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Multi-site phosphorylation of the protein tyrosine phosphatase, PTP1B: identification of cell cycle regulated and phorbol ester stimulated sites of phosphorylation

Andrew J.Flint, Martijn F.G.B.Gebbink[3], B.Robert Franza, Jr[1], David E.Hill[2] and Nicholas K.Tonks

Cold Spring Harbor Laboratory, Cold Spring Harbor, [1]Freeman Laboratory of Cancer Cell Biology, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724 and [2]Oncogene Science, Inc., Cambridge, MA 02142, USA

[3]Present address: Division of Cellular Biochemistry, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

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The non-transmembrane protein tyrosine phosphatase, PTP1B, comprises 435 amino acids, of which the C-terminal 114 residues have been implicated in controlling both localization and function of this enzyme. Inspection of the sequence of the C-terminal segment reveals a number of potential sites of phosphorylation. We show that PTP1B is phosphorylated on seryl residues *in vivo*. Increased phosphorylation of PTP1B is seen to accompany the transition from G_2 to M phase of the cell cycle. Two major tryptic phosphopeptides appear in two-dimensional maps of PTP1B from mitotic cells. One of these comigrates with the peptide generated following phosphorylation of PTP1B *in vitro* at Ser386 by the mitotic protein Ser/Thr kinase $p34^{cdc2}$:cyclin B. The site of phosphorylation that is responsible for the pronounced retardation in the electrophoretic mobility of PTP1B from mitotic cells has been identified by site directed mutagenesis as Ser352. The identity of the kinase responsible for this modification is presently unknown. We also show that stimulation of HeLa cells with the phorbol ester TPA enhances phosphorylation of PTP1B. Two dimensional phosphopeptide mapping reveals that the bulk of the phosphate is in a single tryptic peptide. The site, identified as Ser378, is also the site of phosphorylation by protein kinase C (PKC) *in vitro*. Thus the TPA-stimulated phosphorylation of PTP1B *in vivo* appears to result directly from phosphorylation by PKC. The effect of phosphorylation on the activity of PTP1B has been examined in immunoprecipitates from TPA-treated and nocodazole-arrested cells. TPA treatment does not appear to affect activity directly, whereas the activity of PTP1B from nocodazole-arrested cells is only 70% of that from asynchronous populations.

Key words: cell cycle/kinase/phosphatase/phosphorylation/tyrosine

Introduction

Protein tyrosine phosphorylation is an essential element in the control of fundamental cellular signalling events involved in growth, proliferation and differentiation. This is a dynamic process: the phosphorylation state of a protein *in vivo* reflects the competing action of kinases, responsible for the phosphorylation reaction, and phosphatases that catalyze protein dephosphorylation. The characterization of protein tyrosine phosphatases (PTPases) is therefore necessary to understand the physiological significance of reversible tyrosine phosphorylation. It now appears that the PTPases represent a burgeoning, structurally diverse family of integral membrane receptor-like and non-transmembrane proteins which are ubiquitous in eukaryotes and will play an essential role in cellular signalling responses (for review see Fischer *et al.*, 1991; Charbonneau and Tonks, 1992).

The emergent underlying theme is that the non-catalytic segments of the PTPases appear to control function either by targeting to specific subcellular compartments or by modulating enzymatic activity directly. The non-receptor PTPases comprise a catalytic domain fused to distinct structural motifs of varying lengths at either the N- or C-terminus of the protein. These include segments of homology to the band 4.1-related family of cytoskeletal-associated proteins (Gu *et al.*, 1991; Yang and Tonks, 1991), to retinaldehyde binding protein (Gu *et al.*, 1992) to SH2 domains (Shen *et al.*, 1991; Matthews *et al.*, 1992; Plutzky *et al.*, 1992; Yi *et al.*, 1992) and to PEST sequences (Matthews *et al.*, 1992; Yang *et al.*, 1993). Additionally PTPases have been identified with unique non-catalytic domains, the functions of which remain to be ascertained (Guan and Dixon, 1990; Lombroso *et al.*, 1991; Ottilie *et al.*, 1991; Ota and Varshavsky, 1992).

The best characterized non-transmembrane PTPases remain the ubiquitous enzymes PTP1B and TCPTP. PTP1B was originally isolated from human placenta as a monomeric catalytic subunit of 37 kDa (Tonks *et al.*, 1988a,b). Isolation of cDNA for PTP1B indicated that the placenta protein was derived from a full length enzyme of 435 amino acids (Brown-Shimer *et al.*, 1990; Chernoff *et al.*, 1990; Guan *et al.*, 1990). TCPTP was originally isolated from a human peripheral T cell cDNA library and is a close homologue of PTP1B which displays 74% identity in the catalytic domain (Cool *et al.*, 1989). The cDNA for TCPTP predicts a protein of 48 kDa, also with a C-terminal extension relative to the 37 kDa form of PTP1B purified from placenta. We had previously noted (Brown-Shimer *et al.*, 1990; Cool *et al.*, 1990) that although comparison of the sequences indicated a greater divergence of the C-terminal non-catalytic segments than of the catalytic domains, nevertheless significant structural similarity was retained suggesting that these segments may have related functions. When the 48 kDa form of TCPTP is expressed in BHK cells it is recovered from the particulate fraction of cell lysates, requiring detergent treatment for extraction, whereas a 37 kDa truncated form of TCPTP in which the C-terminal segment has been deleted is no longer predominantly particulate (Cool

et al., 1990). A stretch of hydrophobic residues at the extreme C-terminus of TCPTP is presumably responsible for this localization (Cool *et al.*, 1990). Recently the equivalent segment of ~35 residues at the extreme C-terminus of PTP1B was demonstrated to be both necessary and sufficient for targeting the enzyme to the cytosolic side of the endoplasmic reticulum (Frangioni *et al.*, 1992).

While the non-catalytic C-terminal domains of PTP1B and TCPTP have been shown to be important for subcellular localization and, in the case of TCPTP, regulation of activity (Zander *et al.*, 1991), they may also be targets for interaction with other proteins. Following the development of monoclonal antibodies to PTP1B we have addressed such a role at a molecular level. Inspection of the sequence of these domains reveals that the extreme C-terminal hydrophobic segment is preceded by a stretch of 80–100 residues that is predominantly hydrophilic in nature and bears several putative sites of phosphorylation by Ser/Thr kinases. Thus the state of phosphorylation of PTP1B *in vivo* was ascertained. In this paper we demonstrate that PTP1B is a substrate for PKC *in vitro* and *in vivo*. Furthermore its phosphorylation state is altered in a cell cycle dependent manner. In mitosis PTP1B is phosphorylated by $p34^{cdc2}$ and a yet to be identified Ser-Pro-directed protein serine kinase. These observations highlight a novel physiological interaction between Ser/Thr and Tyr phosphorylation.

