Protein tyrosine phosphatase 1B interacts with and is tyrosine phosphorylated by the epidermal growth factor receptor

Feng LIU* and Jonathan CHERNOFF†[1]

*Chemistry Department, Temple University, Philadelphia, PA 19122, U.S.A., and †Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, PA 19111, U.S.A.

We used a substrate-trapping technique to search for substrates of protein tyrosine phosphatase (PTP) 1B. A catalytically inactive form of this enzyme forms a stable, phosphotyrosine-dependent complex with epidermal growth factor receptor (EGFR) both *in vitro* and in cells. PTP1B also interacts with activated platelet-derived growth factor receptor (PDGFR) but not with colony-stimulating factor 1 receptor (CSF-1R). After binding to EGFR, PTP1B becomes tyrosine-phosphorylated at Tyr-66, a site that conforms to the consensus binding sequence for the Src homology 2 (SH2) domains of the adapter protein Grb2. This tyrosine phosphorylation is correlated with a 3-fold increase in PTP catalytic activity. These findings suggest that PTP1B selectively regulates specific activated receptor protein tyrosine kinases (RPTKs) *in vivo* and might itself be regulated by such receptors.

INTRODUCTION

The phosphorylation of proteins on tyrosine residues is a central mechanism regulating signalling networks that control cell growth and differentiation [1,2]. Tyrosine phosphorylation is a dynamic process resulting from the competing activities of protein tyrosine kinases and protein tyrosine phosphatases (PTPs). As the role of protein tyrosine kinases in signal transduction is now understood in some detail, increasing attention has been focused on the role of PTPs in regulating signalling pathways. To determine the physiological roles of these enzymes, it is important to identify their substrates *in vivo*. One method that has been used for this purpose exploits the ability of certain PTP mutants to bind to, but not dephosphorylate, tyrosine phosphorylated substrates. These PTP mutants are constructed by replacing an essential conserved cysteine residue with serine. Such cysteine to serine (CS) mutations completely abolish the catalytic activity of the enzyme without unduly distorting protein folding [3,4]. This strategy has been used successfully to show interaction between the transmembrane PTP CD45 and its putative substrate, the CD3 ζ chain [5]. Similar complex formation has been observed between a CS mutant of the mammalian dual-specificity PTP MKP1 and $p42^{MAPK}$ [6] and fission yeast PTPs Pyp1 and Pyp2 and the stress-activated protein kinase Spc1 [7,8]. Finally, the CS mutant of human PTP1B, a ubiquitous non-transmembrane phosphatase of unknown function, has been shown to bind to activated epidermal growth factor (EGF) receptor (EGFR) *in vitro* [9]. The structural basis for binding of CS PTP1B to a EGFR-derived phosphotyrosyl peptide has recently been elucidated by X-ray crystallography [3].

On the basis of its effects on mitogenesis and on insulin signalling, PTP1B is thought to have a negative role in signal transduction [10,11], but the mechanism by which this occurs is not understood. PTP1B does not contain obvious targeting motifs, such as Src homology 2 (SH2) or phosphotyrosine-binding (PTB) domains, that mediate binding to tyrosine autophosphorylation sites on RPTKs. As there are few reports of PTP1B–RPTK interactions in intact cells [12], it remains possible that the observed effects of PTP1B on RPTK tyrosine phosphorylation levels and activity are indirect. In this paper we show that CS-PTP1B forms complexes with activated EGFR and platelet-derived growth factor (PDGF) receptor (PDGFR), but not with CSF-1R, in a phosphotyrosine-dependent manner in cultured cells. When bound to EGFR, the CS-PTP1B becomes tyrosine-phosphorylated at Tyr-66; this phosphorylation increases the catalytic activity of PTP1B approx. 3-fold. These findings demonstrate that PTP1B can selectively couple to certain RPTKs and suggest that this phosphatase might regulate and/or serve as a target for these kinases.

MATERIALS AND METHODS

Materials

EGF was purchased from Harlan Bioproducts. EGFR was purified from A431 cells by the procedure of Cohen et al. [13]. PDGF, anti-EGFR and anti-PDGFR antibodies were purchased from Upstate BioTechnology. Monoclonal anti-haemagglutinin (12CA5) and anti-phosphotyrosine (PY20) antibodies were from BabCo and Transduction Laboratories respectively. Human colony-stimulating factor 1 (CSF-1) was a gift from Richard Stanley (Albert Einstein College of Medicine) and antibodies against CSF-1 receptor (CSF-1R) were a gift from Martine Roussel (St Jude's Children's Hospital). Chymotrypsin was obtained from Worthington Biochemical Co., and glutathione agarose was from Sigma.

Expression and purification of glutathione S-transferase (GST) fusion proteins

$PTP1B^{C215S}$ (CS-PTP1B), $PTP1B^{Y66F}$, CS-$PTP1B^{Y66F}$ and CS-$PTP1B^{Y152,153F}$ were constructed by site-directed mutagenesis with standard techniques [14]. Wild-type and mutant cDNA species were subcloned as *Bam*HI, *Eco*RI fragments into pGEX-2T [15]. GST–PTP1B fusion proteins were made and purified by standard methods [15]. In some cases, GST–PTP1B proteins

Abbreviations used: ATP[S], adenosine 5′[γ-thio]triphosphate; CS, cysteine to serine; CSF-1, colony-stimulating factor 1; CSF-1R, CSF-1 receptor; DMEM, Dulbecco's modified Eagle medium; EGF, epidermal growth factor; EGFR, EGF receptor; ER, endoplasmic reticulum; FBS, fetal bovine serum; GAP, GTPase activating protein; GST, glutathione S-transferase; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; PLCγ, phospholipase Cγ; PNPP, *p*-nitrophenyl phosphate; PTB, phosphotyrosine-binding; PTP, protein tyrosine phosphatase; RPTK, receptor protein tyrosine kinase; SH2, Src homology 2; SGB, stacking gel buffer.

