Interaction of protein tyrosine phosphatase (PTP) 1B with its substrates is influenced by two distinct binding domains

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We have shown previously that protein tyrosine phosphatase (PTP) 1B interacts with insulin receptor and negatively regulates insulin signalling by an N-terminal binding domain [Dadke, Kusari and Chernoff (2000) J. Biol. Chem. **275**, 23642–23647] and it also negatively regulates integrin signalling through a proline-rich region present in the C-terminus [Liu, Hill and Chernoff (1996) J. Biol. Chem. **271**, 31290–31295; Liu, Sells and Chernoff (1998) Curr. Biol. **8**, 173–176]. Here we show that PTP1B mutants that are defective in Src homology 3 domain binding fully retain the ability to inhibit insulin signalling, whereas mutants defective in insulin-receptor binding fully retain the ability to inhibit integrin signalling. In contrast, both the C-terminal proline-rich region and the tandem tyrosine residues present in the N-terminal region are required for the activation of Src family kinases. These data show that PTP1B can independently regulate insulin and integrin signals, and that Src might represent a convergence point for regulating signal transduction by this phosphatase.

Key words: protein–protein interaction, signal transduction, tyrosine kinase.

INTRODUCTION

The regulation of tyrosine phosphorylation is a key mechanism governing cellular functions. The state of tyrosine phosphorylation is regulated by the sum of actions of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). Whereas many PTKs have been tied to specific signal-transduction systems, determining the precise function of PTPs has often proven elusive, as these enzymes often display promiscuous activity *in vitro*. Recently, however, it has become clear that many PTPs target their cellular substrates by means of specific binding motifs. For example, mitogen-activated protein kinase (MAPK) phosphatases contain binding motifs that allow them to associate tightly and specifically with their substrates [1,2], while Src-homology-domain-containing proteins (SHPs) 1 and 2 associate with tyrosine-phosphorylated receptor protein tyrosine kinases (RPTKs) and with cytokine receptors via Src homology 2 (SH2) domains [3,4]. The intracellular enzyme PTP1B presents a particularly interesting example in this regard. There is strong evidence supporting a role for PTP1B as a negative regulator of insulin signalling. For example, injection of PTP1B into *Xenopus* oocytes impedes insulin-stimulated maturation [5]. In mammalian cells, overexpression of PTP1B suppresses insulin signalling [6–8], whereas inhibition of this phosphatase enhances insulin signalling [9,10]. Finally, deletion of the *ptp1B* gene in mice causes marked insulin sensitivity and prolonged insulin receptor (IR) autophosphorylation [11,12]. It is thought that PTP1B complexes directly with RPTKs, such as the IR, as 'substrate-trap' mutants of PTP1B can co-immunoprecipitate the IR [13–15]. We have recently shown that the effects of PTP1B overexpression on insulin signalling are mediated, at least in part, by an N-terminal IR-binding domain [15]. Mutation of key residues in this domain reduces the effects of PTP1B on IR autophosphorylation and on downstream events, such as Akt and MAPK activation and glucose uptake. These data suggest that, like several other PTPs, PTP1B targets its substrates by means of a specific binding element.

In addition to its effects on insulin signalling, PTP1B also regulates integrin signalling [16,17]. In this case, PTP1B specifically affects the tyrosine phosphorylation of Src homology 3 (SH3)-domain-containing focal-adhesion proteins, such as $p130^{Cas}$ and focal adhesion kinase (Fak) [16,18,19]. PTP1B contains a proline-rich region near its C-terminus, and deletion or point mutation within this region eliminates the interaction of PTP1B with $p130^{Cas}$ and Fak and other SH3-containing proteins, such as Crk and Grb2, as well as its effects on integrin signalling and transformation. Thus PTP1B contains two distinct targeting motifs, one near its N-terminus that directs it to the IR (and perhaps other RPTKs) [20,21] and one in its C-terminus that directs it to SH3-containing proteins.