Results

Immunoblot analysis of PTP1B from HeLa cell lysates

Using human PTP1B expressed in *Escherichia coli* as antigen, a series of monoclonal antibodies to this PTPase were generated. Of these, FG6 and DH8 (IgG2a and IgG2b subtypes respectively) were used in the experiments described below. A detailed characterization of these antibodies will be reported elsewhere (D.E.Hill *et al.*, in preparation). Both antibodies recognize a single protein of ~53 kDa in immunoblots of HeLa cell lysates (Figure 1). Both antibodies also recognized PTP1B as a protein of ~53 kDa following stable expression of cDNA in 3T3 cells, transient expression in Cos, 293 or Rat 1 fibroblasts, purification from Sf9 cells following expression using recombinant baculovirus or expression in *E.coli* (K.A.Johnson, D.E.Hill, A.J.Flint and N.K.Tonks, unpublished). In all cases the PTP1B band migrates slightly faster than the FG6 antibody heavy chain on SDS–PAGE. The difference between the apparent M_r observed here and that of ~50 kDa reported by Frangioni *et al.* (1992) most likely reflects the choice of molecular weight markers and gel conditions. Interestingly, only the full length form of the enzyme was detected in HeLa cell lysates or in transfected cell lines, in agreement with Frangioni *et al.* (1992), Woodford-Thomas *et al.* (1992) and Brown-Shimer *et al.* (1992). The truncated 37 kDa species that was isolated originally from human placenta (Tonks *et al.*, 1988a,b) was not observed.

Cell cycle dependent changes in electrophoretic mobility of PTP1B

A striking observation is that in lysates of mitotic HeLa cells, arrested in M phase by treatment with nocodazole, the band corresponding to PTP1B displayed a retarded mobility on SDS–PAGE relative to the protein extracted from asynchronous cell populations (Figure 1). As there is precedent for protein phosphorylation causing such retardation in electrophoretic mobility, samples of HeLa cell lysate were treated with calf intestine alkaline phosphatase. As can be seen in Figure 1, a 30 min incubation with buffer alone did not alter the mobility of PTP1B, whereas treatment with alkaline phosphatase increased the mobility of the mitotic form of the phosphatase to that of the protein in asynchronous cell populations. The signal intensity of the bands in the immunoblot was not altered following alkaline phosphatase treatment suggesting that this effect did not result from proteolysis. Thus phosphatase treatment abolished the shift in electrophoretic mobility of PTP1B detected in lysates of mitotic cells.

One possible consequence of the modification of PTP1B in mitotic cells would be translocation from a membrane

Fig. 1. Immunoblot analysis of PTP1B from HeLa cells. Effect of phosphatase treatment on mobility of PTP1B on SDS–PAGE. Each lane contains 30 μg of protein from the Triton-solubilized membrane fraction of non-mitotic (–) or mitotic (+ nocodazole) HeLa cells. Samples were incubated in a buffer comprising 50 mM Tris–Cl pH 9.0, 5 mM $MgCl_2$ in the presence or absence of 20 units of calf intestine alkaline phosphatase (New England Biolabs) for 30 min at 30°C. Reactions were terminated by addition of Laemmli sample buffer and PTP1B was visualized by immunoblotting. Gels were run on a Hoefer SE600 Slab Gel apparatus.

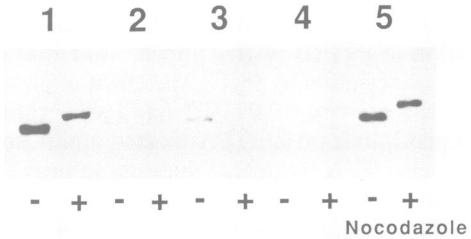

Fig. 2. The mitotic form of PTP1B remains associated with membranes. Lysates of non-mitotic and mitotic HeLa cells were fractionated into cytosolic, nuclear and membrane components as described in Materials and methods. Each lane was loaded with an equal volume of each fraction, an amount equivalent to 10 μg of lysate protein. The fractions obtained were (1) postnuclear supernatant, (2) a second postnuclear supernatant, (3) Triton X-100 extracted nuclear pellet, (4) cytosol, (5) Triton X-100 extracted membranes. PTP1B was visualized by immunoblotting using monoclonal antibody FG6 and the enhanced chemiluminescence detection system (Amersham).

fraction into the cytosol as observed, for example, with the ras-like GTP binding protein rab4 (Van der Sluijs *et al.*, 1992). However, in both asynchronous and mitotic cells, PTP1B was recovered in a crude membrane fraction (high speed pellet) and was not detected in cytosol (high speed supernatant) or nuclear (low speed) pellets (Figure 2). While complete extraction of PTP1B from membranes required solubilization with detergent, a significant quantity (up to 50%) could be extracted with 100 mM Na_2CO_3, pH 11.5. However, the proportion susceptible to alkaline extraction did not vary with the cell cycle (data not shown). Thus the change in electrophoretic mobility in M phase did not result in a global translocation out of the membrane fraction or an increased susceptibility to high pH extraction.

In order to examine whether this apparent mitotic phosphorylation was reversible *in vivo*, cells were arrested with nocodazole, the mitotic cells harvested and replated into drug-free media. At various times lysates were prepared and subjected to immunoblot analysis (Figure 3). Within 4 h of release from nocodazole arrest the bulk of the PTP1B has reverted to the faster migrating form. This conversion does not appear to be a result of synthesis of new phosphatase as the change in mobility is seen in PTP1B from cells cultured in the presence or absence of cycloheximide. Therefore the data suggest that PTP1B is subject to reversible phosphorylation *in vivo*.

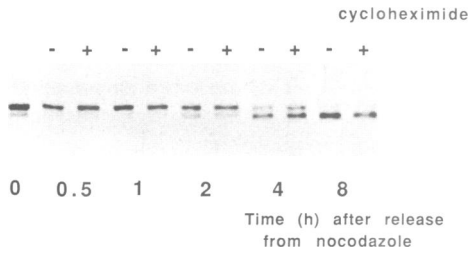

Fig. 3. Dephosphorylation of PTP1B after release of HeLa cells from nocodazole arrest. Mitotic HeLa cells, washed twice with PBS to remove nocodazole, were plated in fresh DMEM, 5% FBS with or without 20 μg/ml cycloheximide. At the indicated times, cells were scraped, rinsed twice with PBS, and lysed in Triton-containing lysis buffer. PTP1B was detected in 10 μg of lysate by immunoblotting with FG6.

PTP1B is a phosphoprotein in vivo

While the changes in electrophoretic mobility of PTP1B that accompany mitosis and the reversal of these effects by phosphatase treatment are consistent with an alteration in phosphorylation state, formal proof was provided by labelling HeLa cells with [^{32}P]orthophosphate for 4 h prior to lysis. A labelled protein that comigrated with PTP1B was immunoprecipitated from asynchronous cells. In mitotic cells this protein displayed both enhanced phosphorylation and retarded electrophoretic mobility (Figure 4A). Phosphoamino acid analyses of these labelled bands indicated that PTP1B from both asynchronous and mitotic cells appeared to be phosphorylated exclusively on seryl residues.