[1] To whom correspondence should be addressed.

were eluted from the beads in 50 mM Tris/HCl (pH 8.0)/5 mM glutathione.

A431 cell lysates

A431 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10 % (v/v) fetal bovine serum (FBS) (Gibco) until they reached 80 % confluence. The cells were starved overnight, treated with EGF (400 ng/ml) for 10 min, then lysed in NP40 lysis buffer [50 mM Tris/HCl (pH 8.0)/137 mM NaCl/10 % (v/v) glycerol/1 % (v/v) NP40/50 mM NaF/10 mM β-glycerophosphate] containing 1 mM sodium vanadate, 1 mM PMSF and 10 μg/ml aprotinin.

Expression plasmids and transfection

pCMV5 EGFR was constructed by cutting LTR-2 EGFR [16] with *Xho*I and transferring the 4.2 kb fragment into *Xho*I-cut pCMV5 [17]. pCMV5-CSF-1R was constructed by cutting pSM-CSF-1R [18] with *Bam*HI and transferring the approx. 3 kb fragment into *Bam*HI-cut pCMV5. pSV40-PDGFR [19] was kindly provided by Phil Tsichlis (Fox Chase Cancer Center). The construction of pJ3H-PTP1B has been described previously [20]. This expression plasmid encodes an N-terminal haemagglutinin-tagged PTP1B. CS-PTP1B, CS-PTP^{Y66F} and CS-PTP^{Y152,153F} were subcloned as *Bam*HI, *Eco*RI fragments into pJ3H. COS-1 cells were grown in DMEM plus 10 % (v/v) FBS until they reached 80 % confluence; they were then transfected with expression plasmids by using Lipofectamine (Gibco/BRL) in accordance with the manufacturer's recommendations. The cells were harvested for analysis 48 h after transfection.

Immunoprecipitations and phosphoamino acid analysis

Cells were starved overnight in 0.5 % FBS in DMEM and labelled for 4 h with 100 μCi/ml [^{35}S]methionine (ICN) in 90 % methionine-free minimal essential medium/10 % DMEM containing 0.5 % (v/v) fetal calf serum, then treated with EGF (400 ng/ml) or PDGF (5 ng/ml) for 10 min, or CSF-1 (10000 i.u./ml) for 2 min before the cells were lysed. For ^{32}P labelling, cells were washed three times with phosphate-free DMEM then incubated at 37 °C for 2 h in phosphate-free DMEM containing 100 μCi/ml carrier-free [^{32}P]P_i (ICN). The cells were stimulated with EGF (400 ng/ml) for 10 min before lysis. Cells were lysed in NP40 lysis buffer containing 1 mM sodium vanadate, 1 mM PMSF and 10 μg/ml aprotinin. Immunoprecipitations were performed with 2 mg/ml anti-EGFR or 2 mg/ml anti-HA antibodies in 100 μl for a minimum of 2 h at 4 °C. Immunocomplexes were washed three times with NP40 lysis buffer and boiled for 5 min in SDS/PAGE sample buffer. The samples were fractionated by SDS/PAGE [10 % (w/v) gel] and transferred to nitrocellulose membranes. The membranes were autoradiographed and also probed with anti-receptor and anti-phosphotyrosine antibodies. Phosphoamino acid analysis was performed by one-dimensional thin-layer electrophoresis as described [21].

Phosphorylation mapping *in vitro*

GST–CS-PTP1B, GST–CS-PTP1B^{Y66F} and GST–CS-PTP-1B$^{Y152/153F}$ fusion proteins were made in *Escherichia coli* and purified on glutathione–agarose beads. EGFR membrane vesicles (10 μg) were mixed with 25 μl of GST fusion protein beads [50 % (v/v) in PBS], incubated at 30 °C for 30 min in kinase buffer [20 mM Tris/HCl (pH 7.5)/10 mM $MgCl_2$] in the presence of 1 mM sodiium vanadate, 300 μM ATP and 20 μCi of [γ-^{32}P]ATP. The beads were washed three times in NP40 lysis buffer, separated by SDS/PAGE [10 % (w/v) gel] and transferred to nitrocellulose membranes. The membranes were exposed to Kodak X-ray film. Phosphoamino acid analysis was performed by one-dimensional thin-layer electrophoresis as described [21].

PTP1B phosphorylation *in vitro*

The kinase reaction mixture included recombinant GST–PTP1B (wild-type PTP1B, PTP1B^{C215S}, PTP1B^{Y66F}) (100 μg), kinase buffer [25 mM Hepes (pH 7.5)/10 mM $MgCl_2$/10 mM $MnCl_2$/0.1 % NP40/100 mM NaCl/10 % (v/v) glycerol], BSA (1.5 μg) and 300 μM adenosine 5′[γ-thio]triphosphate (ATP[S]) (Sigma), and Protein A–Sepharose beads (20 μl) bound to EGFR (immunoprecipitated from A431 cells), in a total reaction volume of 100 μl. The control reaction mixture was identical but did not include ATP[S]. The incubation was performed at 30 °C. Aliquots of supernatant were removed at various time intervals and assayed for PTPase activity.