These results suggest that PTP1B uses two distinct substrate-targeting motifs to regulate two distinct signalling pathways. However, Goldstein et al. [22] recently showed that PTP1B induces dephosphorylation of IR substrate (IRS) 1, which is the major target for phosphorylation by the IR and is central to insulin signalling. IRS-1 complexes with several SH3-containing adaptor proteins, such as Grb2, Nck-2 and Crk, and it is thought that the effects of PTP1B on IRS-1 are mediated by the binding of PTP1B to one or more of these adaptors. Since PTP1B targets certain SH3 proteins, these findings raise the question as to whether SH3–proline interactions are also important for PTP1B's effects on insulin signalling. In the case of adhesion signalling,

Abbreviations used: PTP, protein tyrosine phosphatase; PTK, protein tyrosine kinase; RPTK, receptor protein tyrosine kinase; SH2, Src homology 2; SH3, Src homology 3; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; IR, insulin receptor; IRS, IR substrate; HA, haemagglutinin; DMEM, Dulbecco's modified Eagle's medium; GST, glutathione S-transferase; WT, wild-type; CS, catalytically inactive containing a Cys-215-to-Ser-215 mutation; PA, Pro-309,310-to-Ala mutant; YF, Tyr-152,153-to-Phe mutant; Fak, focal adhesion kinase.

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recent evidence suggests that integrins transactivate RPTKs, such as the epidermal growth factor receptor [23–26]. If so, then PTP1B mutants defective in binding to RPTKs might also be defective in regulating integrin signals. To determine the relationship of PTP1B's substrate-targeting motifs to its effects on signal transduction, we have systematically examined the properties of PTP1B mutants on substrate binding and on insulin and integrin signalling. We present evidence that a PTP1B mutant that is defective in binding to the IR is still able to down-regulate integrin signalling, whereas a PTP1B mutant that is defective in binding SH3-domain-containing proteins remains able to down-regulate insulin signalling. Interestingly, Src-family kinases, which are activated by both integrins and insulin [27–29], can also be activated by PTP1B [17,30], and we show here that the activation of Src-family kinases requires both of these binding motifs. Thus Src-family kinases represent unique targets of PTP1B, regulated via at least two distinct binding motifs. Interference with these different regions of PTP1B might selectively impact insulin versus integrin signalling.

EXPERIMENTAL

Materials

Rat 3Y1 cells were grown to 40 % confluence and transfected using Lipofectamine Plus reagent (Life Technologies) according to the manufacturer's recommendations with pJ3H, pJ3H-PTP-WT, pJ3H-PTP-CS, pJ3H-PTP-PA [18] or pJ3H-PTP-YF [15] together with a plasmid encoding a puromycin-resistance marker. The cells were selected by growth in 2 μg/ml puromycin and colonies were isolated with cloning cylinders. The monoclonal anti-haemagglutinin (HA) antibody 12CA5-J was obtained from Babco. Monoclonal anti-phosphotyrosine PY20, polyclonal anti-IR, monoclonal anti-CrkI and monoclonal anti-p130^{Cas} antibodies were purchased from Transduction Laboratories. Polyclonal anti-p130^{Cas} (used for immunoprecipitations) was obtained from Santa Cruz Biotechnology. Anti-phospho-Src (Tyr-416 and Tyr-527) antibodies were obtained from Cell Signalling. Anti-active-MAPK and anti-active-c-Jun N-terminal kinase (JNK) polyclonal antibodies, and the tyrosine phosphatase assay system were purchased from Promega.

Adhesion assays

The assays were carried out as described previously [16]. Briefly, cells derived from 3Y1 fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) plus 10 % fetal bovine serum and 2 μg/ml puromycin. Cells were starved overnight in serum-free DMEM and dissociated from culture flasks with 0.04 % trypsin/0.53 mM EDTA. The suspended cells were washed once in serum-free DMEM containing 0.1 mg/ml soyabean trypsin inhibitor (Sigma) and twice with serum-free DMEM alone, and held in suspension in DMEM plus 2 % BSA at 37 °C for 30 min with rotation. An equivalent number of cells were then plated on tissue-culture dishes with a 25 μg/ml solution of fibronectin (prepared by adsorbing 25 μg/ml fibronectin to 60 mm tissue-culture dishes at room temperature for 1 h) for 60 min or retained in suspension and incubated at 37 °C for the times indicated in the Figure legends.