To examine this modification in greater detail, immunoprecipitates of PTP1B from ^{32}P-labelled HeLa cells were analysed by 2D gel electrophoresis, resolving proteins on the basis of charge by isoelectric focusing in the first dimension and size by an SDS−polyacrylamide gel second dimension (Figure 4B). At least three distinct charged forms of PTP1B are resolved in asynchronous cells. In immunoprecipitates from nocodazole-arrested cells the distinct retardation in mobility was again observed, coincident with the appearance of two additional species of a more acidic nature, most likely resulting from an altered pattern of phosphorylation within the molecule. Similar patterns of ^{32}P-labelled PTP1B were observed in immunoprecipitates with either mAb FG6 or DH8. These data are clearly consistent with phosphorylation of PTP1B on multiple sites *in vivo*. The shift in electrophoretic mobility was not an artefact of arrest with nocodazole. Similar shifts were observed coincident with transition of a population of cells into G_2/M following release from a hydroxyurea block (data not shown). No change in the pattern of phosphorylated species resolved by 2D gel electrophoresis was detected whether or not the Ser/Thr phosphatase inhibitor okadaic

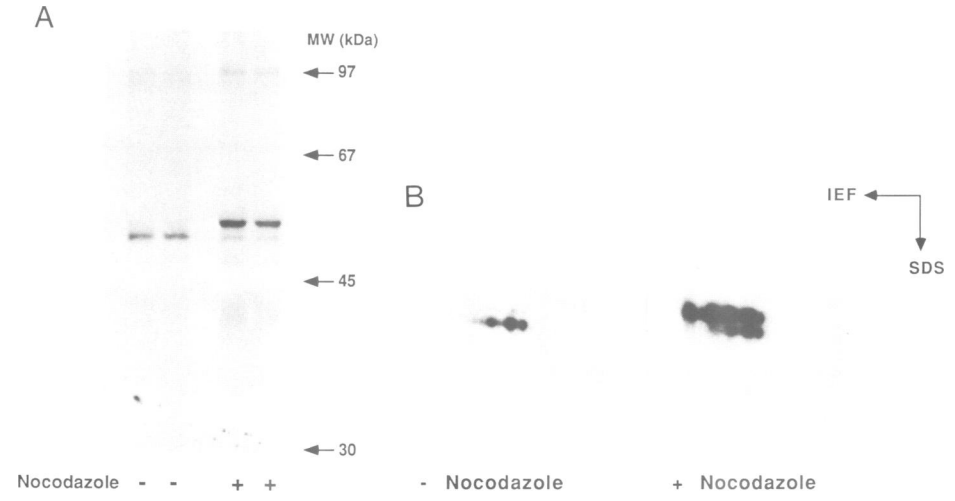

Fig. 4 (A). Immunoprecipitation of PTP1B from ^{32}P-labelled HeLa cells. Each lane depicts an autoradiogram of an immunoprecipitate (FG6) from ~1.3 mg of lysate protein from ^{32}P-labelled HeLa cells. Duplicate samples from cells treated in the presence and absence of 0.1 μg/ml nocodazole are illustrated. **(B)** 2D gel analysis of immunoprecipitates of PTP1B from ^{32}P-labelled HeLa cells. The section of the autoradiogram containing PTP1B is depicted. Each immunoprecipitation was from ~1.9 mg of lysate protein.

acid or the PTPase inhibitor vanadate were included in the lysis buffer.

Immunoprecipitates of PTP1B contained PTPase activity as assessed by dephosphorylation of a synthetic peptide substrate modelled on a site in PTP1B that is phosphorylated by v-*abl in vitro* (Figure 5). Comparison of the activity in immunoprecipitates from asynchronous and nocodazole arrested cells revealed a 30% reduction in activity in the mitotic cells, while the level of protein assessed by immunoblotting (Figure 1) appeared unchanged. Similar data were obtained using RCM-lysozyme as substrate.

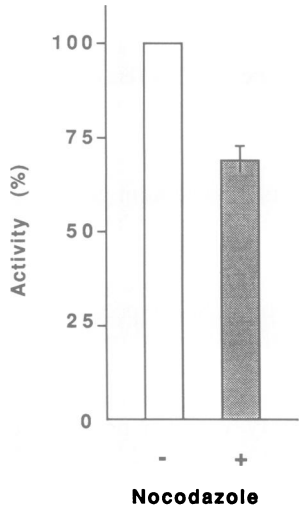

Fig. 5. Effect of nocodazole-arrest on activity of PTP1B. Activity of PTP1B was measured in immunoprecipitates (FG6) using a tyrosine phosphorylated synthetic peptide (EDNDYINASL) as substrate. PTP1B activity from asynchronous cells is represented by the open bar and is defined as 100%. The activity from an equal amount of lysate protein from mitotic cells (+ nocodazole) is shown in the shaded bar and is presented as the mean ± the standard error of the mean for nine determinations, expressed as the percentage of the activity in the companion control assay.

Mutation of Ser352 in PTP1B to Ala abolishes the shift in electrophoretic mobility in mitotic cells

Both localization and functional analyses strongly implicate the C-terminal segment of PTP1B in the regulation of enzyme activity. This segment contains several Ser residues that could be substrates of Ser/Thr kinases, based on similarity to putative consensus recognition sequences (see Kennelly and Krebs, 1991 for review). Therefore in order to glean additional information as to the identity of the kinase that phosphorylates PTP1B in mitotic cells, we utilized site directed mutagenesis and PCR to synthesize templates in which each seryl residue in the segment from Ser295 to Ser393 was individually converted to alanine. We focused on serine, not threonine, on the basis of phosphoamino acid analysis data (Frangioni *et al.*, 1992; N.K.Tonks, unpublished). The nocodazole-induced shift in electrophoretic mobility was then examined following transient expression of wild type PTP1B and each of the point mutants in 293 (human embryonal kidney) cells. These cells were chosen because in preliminary studies with HeLa cells the transfection efficiencies were very low.

Upon expression of the various point mutants in 293 cells it was observed that only the conversion of a single serine to alanine, namely Ser352, was sufficient to abolish the mitotic mobility shift. There was no apparent effect of mutation of serines 295, 363 and 365, 372, 378, 386 or 393 (Figure 6). Serine 352 is surrounded by the sequence EEKGS(352)PLNAA. The most obvious feature of this sequence is the presence of the Pro residue adjacent to the Ser, a feature found in phosphorylation sites recognized by $p34^{cdc2}$ (Moreno and Nurse, 1990).

PTP1B is phosphorylated by $p34^{cdc2}$ and protein kinase C in vitro

In order to assess which enzymes may be involved in the phosphorylation of PTP1B *in vivo* we examined the capacity of several protein Ser/Thr kinases to phosphorylate the