PTPase activity assay

The activity of the enzyme (10 μg) was determined at 30 °C in 0.1 mM acetate/1 mM EDTA (pH 5.5) *p*-nitrophenyl phosphate (PNPP; 4 mM) as substrate. The reaction was incubated for 15 min and quenched by adding 1 ml of 1 M NaOH. The amount of product (*p*-nitrophenol) produced was measured from the absorbance at 405 nm. The non-enzymic hydrolysis of PNPP was corrected for by measuring the increase in absorbance at 405 nm obtained in the absence of enzyme.

Partial proteolytic mapping

The ^{35}S-labelled samples were separated by SDS/PAGE and the gels were exposed to film overnight. The bands corresponding to PTP1B and putative PTP1B (50 kDa) were excised, soaked for 30 min in 1:4 stacking gel buffer (SGB) containing 1 mM EDTA, and inserted into wells of an 11–20 % (w/v) gradient acrylamide gel. Each slice was covered with 10 μl of dye [0.005 % Bromophenol Blue in 1:4 SGB/30 % (v/v) glycerol] and, on top of that, 10 μl of 1:4 SGB containing 10 % (v/v) glycerol and chymotrypsin at 250 μg/ml. The gel was run at 20 mA but was stopped for 30 min as the dye front reached the end of the stacking gel to allow for proteolysis. The gel was dried and exposed to film.

Phosphorylation stoichiometry

COS cells were plated on 60 mm^2 plates and transfected with EGFR and CS PTP1B, then grown in 90 % methionine-free DMEM/10 % (v/v) FBS containing 100 μCi/ml [^{35}S]methionine for 12 h, then in similar medium with 0.5 % (v/v) FBS for 12 h. The cells were treated with EGF (400 ng/ml) for 10 min, then washed three times with PBS and lysed in NP40 lysis buffer. The concentrations of cellular proteins in cell lysates were determined by using a standard bicinchoninic acid protein assay (Pierce) and the cellular proteins were precipitated with 25 % (w/v) trichloroacetic acid on a Millipore filter. Incorporation of [^{35}S]methionine into acid-precipitable material was measured by scintillation counting; the specific activity of cellular protein was then calculated. Anti-HA and anti-EGFR immunoprecipitates were fractionated by SDS/PAGE and the gel was dried without fixation. The pieces corresponding to PTP1B were excised and dissolved in 0.4 ml of 60 % (v/v) perchloric acid and 0.8 ml of 30 % (v/v) H_2O_2 at 60 °C. The amount of radioactivity in each

sample was determined by scintillation counting. The amount of PTP1B in the immunoprecipitate was then calculated. To determine the phosphorylation stoichiometry the cells were labelled with 100 μCi/ml [^{32}P]P_i in phosphate-free DMEM at 37 °C for 6 h. The labelling medium was saved and the concentration of ^{32}P in the medium was determined by scintillation counting. The specific activity of the labelling medium was then calculated. Anti-HA and anti-EGFR immunoprecipitates were fractionated by SDS/PAGE and the PTP1B bands located by autoradiography. Gel pieces containing PTP1B and a representative background level of radioactivity were excised and counted, and the number of moles of phosphate recovered in the protein by immunoprecipitation was calculated.

RESULTS

PTP1B co-immunoprecipitates with EGFR

To investigate potential interaction between PTP1B and EGFR in cells, a transient expression system was employed. COS cells were co-transfected with PTP1B and EGFR expression plasmids and metabolically labelled with [^{35}S]methionine. After stimulation with EGF, the cells were lysed and immunoprecipitated with either anti-EGFR or anti-HA antibodies. The immunocomplexes were fractionated by SDS/PAGE, transferred to nitrocellulose and analysed by anti-EGFR and anti-phosphotyrosine immunoblot (Figures 1A and 1B) and by autoradiography (Figure 1C). In cells transfected with catalytically inactive CS-PTP1B, anti-HA antibodies co-precipitate a protein of approx. 180 kDa that is recognized by EGFR antibody (Figure 1A, lanes 2 and 4). Reprobing the same blot with anti-phosphotyrosine antibody revealed that this 180 kDa protein is tyrosine-phosphorylated in a ligand-dependent manner (Figure 1B). A similar band of approx. 180 kDa is seen in anti-HA immunoprecipitates from cells transfected with CS-PTP1B alone (results not shown), which is likely to represent the endogenous EGFR (see, for example, Figure 1A, lane 6, and Figure 1B, lane 2). Association of EGFR with PTP1B is at least partly ligand-dependent, as evidenced by the increased EGFR signal detected in growth factor-treated cells. Binding of unstimulated EGFR to CS-PTP1B might be due to protection of the EGFR tyrosine phosphorylation sites by inactive PTP1B. A similar phenomenon has been observed with p42^{MAPK} in cells expressing a catalytically inactive form of MKP1 [6]. EGFR was not detected in immunoprecipitates of PTP1B-transfected cells by using non-immune IgG (Figure 1A, lane 5), or immunoprecipitates of untransfected cells by using anti-HA antibodies (lane 7). These results demonstrate that the observed interaction between CS-PTP1B and the EGFR receptor is specific.