Transient transfection

Rat 3Y1 cells were transiently transfected with pCMV6H-PTP1B mutants harbouring different point mutations using Lipofectamine Plus reagent according to the manufacturer's recommendations. After transfection (48 h) cells were harvested for analysis.

In vitro binding assay

Transfected 3Y1 cells were lysed in 1 % Nonidet P40 lysis buffer (50 mM Tris/HCl, pH 8.0, 137 mM NaCl, 10 % glycerol, 1 % Nonidet P-40, 50 mM NaF and 10 mM β-glycerol phosphate) containing 1 mM sodium vanadate, 1 mM PMSF and 10 μg/ml aprotinin. Lysate protein concentrations were measured using bicinchoninic acid (Pierce). We incubated 500 μg of total cell lysates with 20 μl of glutathione S-transferase (GST) alone or GST-SH3-domain fusion protein beads at 4 °C for 2 h. The beads were washed three times with Nonidet P40 lysis buffer and then boiled in SDS sample buffer. The samples were fractionated by SDS/PAGE (12 % gel), transferred to nitrocellulose membranes and probed with anti-HA antibody 12CA5.

Immunoprecipitation and immunoblotting

For immunoprecipitation, 1 mg of cell lysate was immunoprecipitated with 2 μg either of anti-HA or anti-p130^{Cas} antibody. Immunocomplexes were washed three times with Nonidet P40 lysis buffer and boiled for 5 min in SDS sample buffer. The samples were fractionated by SDS/PAGE and transferred to nitrocellulose membranes. Immunoblots were developed by a chemiluminescence method (Pierce) using alkaline phosphatase-conjugated secondary antibodies.

PTP activity assay

Activity of the GST fusion proteins GST-PTP-WT [containing wild-type (WT) PTP], GST-PTP-PA (with PTP containing a double Pro-309,310-to-Ala mutation), GST-PTP-YF (with PTP containing a double Tyr-152,153-to-Phe mutation) and GST-PTP-CS (with PTP containing a Cys-215-to-Ser mutation) was measured using the tyrosine phosphatase assay system from Promega as per the manufacturer's recommendations.

RESULTS

Separable interaction of PTP1B with its substrates

PTP1B contains at least two regions that affect its ability to associate with substrates. Near the N-terminus, a di-tyrosine (YY) motif is required for efficient IR binding [15], whereas a C-terminal proline-rich (PP) region is required for binding SH3 proteins [18]. The catalytic Cys-215 residue also affects substrate binding [20,21] (Figure 1A). We established stable clones of rat 3Y1 cells expressing HA-tagged WT PTP1B, catalytically inactive (CS) PTP1B (containing a Cys-215-to-Ser mutation), a Pro-309,310-to-Ala mutant (PA) PTP1B and a defective IR-binding mutant, Tyr-152,153 to Phe (YF) PTP1B [15,16] (Figure 1B). Consistent with our previous studies, the PA mutant was severely impaired in its ability to bind SH3-domain-containing proteins. Binding to the SH3 domain of p130^{Cas} was completely abolished, as was binding to the SH3 domain of Grb2 and Crk1. Binding to the SH3 domain of Src, although not completely abolished, was greatly reduced (Figure 1C).