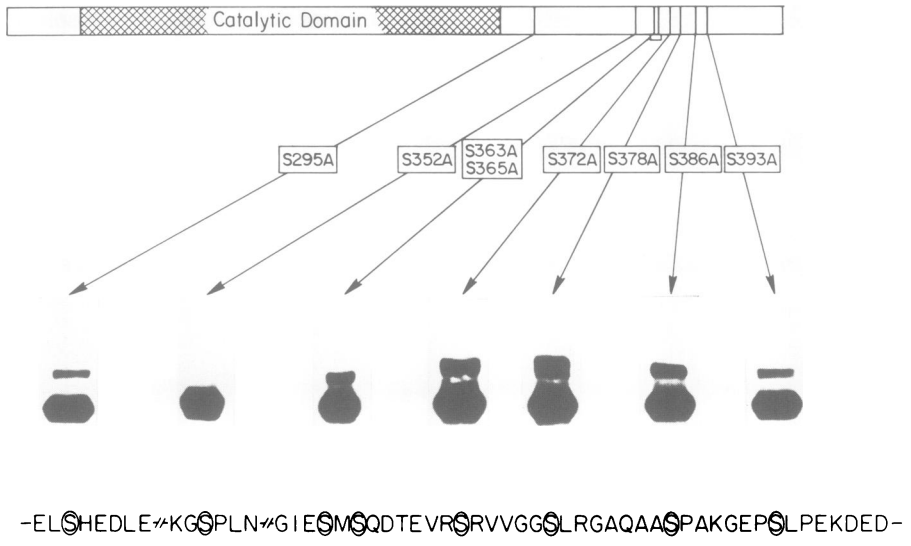

Fig. 6. Transient expression of PTP1B in 293 cells: identification of a site of mitotic phosphorylation by site directed mutagenesis. Each of the mutant forms of PTP1B was expressed in 293 cells which were then arrested with nocodazole. The electrophoretic mobility of the PTP1B mutants in cell lysates was visualized by immunoblot analysis with monoclonal antibody FG6. The schematic representation of PTP1B is drawn in proportion to the number of residues in the molecule to indicate the position of the various mutated Ser residues. The sequence surrounding each Ser residue that has been mutated is presented beneath each immunoblot. Gels were run in a Hoefer SE600 apparatus to maximize the resolution of faster and slower migrating forms of PTP1B.

phosphatase *in vitro*. To this end PTP1B was expressed in Sf9 cells using a recombinant baculovirus and purified to apparent homogeneity, in a catalytically active form, by ion exchange chromatography (A.J.Flint and N.K.Tonks, in preparation). Figure 7 indicates that purified PTP1B was readily phosphorylated *in vitro* by $p34^{cdc2}$ and protein kinase C (PKC).

To identify the sites of phosphorylation, the individual Ser→Ala point mutants of PTP1B were immunoprecipitated and phosphorylated by either $p34^{cdc2}$ or PKC (Figure 8).

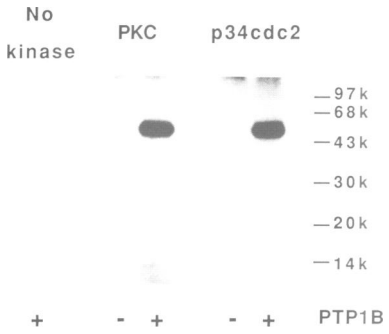

Fig. 7. Phosphorylation of PTP1B by Ser/Thr kinases *in vitro*. PTP1B purified from Sf9 cells infected with recombinant baculovirus was phosphorylated by the indicated kinases *in vitro*. The figure depicts an autoradiogram of an SDS gel. In each case the pattern of phosphorylation in the absence (−) and presence (+) of PTP1B is presented. The molecular weight standards were from Bio-Rad.

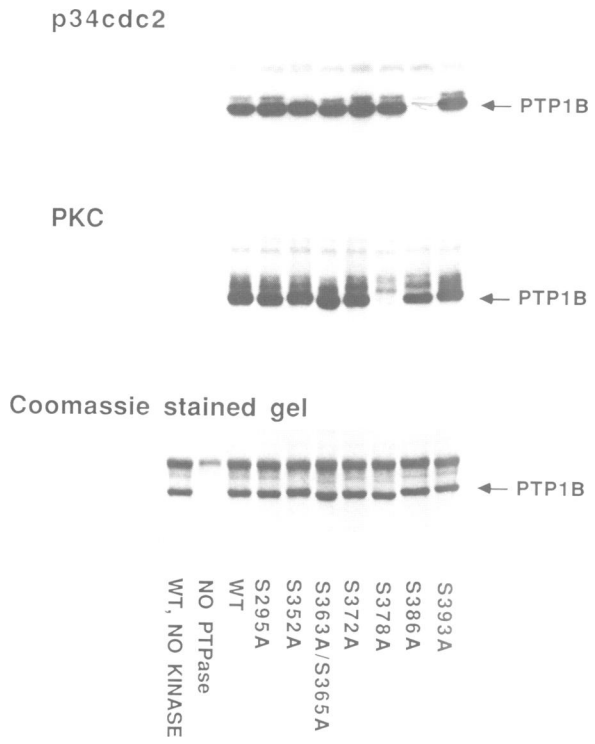

Fig. 8. Phosphorylation of mutant PTP1B proteins *in vitro*. 293 cells were transfected with plasmids encoding each mutant of PTP1B and immunoprecipitates were prepared using 4 μg of FG6. Each immunoprecipitate was divided in half, phosphorylated with either PKC or $p34^{cdc2}$ and subjected to SDS−PAGE. The gel was stained with Coomassie blue and exposed to X-ray film. The panel showing the Coomassie blue-stained gel indicates that approximately equal amounts of each mutants were immunoprecipitated. Lanes containing wild type (WT) or each mutant PTP1B are marked.

For both kinases mutation of a single Ser to Ala severely diminished the phosphorylation. The S386A mutant was no longer susceptible to phosphorylation by $p34^{cdc2}$, whereas PKC did not phosphorylate the S378A mutant. Interestingly mutation of Ser352→Ala, the site of phosphorylation responsible for the shift in electrophoretic mobility of PTP1B in mitotic cells, did not affect phosphorylation by either kinase.

PTP1B is phosphorylated on Ser386 by $p34^{cdc2}$ in vivo

PTP1B, isolated from ^{32}P-labelled mitotic HeLa cells by immunoprecipitation, was subjected to 2D tryptic phosphopeptide mapping. Two major mitotic phosphopeptides were resolved (Figure 9). A similar pattern was detected in PTP1B from 293 cells. A synthetic peptide modelled on the sequence surrounding S386 in PTP1B was phosphorylated *in vitro* by $p34^{cdc2}$ and was subjected to the same tryptic mapping procedure. A single labelled product was observed that precisely comigrated with one of the phosphopeptides recovered from the mitotic form of PTP1B. Thus PTP1B appears to be phosphorylated on S386 by $p34^{cdc2}$ *in vivo*. In consideration of the facts that mutation of Ser352→Ala abolishes the mitotic electrophoretic mobility shift in PTP1B and that this shift is sensitive to alkaline phosphatase treatment, we propose that the second tryptic phosphopeptide contains this site.

PTP1B is phosphorylated on Ser378 by PKC in vivo

In view of the susceptibility of PTP1B to phosphorylation by PKC *in vitro*, we examined whether PTP1B was phosphorylated *in vivo* following stimulation of ^{32}P-labelled HeLa cells with the phorbol ester TPA, which activates PKC. This treatment enhanced the phosphorylation of PTP1B (Figure 10). Tryptic phosphopeptide mapping revealed that this additional phosphorylation occurs primarily on a single peptide (Figure 11) which is partially phosphorylated in the untreated cells. Elevated phosphorylation of a unique site is consistent with the observation that mutation of Ser378 alone to Ala abolished phosphorylation *in vitro* (Figure 8). This peptide precisely comigrated with the single phosphopeptide generated in tryptic maps of PTP1B phosphorylated by PKC *in vitro* (Figure 11). Thus the enhanced phosphorylation of PTP1B in response to TPA is mediated by PKC acting on the phosphatase directly, phosphorylating Ser378. No effect of this phosphorylation on the activity of PTP1B was detected with either RCM-lysozyme or the synthetic peptide substrate.