As PTP1B co-migrates with IgG heavy chains on SDS/PAGE, making identification by immunoblot difficult, we used autoradiography of ^{35}S-labelled proteins to examine whether anti-EGFR immunoprecipitates contain PTP1B. In untransfected cells, neither anti-EGFR nor anti-HA antibodies immunoprecipitated proteins in the 50 kDa range (Figure 1C, lanes 1 and

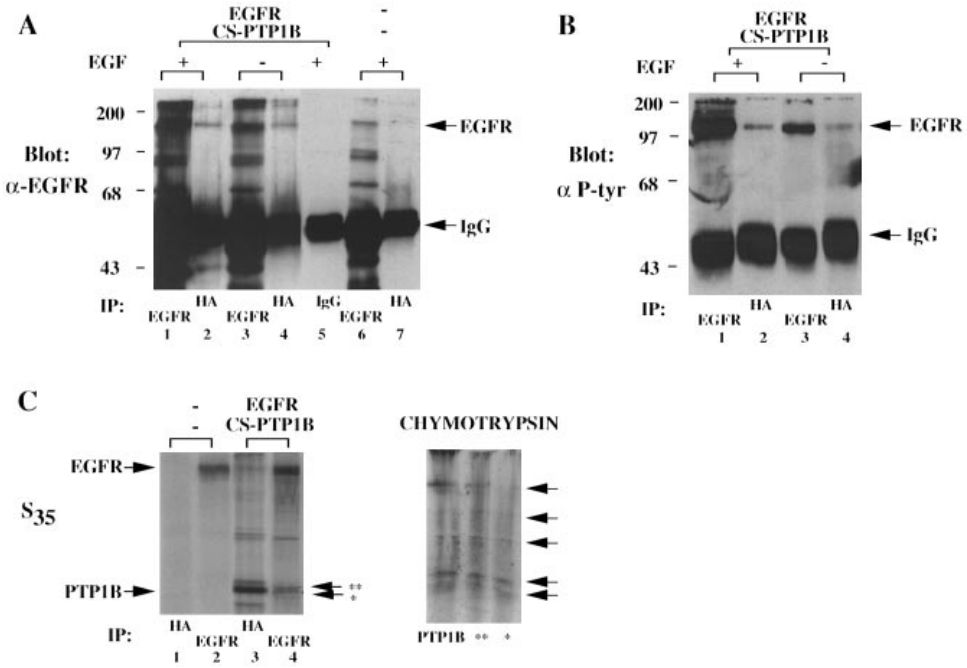

Figure 1 PTP1B binds to EGFR in cells

(**A**) COS cells were transfected with pCMV5-EGFR and pJ3H-CS-PTP1B (lanes 1–5) or empty vectors (lanes 6 and 7); 24 h after transfection the cells were starved overnight in 0.5% (v/v) FBS in DMEM. The cells were then left untreated or stimulated with EGF for 10 min, then lysed and immunoprecipitated by either anti-EGFR (lanes 1, 3 and 6) or anti-HA (lanes 2, 4 and 7) antibody. The immunocomplexes were fractionated by electrophoresis, transferred to a nitrocellulose membrane and probed with anti-EGFR antibody. Control immunoprecipitation, with non-immune IgG (lane 5) was also performed. The positions of the EGFR and IgG heavy chains are indicated by arrows. These experiments were repeated at least four times, with essentially identical results. (**B**) COS cells were transfected and processed as in (**A**), then immunoprecipitated with either anti-EGFR (lanes 1 and 3) or anti-HA (lanes 2 and 4). Immunoblots were performed with anti-phosphotyrosine (α-P-Tyr) antibody. (**C**) COS cells were transfected with empty vectors or vectors expressing EGFR and CS-PTP1B as in (**A**) and metabolically labelled with [^{35}S]methionine for 4 h. Lysates were immunoprecipitated with anti-EGFR or anti-HA. An autoradiogram of immunoprecipitated samples is shown (left panel). The PTP1B band in the anti-HA immunocomplex (third lane) and the 50 kDa doublets in the anti-EGFR immunocomplex (fourth lane, asterisks) were excised, subjected to chymotrypsin partial digestion, and separated by SDS/PAGE [11–20% (w/v) gradient gel]. An autoradiogram of the dried gel is shown (right panel). Abbreviation: IP, immunoprecipitate.