Since the YF mutation lies within the catalytic domain of PTP1B, we assessed the effect of this and other mutations on the catalytic function of PTP1B. As shown in Figure 1(D), WT, YF and PA PTP1B dephosphorylate a model phosphotyrosyl peptide substrate with essentially identical kinetics. Similar results were obtained when *p*-nitrophenol phosphate was used as substrate (results not shown). Since the WT and mutant forms of

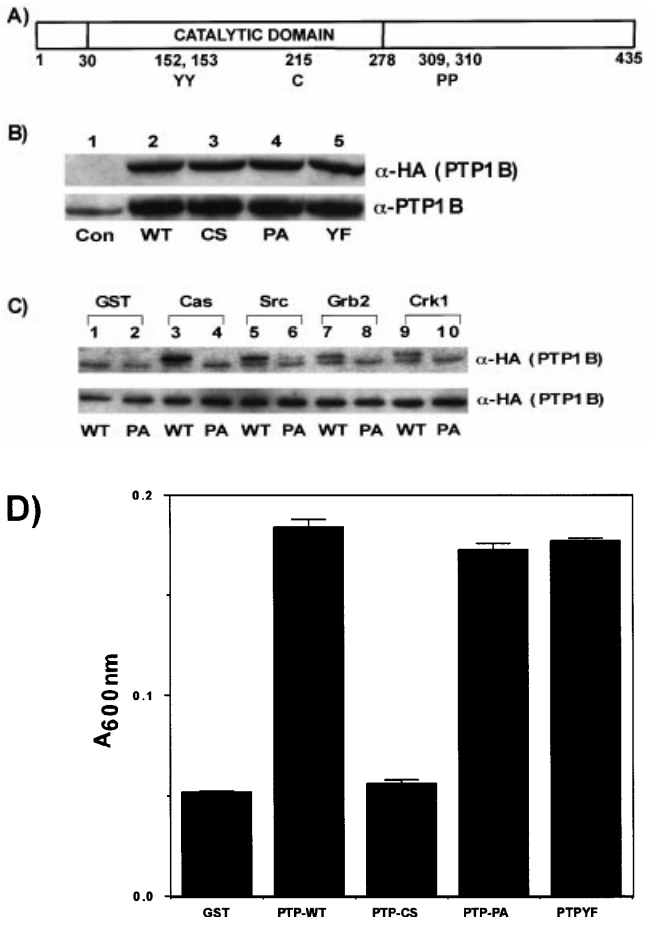

Figure 1 Characterization of cells expressing PTP1B

(**A**) PTP1B constructs. YY denotes Tyr residues 152 and 153, C denotes Cys residue 215 and PP denotes Pro residues 309 and 310. (**B**) Expression of PTP1B. Immunoblot to monitor the expression of HA-epitope-tagged WT, CS, PA and YF PTP1B in 3Y1 stable cell lines. Con, control. (**C**) Binding of PTP1B to SH3 domains. SH3 domains from p130^{Cas} (Cas), Src, Grb2 and Crk1 were purified as GST fusions. Lysates from cells expressing WT and PA PTP1B were incubated with the gluthione–Sepharose beads bound to the GST-fusion proteins. Following extensive washes, the proteins were eluted into SDS sample buffer and analysed by immunoblotting (upper panel). Whole-cell lysates were also analysed for PTP1B expression (lower panel). (**D**) Activity of PTP1B. WT, CS, PA and YF proteins were produced in *Escherichia coli*, purified by glutathione–Sepharose chromatography and assayed for activity towards a model phosphotyrosyl peptide. Assays were carried out in triplicate. A representative example of a single assay is depicted.

PTP1B behave identically in this assay, any differences in the effects of these two mutants (YF and PA) on either insulin or integrin signalling can be attributed to differences in their ability to interact with substrates such as the IR or p130^{Cas} rather than to impaired catalytic function.