Discussion

Phosphorylation of the PTPases themselves may prove to be a mechanism of general importance in control of the activity of these enzymes. For example there have been reports of phosphorylation of tyrosyl residues in CD45 in Jurkat T cells following stimulation of the cells with the mitogenic lectin phytohemagglutinin or anti-CD3 antibodies (Stover *et al.*, 1991). Phorbol ester stimulation of peripheral T cells induces a rapid phosphorylation of CD45 (Autero and Gahmberg, 1987) whereas ionomycin treatment, to elevate the level of intracellular Ca^{2+}, leads to a decrease in phosphorylation of Ser residues in CD45 in a variety of T cell lines (Ostergaard and Trowbridge, 1991). Brautigan and Pinault (1991) have also reported the stimulation of an unidentified membrane-bound PTPase of ~55 kDa in CV-1

kidney cells following activation of cAMP dependent protein kinase or PKC, or inhibition of protein Ser/Thr phosphatases by treatment of the cells with okadaic acid. While these data are consistent with a role of Ser/Thr phosphorylation in stimulating phosphatase activity, direct phosphorylation of the PTPase was not demonstrated. Frangioni *et al.* (1992) have reported the presence of phosphoserine in PTP1B *in vivo*. However, our studies not only establish the phosphorylation of PTP1B *in vivo* but also identify the sites at which its phosphorylation state is modulated both in a cell cycle dependent manner and in response to phorbol ester treatment.

We show that the phosphorylation of PTP1B *in vivo* occurs at multiple sites. In attempting to identify the kinases involved in phosphorylating PTP1B we focused on the C-terminal segment because of its apparent regulatory importance. Upon inspection of the amino acid sequence of that segment, several putative sites of phosphorylation sites were noted. For example, serines 286, 295, 365 and 393 all possess at least the minimum acidic residue at the +3 (C-terminal) position required for sites of phosphorylation by casein kinase 2 (Kuenzel *et al.*, 1987). Serine 386 in the sequence -SPAK- displays features of a $p34^{cdc2}$ phosphorylation site; furthermore Ser352 also has an adjacent C-terminal Pro residue, the minimum requirement for phosphorylation by $p34^{cdc2}$ (Moreno and Nurse, 1990).

PTP1B, purified to homogeneity as an active PTPase following expression in Sf9 cells from recombinant baculovirus, was used as substrate to demonstrate phosphorylation by $p34^{cdc2}$ and PKC *in vitro* (Figure 7). Phosphorylation of PTP1B *in vivo* was also demonstrated in response to TPA (Figure 10). The combination of tryptic phosphopeptide mapping of PTP1B following phosphorylation *in vitro* and in response to TPA *in vivo* (Figure 11) and site directed mutagenesis (Figure 8) point to Ser378 as the single major site of phosphorylation by PKC. However, TPA treatment did not induce the shift in electrophoretic mobility of PTP1B that is seen in extracts of mitotic cells (Figure 1). Thus it appears that the phosphorylation state of PTP1B is responsive to signalling pathways that involve PKC in addition to kinases that alter PTP1B during transition from G_2 into M phase of the cell division cycle.

Without knowing the kinase responsible for the electrophoretic mobility shift, we used a genetic approach to dissect which amino acid residues were responsible. By systematically introducing single point mutations into PTP1B to convert Ser residues to Ala, then expressing the mutants in 293 kidney cells, we demonstrated that mutation of a single Ser,

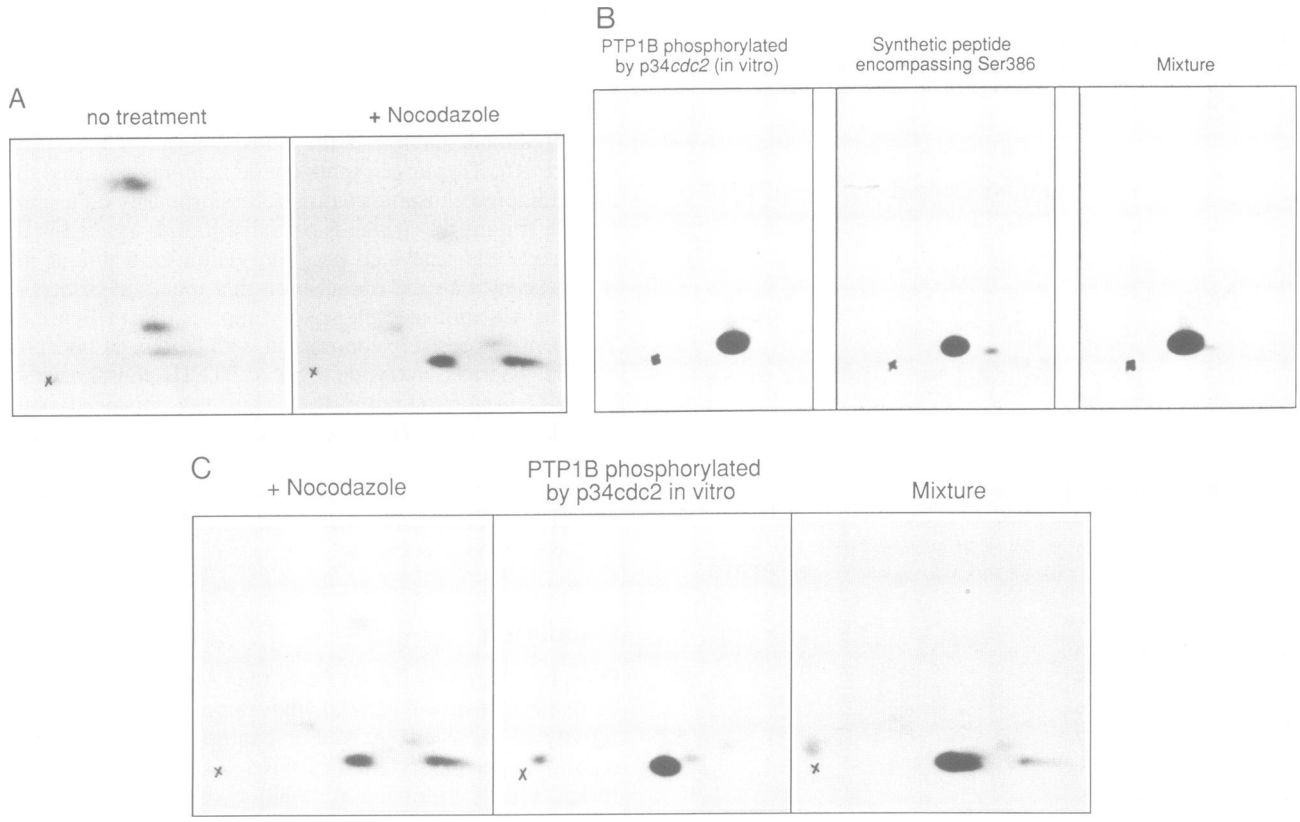

Fig. 9. Two dimensional tryptic phosphopeptide maps of PTP1B. (**A**) Tryptic phosphopeptides of PTP1B immunoprecipitated from asynchronous (no treatment) or mitotic (+ nocodazole) ^{32}P-labelled HeLa cells. (**B**) Phosphorylation of PTP1B *in vitro* at Ser386 by $p34^{cdc2}$. Tryptic peptides were prepared from PTP1B that had been phosphorylated by $p34^{cdc2}$ and subjected to SDS−PAGE as described in Figure 7. Peptide CSH278 (RRRGAQAASPAKGE) encompassing Ser386 was phosphorylated, purified on P81 paper, eluted and digested with trypsin. An equal number of c.p.m. from each sample were mixed together before separation (panel labelled mixture). (**C**) Identification of Ser386 as a mitotic phosphorylation site. Tryptic phosphopeptides of PTP1B from mitotic cells (+ nocodazole) were obtained as in (**A**). The tryptic peptides from PTP1B phosphorylated *in vitro* by $p34^{cdc2}$ were generated as in (**B**). A mixture of the two samples was separated together (mixture). Peptides were separated horizontally by electrophoresis at pH 1.9 with the anode on the left and vertically by ascending chromatography. An 'x' marks the origin where the samples were spotted. TLC plates were exposed to film for 3−10 days at −70°C.