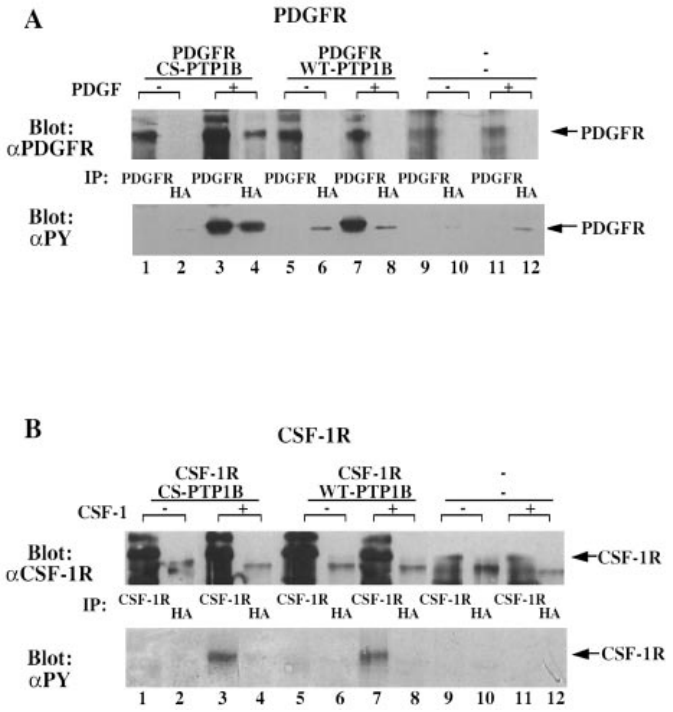

Figure 2 PTP1B binds selectively to RPTKs

(**A**) COS cells were transfected with pSV-PDGFR and pJ3H-PTP1B; 48 h after transfection the cells were stimulated with PDGF for 10 min, then lysed and immunoprecipitated by either anti-PDGFR or anti-HA antibody. The immunocomplexes were fractionated by electrophoresis, transferred to nitrocellulose membranes and probed with anti-PDGFR (αPDGFR) (upper panel) or anti-phosphotyrosine (αPY) antibodies (lower panel). The odd-numbered lanes were immunoprecipitated (IP) with anti-PDGFR and the even-numbered lanes with anti-HA antibody. Lanes 1–4, COS cells transfected with pSV-PDGFR and pJ3H-CS-PTP1B; lanes 5–8, COS cells transfected with pSV-PDGFR and pJ3H-wild-type (WT)-PTP1B; lanes 9–12, untransfected COS cells (negative control). The positions of PDGFR, PTP1B and IgG are indicated. (**B**) COS cells were transfected with pCMV5-CSF-1R and pJ3H-PTP1B. After stimulation with CSF-1 for 2 min, the cells were lysed and processed as above. The odd-numbered lanes were immunoprecipitated with anti-CSF-1R antibody and the even-numbered lanes with anti-HA antibody.

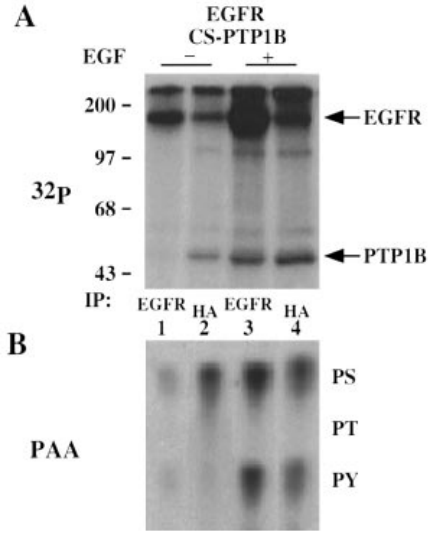

Figure 3 PTP1B becomes tyrosine-phosphorylated on binding to EGFR

(**A**) Expression vectors bearing EGFR and CS were co-transfected into COS cells. The cells were starved overnight in 0.5% (v/v) FBS in DMEM, labelled for 2 h with [^{32}P]P_i, stimulated with EGF for 10 min, then lysed and immunoprecipitated by either anti-EGFR (odd-numbered lanes) or anti-HA antibody (even-numbered lanes) as indicated. The immunocomplexes were fractionated by SDS/PAGE and autoradiographed. (**B**) Phosphoamino acid analysis (PAA) of the 50 kDa phosphorylated bands from anti-HA and anti-EGFR immunocomplexes [as shown in (**A**)]. Phosphoamino acids were resolved in one dimension with a pH 2.5 buffer. Abbreviations: PS, phosphoserine; PT, phosphothreonine; PY, phosphotyrosine. The positions of EGFR and PTP1B are indicated.

2). However, in CS-PTP1B-expressing cells, anti-EGFR immunoprecipitates contained a prominent radiolabelled doublet band with approximately the same mobility as PTP1B, whereas anti-HA immunoprecipitates contained a band that co-migrated with EGFR (lanes 3 and 4). To determine whether the approx. 50 kDa protein bands that co-precipitated with EGFR were indeed PTP1B (Figure 1C, lane 4, arrows labelled with asterisks), partial chymotryptic mapping was performed on the authentic and presumptive PTP1B bands. Both of the doublet bands that co-precipitated with EGFR had similar chymotryptic profiles, identical with that of authentic PTP1B (Figure 1C, right panel). Although not examined in detail, we assume that the upper band of the doublet represents a more highly phosphorylated form of PTP1B. These results indicate that the 50 kDa protein that complexed with EGFR is likely to be PTP1B.

PTP1B interacts selectively with specific RPTKs

To determine whether the PTP1B/EGFR interaction is unique to this receptor, we also tested whether PTP1B binds to β-PDGF and CSF-1 receptors. These two receptors are similar in structure and belong to the same subfamily of RPTKs, but have significantly different autophosphorylation sites. For example, in contrast with β-PDGFR, CSF-1R lacks binding sites for phospholipase Cγ (PLCγ) and GTPase activating protein (GAP) [22,23]. COS cells were transfected with either a PDGFR or CSF-1R expression plasmid plus wild-type or catalytically inactive (CS) PTP1B. The transfected cells were starved overnight, then treated with the appropriate growth factor. Reciprocal immunoprecipitations were performed using anti-receptor and anti-HA antibodies. The immunoprecipitates were fractionated by SDS/PAGE and transferred to nitrocellulose membranes, then probed with anti-receptor or anti-phosphotyrosine antibody. These experiments revealed that PDGFR co-immunoprecipitates with CS-PTP1B (Figure 2A, upper panel, lane 4), and that this association is phosphotyrosine-dependent (Figure 2A, lower panel; compare lanes 2 and 4). Although the anti-HA antibody appeared to precipitate non-specifically a small amount of tyrosine-phosphorylated protein with the same mobility as PDGFR (Figure 2A, lower panel, lanes 10 and 12), the amount of genuine PDGFR increased markedly in anti-HA immunoprecipitates from CS-PTP1B-transfected cells (Figure 2A, lane 4). With CSF-1R, co-precipitation was not seen in anti-HA immunoprecipitates in either unstimulated or stimulated cells (Figure 2B). These results indicate that tyrosine phosphorylated PDGFR, but not CSF-1R, co-precipitates with catalytically inactive PTP1B.

