular cloning of a novel protein-tyrosine phosphatase SH-PTP3 with sequence similarity to the Src-homology region 2. *FEBS Lett.* **314**, 335–339.
- Plutzky, J., Neel, B. G. and Rosenberg, R. D. (1992) Isolation of a Src homology 2-containing tyrosine phosphatase. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 11239–11243.
- Ahmad, S., Banville, D., Zhao, Z., Fischer, E. H. and Shen, S.-H. (1993) A widely expressed human protein-tyrosine phosphatase containing Src homology 2 domain. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 2197–2201.
- Vogel, W., Lammers, R., Huang, J. and Ullrich, A. (1993) Activation of a phosphotyrosine phosphatase by tyrosine phosphorylation. *Science* **259**, 1611–1614.
- Feng, G. S., Hui, C. C. and Pawson, T. (1993) SH2-containing phosphotyrosine phosphatase as a target of protein-tyrosine kinases. *Science* **259**, 1607–1611.
- Perkins, L. A., Larsen, I. and Perrimon, N. (1992) Corkscrew encodes a putative protein tyrosine phosphatase that functions to transduce the terminal signal from the receptor tyrosine kinase torso. *Cell* **70**, 225–236.
- Lechleider, R. J., Sugimoto, S., Bennett, A. M., Kashishian, A. S., Cooper, J. A., Shoelson, S. E., Walsh, C. T. and Neel, B. G. (1993) Activation of the SH2-containing phosphotyrosine phosphatase SH-PTP2 by its binding site, phosphotyrosine 1009, on the human platelet-derived growth factor receptor. *J. Biol. Chem.* **268**, 21478–21481.
- Pluskey, S., Wandless, T. J., Walsh, C. T. and Shoelson, S. E. (1995) Potent stimulation of SH-PTP2 phosphatase activity by simultaneous occupancy of both SH2 domains. *J. Biol. Chem.* **270**, 2897–2900.
- Kharitonkov, A., Schnekenburger, J., Chen, Z., Knyazev, P., Ali, S., Zwick, E., White, M. and Ullrich, A. (1995) Adapter function of protein-tyrosine phosphatase 1D in insulin receptor/insulin receptor substrate-1 interaction. *J. Biol. Chem.* **270**, 29189–29193.
- Manes, S., Mira, E., Gomez-Mouton, C., Zhao, Z. J., Lacalle, R. A. and Martinez, A. C. (1999) Concerted activity of tyrosine phosphatase SHP-2 and focal adhesion kinase in regulation of cell motility. *Mol. Cell. Biol.* **19**, 3125–3135.
- Tsuda, M., Matozaki, T., Fukunaga, K., Fujioka, Y., Imamoto, A., Noguchi, T., Takada, T., Yamao, T., Takeda, H., Ochi, F. et al. (1998) Integrin-mediated tyrosine phosphorylation of SHPS-1 and its association with SHP-2. Roles of Fak and Src family kinases. *J. Biol. Chem.* **273**, 13223–13229.
- Saxton, T. M. and Pawson, T. (1999) Morphogenetic movements at gastrulation require the SH2 tyrosine phosphatase Shp2. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 3790–3795.
- Mountjoy, K. G., Robbins, L. S., Mortrud, M. T. and Cone, R. D. (1992) The cloning of a family of genes that encode the melanocortin receptors. *Science* **257**, 1248–1251.
- Masui, H. and Garren, L. D. (1971) Inhibition of replication in functional mouse adrenal tumor cells by adrenocorticotrophic hormone mediated by adenosine 3':5'-cyclic monophosphate. *Proc. Natl. Acad. Sci. U.S.A.* **68**, 3206–3210.
- Hornsby, P. J. (1985) Cytochrome P-450/pseudosubstrate interactions and the role of antioxidants in the adrenal cortex. *Endocrinol. Res.* **10**, 259–281.
- Kimura, E. and Armelin, H. A. (1990) Phorbol ester mimics ACTH action in corticoadrenal cells stimulating steroidogenesis, blocking cell cycle, changing cell shape, and inducing c-fos proto-oncogene expression. *J. Biol. Chem.* **265**, 3518–3521.
- Weidman, R. E. and Gill, G. N. (1977) Differential effects of ACTH or 8-Dr-cAMP on growth and replication in a functional adrenal tumour cell line. *J. Cell. Physiol.* **90**, 91–103.
- Chabre, O., Cornillon, F., Bottari, S., Chambaz, E. M. and Vilgrain, I. (1995) Hormonal regulation of mitogen-activated protein kinase activity in bovine adrenocortical cells: cross-talk between phosphoinositides, adenosine 3',5'-monophosphate, and tyrosine kinase receptor pathways. *Endocrinology* **136**, 956–964.
- Rainey, W., Hornsby, P. J. and Shay, J. (1983) Morphological correlates of adrenocorticotropin-stimulated steroidogenesis in cultured adrenocortical cells: differences between bovine and human adrenal cells. *Endocrinology* **113**, 48–54.