Ser352, was sufficient to block the shift in electrophoretic mobility in response to nocodazole (Figure 6). Band migration upon SDS−PAGE was unaffected by mutation of the putative sites of phosphorylation by casein kinase 2, or Ser386 in the sequence -SPAK- which bears the hallmark of a $p34^{cdc2}$ phosphorylation site. It was hoped that the features of the site identified would provide clues to the identity of the kinase involved. The principle feature that characterizes the sequence surrounding Ser352 is the presence of an adjacent, C-terminal Pro residue (Figure 6). Thus this site has at least the minimum requirements for phosphorylation by $p34^{cdc2}$. Nevertheless the major site of phosphorylation by $p34^{cdc2}$, both *in vitro* and *in vivo*, is Ser386 (Figures 8 and 9) and it would appear that a distinct, as yet unidentified kinase recognizes Ser352. In summary (Figure 12) PKC contributes primarily to the phosphorylation of PTP1B in interphase at Ser378. Mitosis is accompanied by an increase and redistribution of phosphate in PTP1B. The phosphate content in Ser378 is decreased, while there is enhanced phosphorylation of Ser352 and Ser386. Whether this decreased phosphorylation at Ser378 results from increased phosphatase activity in mitosis, decreased PKC activity or accessibility of PTP1B to either modifying enzyme is not yet known. These observations are important because they illustrate both a novel interplay between Ser/Thr and Tyr phosphorylation as well as a new potential tier of control of the level of cellular phosphotyrosine.

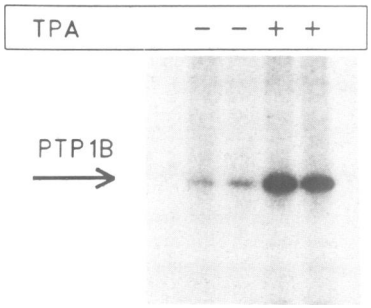

Fig. 10. Immunoprecipitation of PTP1B from ^{32}P-labelled HeLa cells treated with phorbol esters. Cells were stimulated with either 200 nM TPA (Sigma) or with DMSO (solvent control) for 10 min prior to lysis. After lysis, preclearing and immunoprecipitation (FG6) the immune complex was analyzed by gel electrophoresis and autoradiography.

What are the effects of these modifications on the activity of PTP1B? As anticipated, immunoprecipitates of PTP1B contained tyrosine phosphatase activity. Comparison of activity in immunoprecipitates from asynchronous and mitotic cells revealed a 30% decrease in activity in the latter (Figure 5). This modest effect could reflect an inappropriate choice of substrate. At the present time there are no data describing physiologically relevant substrates for PTP1B. As such proteins are identified it will be pertinent to reassess this issue as more profound effects may then be detected.

Attempts to reconstitute the effects on activity *in vitro* have been unsuccessful due to several technical difficulties. It was not possible to discern the effects on activity of dephosphorylation of the phosphorylated form or PTP1B because phosphorylation was not reversed by treatment with the catalytic subunits of the Ser/Thr phosphatases PP1 and PP2A (which are readily inhibitable by okadaic acid) and use of either alkaline or acid phosphatases left too much contaminating activity to assess an effect on PTP1B accurately. At the present time it is not possible to determine which of the sites of phosphorylation that are altered in mitotic cells are responsible for the effects on activity. The phosphatase responsible for dephosphorylation of Ser378 and the kinase that phosphorylates Ser352 are unknown and thus their effects *in vitro* cannot be tested at present. In addition we were unable to achieve stoichiometries of phosphorylation of PTP1B *in vitro*, by either PKC (Ser378) or $p34^{cdc2}$ (Ser386), of >20%. Thus it is not possible to test directly whether such phosphorylation exerted a significant effect on the activity of PTP1B.

Two recent studies have demonstrated that PTP1B is associated with the endoplasmic reticulum (Frangioni *et al.*, 1992; Woodford-Thomas *et al.*, 1992) but the function of the enzyme remains unknown. Such spatial restriction within the cell would be expected to limit the spectrum of substrates to which the enzyme has access. The identification of Ltk as an endoplasmic reticulum-localized PTK (Bauskin *et al.*, 1991) provides potential targets, both the kinase itself and its substrates, for the action of PTP1B. In addition, PTP1B could serve in controlling the activity of other receptor PTKs in transit to the plasma membrane via the endoplasmic

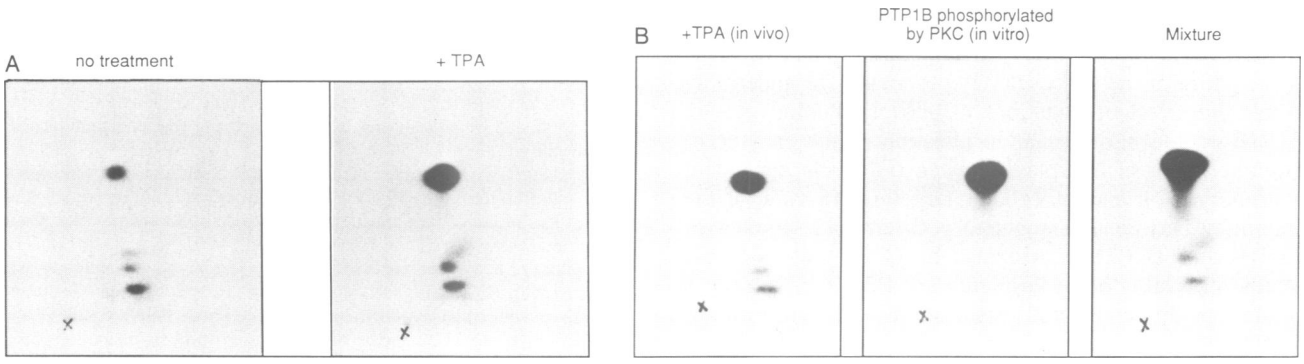

Fig. 11. Tryptic phosphopeptide maps of PTP1B phosphorylated by PKC *in vitro* and in response to TPA *in vivo*. (**A**) Tryptic peptides of PTP1B immunoprecipitated from ^{32}P-labelled HeLa cells with (+ TPA) or without (no treatment) phorbol ester stimulation (as in Figure 10). Separation of the peptides was performed as in Figure 9. (**B**) Comigration of phosphopeptide from TPA stimulation *in vivo* and PKC phosphorylation *in vitro*. Tryptic peptides of PTP1B phosphorylated in response to TPA *in vivo* were obtained as in (A). Tryptic peptides were derived from PTP1B phosphorylated *in vitro* by PKC (as described in Figure 7). Separation of a mixture of the two samples yielded the panel labelled 'mixture'.