PTP1B becomes tyrosine phosphorylated on binding to EGFR

Previous studies have reported that PTP1B is phosphorylated primarily at serine residues in cells [24–26]. To investigate the phosphorylation state of EGFR-bound PTP1B, we performed reciprocal immunoprecipitations of CS-PTP1B and EGFR from metabolically ^{32}P-labelled transfected COS cells. Anti-EGFR

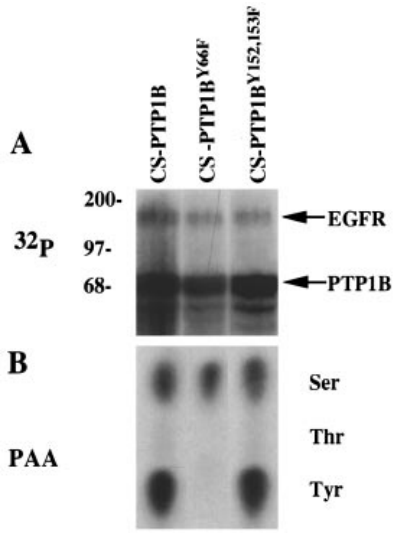

Figure 4 Tyr-66 is the major site on PTP1B for phosphorylation by EGFR *in vitro*

(**A**) Glutathione–agarose bound GST–CS-PTP1B, GST–PTP1B^{Y66F} or GST–PTP1B^{Y152,153F} were incubated with purified A431 membranes in the presence of [γ-^{32}P]ATP as described in the Materials and methods section. After extensive washing, the beads were dissolved in SDS/PAGE sample buffer, fractionated by SDS/PAGE [10% (w/v) gel], dried and autoradiographed. The positions of EGFR and PTP1B are indicated. (**B**) Phosphoamino acid analysis (PAA) of PTP1B was performed in one dimension with a pH 2.5 buffer. Abbreviations: Ser, phosphoserine; Thr, phosphothreonine; Tyr, phosphotyrosine.

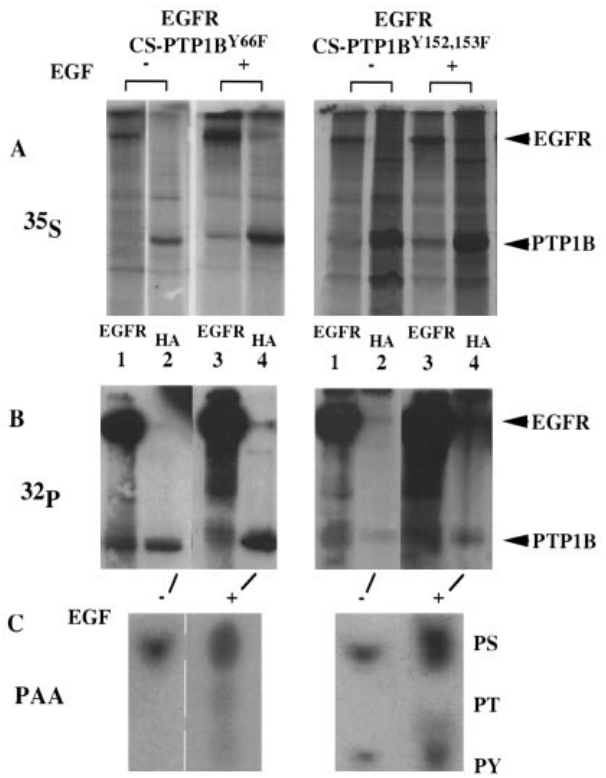

Figure 5 Tyr-66 is the major site on PTP1B for phosphorylation by EGFR *in vivo*

COS cells were co-transfected with the expression plasmids pJ3H-CS-PTP1B^{Y66F} or pJ3H-CS-PTP1B^{Y152,153F} plus pCMV5-EGFR. The cells were labelled with either (**A**) ^{35}S or (**B**) ^{32}P and immunoprecipitated as described in the legend to Figure 4. Odd-numbered lanes were immunoprecipitated with anti-EGFR antibody and even-numbered lanes with anti-HA (PTP1B) antibody. The positions of EGFR and PTP1B are indicated by arrows. (**C**) Phosphoamino acid analysis (PAA) of the 50 kDa phosphorylated bands in anti-HA immunocomplexes. PS, phosphoserine; PT, phosphothreonine; PY, phosphotyrosine.

immunoprecipitates contain a 50 kDa phosphoprotein that co-migrates with PTP1B, and anti-HA immunoprecipitates contain a 180 kDa phosphoprotein that co-migrates with EGFR (Figure 3A). The 50 kDa ^{32}P-labelled CS-PTP1B (Figure 3A, lanes 2 and 4) as well as the putative CS-PTP1B (i.e. the 50 kDa band in anti-EGFR immunocomplex, lanes 1 and 3) were excised from the gel, eluted and subjected to phosphoamino acid analysis. This analysis revealed that without EGF stimulation, CS-PTP1B is mostly serine-phosphorylated, whereas with EGF stimulation, serine phosphorylation content increases and there is also a substantial amount of tyrosine phosphorylation (Figure 3B). These results suggest that activated EGFR tyrosine-phosphorylates bound PTP1B. The stoichiometry of phosphorylation of mutant PTP1B was determined by parallel equilibrium labelling of transfected COS cells with [^{35}S]methionine and [^{32}P]P_i and subsequent immunoprecipitation of PTP1B and EGFR. The amount of radioactivity in the PTP1B bands was determined and the stoichiometry of phosphorylation of PTP1B was calculated. Before EGF treatment 5.8% of total CS-PTP1B was serine-phosphorylated and 0.2% was tyrosine-phosphorylated (lane 2). After EGF treatment the serine and tyrosine phosphorylation levels increased to 21.9% and 12% respectively (lane 4).