- Vilgrain, I., Chinn, A., Gaillard, I., Chambaz, E. M. and Feige, J. J. (1998) Hormonal regulation of focal adhesions in bovine adrenocortical cells: induction of paxillin dephosphorylation by adrenocorticotrophic hormone. *Biochem. J.* **332**, 533–540.
- Zoller, M. J., Kerlavage, A. R. and Taylor, S. (1979) Structural comparisons of cAMP-dependent protein kinases I and II from porcine skeletal muscle. *J. Biol. Chem.* **254**, 2408–2412.
- Dupperray, A. and Chambaz, E. M. (1980) Effect of prostaglandin E1 and ACTH on proliferation and steroidogenic activity of bovine adreno-cortical cells in primary culture. *J. Steroid Biochem.* **13**, 1359–1364.
- Burridge, K. and Nelson, A. (1995) An in-gel assay for protein tyrosine phosphatase activity: detection of widespread distribution in cells and tissues. *Anal. Biochem.* **232**, 56–64.
- Rocchi, S., Tartare-Deckert, S., Mothe, I. and Van Obberghen, E. (1995) Identification by mutation of the tyrosine residues in the insulin receptor substrate-1 affecting association with the tyrosine phosphatase 2C and phosphatidylinositol 3-kinase. *Endocrinology* **136**, 5291–5297.
- Hunter, T. and Sefton, B. M. (1980) Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1311–1315.
- Kerlavage, A. R. and Taylor, S. S. (1982) Site-specific cyclic nucleotide binding and dissociation of the holoenzyme of cAMP-dependent protein kinase. *J. Biol. Chem.* **257**, 1749–1754.
- Yu, D.-H., Qu, C.-K., Henegariu, O., Lu, X. and Feng, G.-S. (1998) Protein-tyrosine phosphatase Shp-2 regulates cell spreading, migration, and focal adhesion. *J. Biol. Chem.* **273**, 21125–21130.
- Lahav, M., Rennert, H. and Barzilai, D. (1986) Inhibition by vanadate of cyclic AMP production in rat corpora lutea incubated *in vitro*. *Life Sci.* **39**, 2557–2564.
- Aharoni, D., Dantes, A. and Amsterdam, A. (1993) Cross-talk between adenylate cyclase activation and tyrosine phosphorylation leads to modulation of the actin cytoskeleton and to acute progesterone secretion in ovarian granulosa cells. *Endocrinology* **133**, 1426–1436.
- Russell, D. L. and Richards, J. S. (1999) Differentiation-dependent prolactin responsiveness and stat (signal transducers and activators of transcription) signaling in rat ovarian cells. *Mol. Endocrinol.* **13**, 2049–2064.
- Flint, A. J., Gebbink, M. F. G. B., Franza, Jr., B. R., Hill, D. E. and Tonks, N. K. (1993) Multi-site phosphorylation of the protein tyrosine phosphatase, PTP1B: identification of cell cycle regulated and phorbol ester stimulated sites of phosphorylation. *EMBO J.* **5**, 1937–1946.
- Peraldi, P., Zhao, Z., Filloux, C., Fisher, E. and Van Obberghen, E. (1994) Protein-tyrosine-phosphatase 2C is phosphorylated and inhibited by 44-kDa mitogen-activated protein kinase. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 5002–5006.
- Pan, M. G., Florio, T. and Stork, P. J. (1992) G protein activation of a hormone-stimulated phosphatase in human tumour cells. *Science* **256**, 1215–1217.
- Florio, T., Rim, C., Hershberger, R. E., Loda, M. and Stork, P. J. (1994) The somatostatin receptor SSTR1 is coupled to phosphotyrosine phosphatase activity in CHO-K1 cells. *Mol. Endocrinol.* **8**, 1289–1297.
- Florio, T., Pan, M. G., Newman, B., Hershberger, R. E., Civitelli, O. and Stork, P. J. (1992) Dopaminergic inhibition of DNA synthesis in pituitary tumor cells is associated with phosphotyrosine activity. *J. Biol. Chem.* **267**, 24169–24172.
- Mauro, L. J., Olmsted, E. A., Skrobacz, B. M., Mourey, R. J., Davis, A. R. and Dixon, J. E. (1994) Identification of a hormonally regulated protein tyrosine phosphatase associated with bone and testicular differentiation. *J. Biol. Chem.* **269**, 30659–30667.
- Brautigan, D. L. and Pinault, F. M. (1991) Activation of membrane protein-tyrosine phosphatase involving cAMP- and Ca²⁺/phospholipid-dependent protein kinases. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 6696–6700.
- Graves, L. M. and Lawrence, J. C. (1996) Insulin, growth factors, and cAMP: antagonism in the signal transduction pathways. *Trends Endocrinol. Metab.* **7**, 43–50.
- Chang, J. S., Iwashita, S., Lee, Y. H., Kim, M. J., Ryu, S. H. and Suh, P. G. (1999) Transformation of rat fibroblasts by phospholipase C-γ1 overexpression is accompanied by tyrosine dephosphorylation of paxillin. *FEBS Lett.* **460**, 161–165.

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