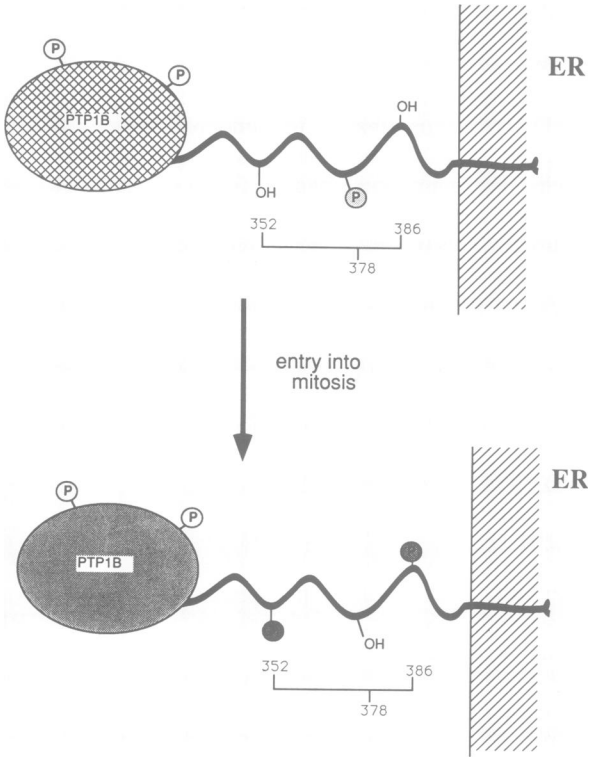

Fig. 12. Schematic depiction of the phosphorylation state of PTP1B. PTP1B is drawn with a globular catalytic domain connected by a hydrophilic spacer of ~80 amino acids to its hydrophobic ER anchoring sequence. The three major sites of phosphorylation are Ser352, Ser378 and Ser386. Two unidentified minor phosphopeptide species observed in the 2D maps are represented by the phosphates on the globular domain. The majority of the phosphorylation of PTP1B in unsynchronized HeLa cells occurs on Ser378. The phosphorylation state of this site is substantially increased in response to TPA treatment of the cells. However, in cells arrested in mitosis with nocodazole phosphate is apparently lost from this site while two new major phosphopeptides are detected. These sites of phosphorylation correspond to Ser386 which is the primary site of $p34^{cdc2}$ phosphorylation *in vitro*, and to Ser352 which is required for the altered electrophoretic mobility of PTP1B isolated from mitotic cells. The kinase responsible for the phosphorylation of Ser352 has not been identified but appears not to be the $p34^{cdc2}$:cyclin B complex.

reticulum. Alternatively PTP1B could be involved in controlling the structural changes in microtubules and the endoplasmic reticulum (Tarasaki *et al.*, 1986) that are associated with the cell cycle. Interestingly Woodford-Thomas *et al.* (1992) report a more diffuse pattern of immunofluorescent staining with antibodies to PTP1B at the G_2/M phase of the cell cycle. Experiments are under way to test the effect of mutations of phosphorylation sites in PTP1B on the intracellular localization of the enzyme to examine potential interplay between cell cycle dependent phosphorylation and subcellular targeting. In view of the homology between the regulatory C-terminal segment of PTP1B and TCPTP it will also be of interest to assess whether this other non-transmembrane PTPase is subject to similar modification and association with intracellular membranes.

Materials and methods

Culture and labelling of HeLa cells

HeLa cells were cultured on plates at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum. For harvesting, medium was aspirated and after one wash the cells were scraped into ice cold PBS (2 ml per 10 cm plate). The cell pellet was collected by centrifugation at 1000 *g* for 3 min, washed once with ice cold PBS then lysed in a buffer mixture comprising 50 mM HEPES pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM benzamidine, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 10% (v/v) glycerol, 1% (v/v) Triton X-100. For isolation of M phase-arrested cells, nocodazole (Janssen) was added at either 0.1 or 1 μg/ml for 18–22 h, which resulted in at least 90% of the cell population displaying the rounded phenotype of mitotic cells. The mitotic cells were harvested from the surface of the dish by squirting with a stream of PBS. Non-rounded cells were left attached to the dish. In harvesting non-mitotic cells from asynchronously growing populations, rounded cells were first washed off the surface of the dish.

In experiments in which the distribution of PTP1B between soluble and particulate fractions was examined (Figure 2) cells were lysed by homogenization in a glass Dounce (20 strokes) after 20 min of swelling in a hypotonic buffer (20 mM Tris–Cl pH 7.6, 5 mM KCl, 1.5 mM $MgCl_2$, 2 mM DTT, 5 μg/ml aprotinin and leupeptin, 2 μM pepstatin A and 1 mM benzamidine). After addition of sucrose and EDTA to 0.25 M and 5 mM respectively, nuclei were pelleted by centrifugation at 1000 *g* for 5 min. The low speed pellet was subjected to the hypotonic swelling and Dounce homogenization procedure to rupture any remaining intact cells. The low speed pellet following this second homogenization was extracted with 1% Triton X-100. An aliquot of this post-nuclear supernatant was retained for subsequent analysis and the rest centrifuged at ~300 000 *g* for 10 min. The cytosol (supernatant) was removed and stored on ice while the membrane pellet was re-extracted in the same volume of lysis buffer containing 1% (v/v) Triton X-100. Insoluble material was precipitated by high speed centrifugation as above. Samples of the post-nuclear supernatant, cytosol and Triton-solubilized membrane fractions were treated at 95°C for 3 min in SDS sample buffer in preparation for SDS–PAGE and immunoblot analysis.

For labelling of proteins to identify targets of phosphorylation *in vivo*, HeLa cells were rinsed twice with phosphate-free DMEM then incubated in 4 ml phosphate-free DMEM + 5% FBS (+/−nocodazole) for 30–60 min prior to addition of 1–3 mCi of [^{32}P]inorganic phosphate (NEN or ICN) per 10 cm plate for 4–5 h prior to harvesting. To examine phorbol ester stimulated phosphorylation, TPA was added to 270 nM, 10 min prior to cell lysis.

Immunoprecipitation, immunoblotting and immune complex kinase and PTPase assays

Anti-PTP1B monoclonal antibodies FG6 and DH8 were raised against bacterially expressed PTP1B. For immunoprecipitation, Triton lysates of HeLa cells were first precleared by addition of IgGsorb (Enzyme Centre) reconstituted in lysis buffer containing 1% (v/v) Triton X-100 (100 μl per 10 cm plate), rocking at 4°C for up to 1 h then centrifugation at 15 000 *g* (4°C) for 5 min. Precleared lysate was added to a fresh tube containing 2–4 mg of antibody coupled to protein A–Sepharose. After rocking at 4°C for 2–8 h, immune complexes were collected by centrifugation for 15 s at 1000 *g*, the supernatant discarded and the beads then washed five times with Triton X-100-containing lysis buffer.