Mapping the major tyrosine phosphorylation site *in vitro* on PTP1B

Most RPTKs phosphorylate substrates at tyrosine residues that are preceded by acidic amino acids [27]. We examined the sequence of PTP1B for such potential tyrosine phosphorylation sites. Only three tyrosine residues fitted this consensus: Tyr-66 (QEDNDY) and Tyr-152 and Tyr-153 (EDIKSYY); these were each replaced with phenylalanine residues. To study the effects of such mutations on PTP1B binding and phosphorylation by EGFR, we examined the ability of this kinase to phosphorylate such tyrosine-to-phenylalanine PTP1B mutants *in vitro*. GST–CS-PTP1B^{Y66F} and GST–CS-PTP1B^{Y152,153F} were expressed in *E. coli* and purified by glutathione–agarose chromatography. These proteins were then incubated with membranes isolated from A431 cells in kinase buffer in the presence of EGF and [γ-^{32}P]ATP. The ^{32}P-labelled GST–CS-PTP1B, GST–CS-PTP1B^{Y66F} and GST–CS-PTP1B^{Y152,153F} bands were excised from the gel, eluted and subjected to phosphoamino acid analysis. Although CS-PTP1B^{Y66F}/EGFR binding was unaffected (Figure 4A), the tyrosine phosphorylation of CS-PTP1B^{Y66F} was almost completely eliminated (Figure 4B). Replacement of tyrosine residues 152 and 153 with phenylalanine did not affect either binding or tyrosine phosphorylation of PTP1B. These results indicate that Tyr-66 is the major site on PTP1B for tyrosine phosphorylation by EGFR *in vitro*.

Tyr-66 is the major EGF-stimulated tyrosine phosphorylation site on PTP1B *in vivo*

To confirm that the mapping results obtained *in vitro* correspond to intracellular events, COS cells expressing CS-PTP1B^{Y66F} and CS-PTP1B^{Y152,153F} were metabolically labelled with ^{35}S or ^{32}P,

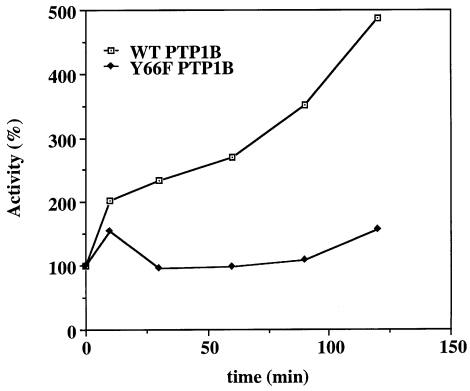

Figure 6 Activity enhancement of phosphorylated PTP1B

The enzyme was incubated at 30 °C with immunoprecipitated EGFR in the presence of ATP[S]. At zero time and at 10, 30, 60, 90 and 120 min, aliquots were sampled from the reaction mixture and assayed for their PTPase activity with PNPP as substrate. The activity was evaluated with respect to a control reaction mixture that did not include ATP[S] and whose activity was given as 100% at any time. Similar results were obtained in three independent experiments.

stimulated with or without EGF, and immunoprecipitated with either anti-EGFR or anti-HA antibodies. The 50 kDa ^{32}P-labelled CS-PTP1B^{Y66F} and CS-PTP1B^{Y152,153F} bands were excised from the gel, eluted and subjected to phosphoamino acid analysis. Although CS-PTP1B^{Y66F}/EGFR binding was unaffected (Figures 5A and 5B), the tyrosine phosphorylation level of CS-PTP1B^{Y66F} was only about one-tenth of that of CS-PTP1B^{Y152,153F} on EGF stimulation, as determined by phosphorimaging analysis (Figure 5C). Replacement of tyrosine residues 152 and 153 with phenylalanine did not affect either binding or tyrosine phosphorylation of PTP1B. These results indicate that Tyr-66 is the major site on PTP1B for tyrosine phosphorylation by EGFR *in vivo*.

Phosphorylation of PTP1B by EGFR enhances its catalytic activity

To assess whether tyrosine phosphorylation of PTP1B modulates its activity, we preincubated GST–PTP1B with EGFR (immunoprecipitated from A431 cells) for various lengths of time in kinase buffer, with ATP[S] in place of ATP. The resulting thiophosphorylated residues are relatively resistant to PTP activity [28], thus preventing rapid auto-dephosphorylation of PTP1B. EGFR was removed and the activity of thio-phosphorylated PTP1B was assayed by using PNPP as substrate. We observed an activation that was correlated with time of incubation with EGFR. As can be seen in Figure 6, the activity of the thio-phosphorylated wild-type enzyme reached 360% activity on PNPP, taking the activity of the control mixture containing the unphosphorylated enzyme as 100%. In contrast, the activity of PTP1B^{Y66F}, in which the target tyrosine residue had been mutated to phenylalanine, was not affected by pre-incubation with EGFR plus ATP[S]. As an additional control, catalytically inactive PTP1B^{C215S} was used to show that the aliquots taken from EGFR/PTP1B kinase mixture had not been significantly contaminated with any other PTPs.

DISCUSSION

PTP1B was originally purified as a 37 kDa protein from human placenta [29]. The subsequent cloning of this gene indicated that the purified placental protein was a proteolytic fragment of a 50 kDa protein [30,31]. This full-length PTP1B (435 amino acid residues) protein has at least three distinct structural features: the phosphatase domain, which comprises approximately residues 40–276, a proline-rich region located just C-terminal to the phosphatase domain, which directs interactions with SH3-containing proteins [32], and a highly hydrophobic domain at the C-terminus, which localizes PTP1B to the endoplasmic reticulum (ER) [25]. The ubiquitous tissue distribution and abundant expression of PTP1B suggests that it has a role in controlling some general cellular function. However, at present, neither the physiological substrates for PTP1B nor its cellular functions are known.

Milarski et al. [9] showed that PTP1B, which does not have recognizable SH2 or PTB domains, binds to activated EGFR *in vitro*. Although this PTP is able to bind to several different phosphotyrosine residues on EGFR, there is a marked preference for certain sites, implying that the binding occurs in a sequence-specific manner. Labelled PTP1B could be displaced from EGFR by the SH2 domains from GAP and PLCγ with IC_{50} values of 0.18 and 0.10 μM respectively. These values are similar to the IC_{50} for unlabelled CS-PTP1B [9]. These authors also demonstrated that mutations within two of the PLCγ sites severely affected the ability of PTP1B to associate with EGFR. These results suggest that PTP1B binds specifically to certain phosphotyrosine residues on EGFR. We showed that, in tissue culture cells, PTP1B binds to EGF and PDGF but not CSF-1R. It is interesting to note that CSF-1R, unlike EGFR and PDGFR, lacks binding sites for the GAP and PLCγ SH2 domains [22,23]. These results underscore the specificity of the interaction between PTP1B and RPTKs, and lead to the prediction that this phosphatase interacts preferentially with receptors that contain GAP and/or PLCγ SH2 binding sites. Although PTP1B does not contain recognizable SH2 or PTB domains, it does contain the sequence FKVRESGS 19 residues N-terminal to the catalytically essential Cys-215 [30,31]. A similar motif, FLVRESES, is a core element found in SH2 domains [33,34]. Indeed, a peptide comprising these eight residues is able to bind to tyrosine-phosphorylated peptides [35]. It is possible that the interaction between PTP1B and the EGFR is mediated through this sequence.

On association with activated EGFR, PTP1B becomes phosphorylated at Tyr-66. The sequence pY^{66}INA at this tyrosine residue fits the consensus sequence (YXNX) for binding of the adapter protein GRB2 [36]. Several other PTPs have been shown to become tyrosine-phosphorylated at similar sites and to bind GRB2. For example, on stimulation with PDGF, SHP2 binds to the PDGFR and becomes tyrosine-phosphorylated at Tyr-542 (pY^{542}TNI). Tyrosine-phosphorylated SHP2 binds GRB2, which by its association with SOS activates Ras [37]. This indicates that the phosphorylation of SHP2 couples GRB2 to the PDGFR, providing a mechanism for positive signalling via SHP2. However, RPTPα, which is phosphorylated on Tyr-789 (pY^{789}ANF) and like SHP2 forms a complex with GRB2 *in vivo*, is thought to inhibit signalling from RPTKs [38]. Unlike SHP2, the RPTPα/GRB2 complex does not include SOS, which suggests that RPTPα could attenuate growth factor signalling by competing with RPTKs for GRB2. Therefore, if PTP1B does associate with adapter proteins such as GRB2 after phosphorylation at Tyr-66, it is uncertain whether these complexes would serve to activate or to inhibit signalling. It is also worth noting that the YINA motif is conserved among PTPs, and that the substitution of a phenylalanine residue for tyrosine within this region renders certain PTPs, such as LAR [39] and PTP1B (J. Chernoff, unpublished work) temperature sensitive. Therefore it might be expected that phosphorylation of this tyrosine would affect PTP activity and/or conformation. Indeed, we have shown

that incubation of wild-type PTP1B, but not a point mutant in which the primary target site for tyrosine phosphorylation has been changed to phenylalanine ($PTP1B^{Y66F}$), increases its catalytic activity substantially. This enhanced PTP activity might serve as a feedback mechanism to deactivate RPTKs or other tyrosine-phosphorylated proteins. Another possibility is that the enhanced activity causes rapid auto-dephosphorylation, thereby ensuring that any PTP1B/SH2-protein-containing complexes are short-lived.

Lammers et al. [12] reported that PTP1B preferentially dephosphorylates precursor forms of certain RPTKs such as β-PDGFR and insulin receptor when co-expressed with these kinases in human 293 cells [12]. Because PTP1B is an ER-associated protein, it is conceivable that its interaction with RPTKs occurs while these proteins are being exported through the ER to the plasma membrane. Our results are consistent with this hypothesis, although we cannot definitely rule out RPTK/PTP1B interactions in other cellular compartments. As suggested by Frangioni et al. [25], PTP1B might serve to dephosphorylate such RPTKs to prevent the premature activation of RPTKs *in vivo*. It is also possible that, like SHP2, PTP1B serves a positive signalling function, but in a different cellular location. In budding yeast, an ER-to-nucleus signal transduction system has been described that is activated by certain cellular stressors [40]. It is conceivable that ER signalling pathways use molecular elements similar to those found in the transduction of extracellular growth factor signals at the plasma membrane, and that RPTKs and PTP1B are part of this signalling apparatus.

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