In 2D gel analysis the immune complexes were subjected to one further wash in PBS followed by addition of 2 μl of DNase/RNase (1 mg/ml) and incubation on ice for 2 min. The beads were then resuspended in 40 μl dSDS (0.3% SDS, 1% 2-mercaptoethanol, 50 mM Tris–Cl pH 8) and incubated at 37°C for 3 min. The beads were pelleted for 5 min in a microfuge and the supernatant aspirated, dried by Speedvac then resuspended in 40 μl of sample buffer containing 9.95 M urea, 4% NP40, 2% ampholytes (pH 6–8) and 0.1 M dithiothreitol. The 2D gels consisted of an isoelectric focusing first dimension (pH 3.5–10) and a 10% polyacrylamide gel second dimension, and were performed by the Cold Spring Harbor Laboratory 2D Gel Facility.

For 1D gel analysis the Triton buffer-washed immune complex was washed once with PBS then treated with Laemmli sample buffer. In immunoblotting procedures proteins were transferred to nitrocellulose membranes which were then blocked with 5% non-fat dry milk in TTBS and subsequently incubated with the appropriate antibody. Antigen-bound antibody was detected with HRP-conjugated sheep anti-mouse secondary antibody and visualized with enhanced chemiluminescence (Amersham).

Before assays of PTPase activity, Triton buffer-washed immune complexes were first washed two additional times with assay buffer (25 mM imidazole HCl pH 7.2, 1 mg/ml BSA, 0.1% 2-mercaptoethanol) then incubated with a ^{32}P-labelled phosphotyrosyl peptide or RCM-lysozyme in a total volume of 60 μl of assay buffer. The peptide substrate was modelled on a site in PTP1B that is phosphorylated *in vitro* by v-*abl* (EDNDYINASL, N.K.Tonks, H.Charbonneau, K.A.Walsh and E.H.Fischer, unpublished observations, D.E.Cool and E.H.Fischer, personal communication). The

reaction was terminated by addition of 290 μl of a 10% suspension of NoritA charcoal in 0.9 M HCl, 90 mM $Na_4P_2O_7$, 2 mM NaH_2PO_4. Samples were centrifuged at 15 000 *g* for 10 min and 250 μl of supernatant counted in scintillant to estimate release of [^{32}P]inorganic phosphate.

Phosphorylation of PTP1B in vitro

Protein kinase C and p34^{cdc2} were generously provided by Dr Angus Nairn (Rockefeller University, NY), and Dr Dan Marshak (Cold Spring Harbor Laboratory), respectively.

The general phosphorylation conditions utilized an assay buffer comprising 50 mM Tris−Cl pH 7.6, 10 mM $MgCl_2$ and 1 mM DTT, [γ^{32}P]ATP at 100 μM and ~2500 c.p.m./pmol, ~2.5 μg PTP1B per assay and the appropriate kinase at 1−5 mU (1 unit = 1 nmol ^{32}P incorporated per min into histone H1) per assay. The kinases were diluted appropriately into 50 mM Tris−Cl pH 7.6, 0.1 mg/ml BSA, 1 mM DTT following assay versus histone. For PKC 50 μg phosphatidyl serine, 100 nM TPA and 1.5 mM $CaCl_2$ were added to the assay. Phosphorylation of PTP1B was allowed to proceed for 20 min at 30°C. The reaction was terminated by addition of Laemmli sample buffer and phosphorylation visualized by SDS−PAGE and autoradiography. The phosphorylation of peptide CSH278 (RRRGAQAASPAKGE) by p34^{cdc2} was performed using identical conditions. The reaction was terminated by spotting onto P81 paper and washing in 0.4% (v/v) H_3PO_4.

Preparation of tryptic digests and analysis by 2-dimensional mapping

Phosphopeptide mapping was performed essentially as described by Boyle *et al.* (1991) except that the phosphorylated PTP1B was digested directly in the macerated gel slice instead of first being extracted and precipitated. Two consecutive digestions in 0.4 ml of 50 μg/ml trypsin, 0.1 M NH_4HCO_3 routinely yielded 85−95% recovery of the total c.p.m. as peptides. Peptides were separated electrophoretically at either pH 1.9 or 4.72 for 30 min at 1000 V using a Pharmacia/LKB Multiphor II electrophoresis unit cooled to 5°C followed by ascending chromatography in butanol:pyridine:acetic acid:H_2O (15:10:3:12).

Phosphorylated peptide CSH278 bound to P81 paper was washed with 0.1% v/v TFA to remove nonvolatile phosphoric acid, dried under vacuum, and eluted with two sequential extractions in 0.1 M NH_4HCO_3. The extracted, lyophilized phosphopeptide was subsequently digested with trypsin.

Construction of mutations in PTP1B

Site specific mutagenesis of serine residues that were potential sites of phosphorylation was accomplished through use of a two step polymerase chain reaction (PCR) procedure. In the first step, two complementary oligonucleotides that overlap the targeted amino acid and encode the desired mutation were each used independently in a PCR with oligonucleotides corresponding to the 5′ end of the coding sequence and a sequence of 75 bp downstream of the stop codon. Additional 5′ nucleotides specifying restriction endonuclease cleavage sites were included in these flanking primers. For each mutant, these two PCRs produced DNA fragments that were complementary at the end containing the primers that specify the mutation. These two fragments were isolated from a low melting point agarose gel and used as template in a PCR containing only the 5′ and 3′ flanking primers. The resulting 1.3 kb sequence encoding human PTP1B was digested with *SalI* and *XbaI* and cloned into the pMT2 expression vector which had been modified by the inclusion of a polylinker at the unique *PstI* site. An unmodified clone of PTP1B also was produced in a PCR using the flanking primers and a plasmid template. Mutagenic oligonucleotides routinely contained silent substitutions that introduced diagnostic sites to facilitate identification of plasmids harboring the desired mutation. To confirm the presence of the desired substitution and to eliminate clones that had acquired additional mutations during the PCR, all cDNAs introduced into pMT2 were sequenced completely. After verifying the sequence of wild type PTP1B in pMT2, some mutants were cloned into the expression vector by replacement of the wild type *XhoI*−*XbaI* fragment.

Analysis of mutant PTP1B cDNA

CsCl-purified plasmid DNA was introduced into human embryonic kidney 293 cells or COS cells by calcium phosphate mediated transfection (20 μg DNA per 6 cm dish). Precipitate was removed after 12−16 h, and 4−12 h later, the medium was replaced and supplemented with 0.1 μg/ml nocodazole as required. After 18−24 h in nocodazole-containing medium, cells were washed gently twice with PBS and then removed from the surface of the dish by vigorous pipetting with PBS. Harvested cells were kept on ice, pelleted by centrifugation for 5 min at 1000 *g*, and lysed in 300 μl of 50 mM Tris−Cl pH 7.6, 5 mM EDTA, 2 mM DTT, 1% Triton X-100, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM benzamidine, 3 mM pepstatin A. Nuclei were removed by centrifugation at 1000 *g* and a portion of the supernatant fraction was heated to 95°C for 3 min in Laemmli sample buffer for analysis on SDS−PAGE. Large Hoefer slab gels (0.75 mm thick, 10% polyacrylamide) were run for 3 or 4 h at 40 mA/gel at 4°C to maximize the separation of the differently migrating forms of PTP1B. Proteins (5−10 μg of lysate protein per lane) were transferred to nitrocellulose membranes for 60 min at 15 V using a semi-dry transfer apparatus (Bio-Rad) and PTP1B was visualized by blotting with monoclonal antibodies FG6 or DH8.

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