Using these cells, we then examined the ability of either WT or mutant PTP1B to bind to either p130^{Cas} or IR. Lysates from cells expressing HA-tagged PTP1B were immunoprecipitated using anti-HA antibody, followed by immunoblotting with anti-Cas antibody. WT, CS and YF PTP1B associate with p130^{Cas}, whereas the PA PTP1B mutant does not bind to p130^{Cas} (Figure 2A). These results show that the proline residues at positions 309 and 310 in PTP1B are required for binding to p130^{Cas}, but that catalytic activity is not required, and nor is the N-terminal element that we showed previously to regulate IR binding [15]. We next wished to carry out the converse experiments regarding

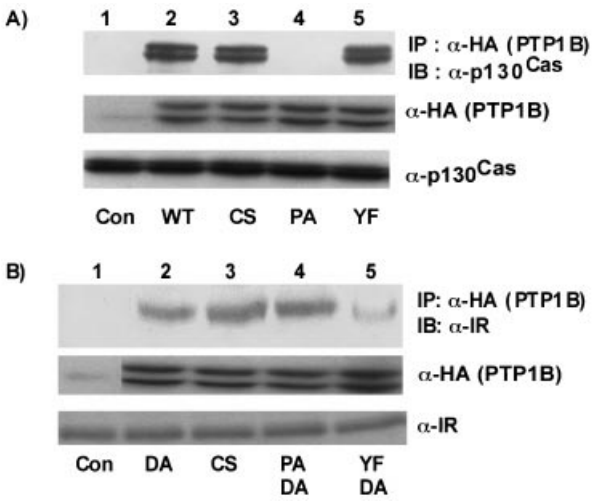

Figure 2 *In vivo* interaction of PTP1B with the p130^{Cas} and IR

(**A**) Control cells and 3Y1 stable cell lines expressing WT, CS, PA and YF PTP1B were lysed, immunoprecipitated (IP) with anti-HA antibody and immunoblotted (IB) with anti-p130^{Cas} antibody (upper panel). Anti-HA (middle panel) and anti-p130^{Cas} (lower panel) immunoblots are also shown. (**B**) 3Y1 cells were transiently transfected with the substrate-trap forms [DA (containing a Asp-181-to-Ala mutation) or CS] of PTP1B to which either PA or YF mutations were added. Proteins were immunoprecipitated with anti-HA antibody and immunoblotted with anti-IR antibody (upper panel). Anti-HA (middle panel) and anti-IR (lower panel) immunoblots are also shown. Con, control.

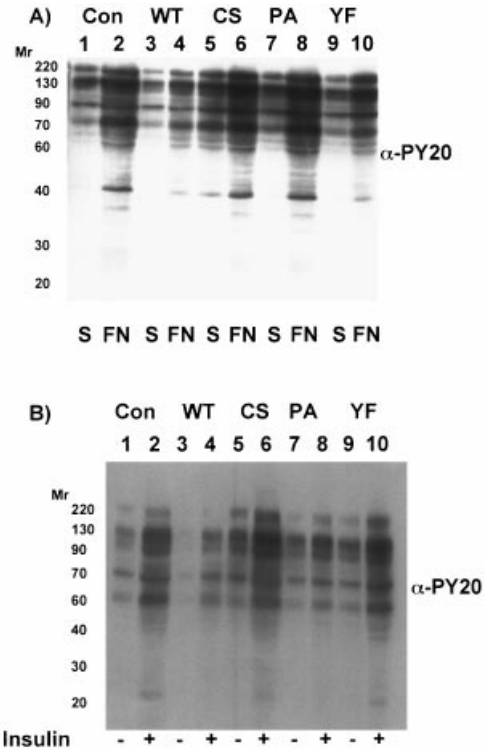

Figure 3 Effect of PTP1B on cell-adhesion-induced or insulin-stimulated total tyrosine phosphorylation

(**A**) Lysates of 3Y1 stable cell lines expressing the indicated forms of PTP1B were prepared from cells in suspension (S) or cells plated on fibronectin (FN), and immunoblotted with anti-phosphotyrosine antibody (PY20). (**B**) Cell lysates were prepared from serum-starved (−) or insulin-stimulated (100 nM, 10 min; +) cells, and immunoblotted with anti-phosphotyrosine antibody. Con, control.

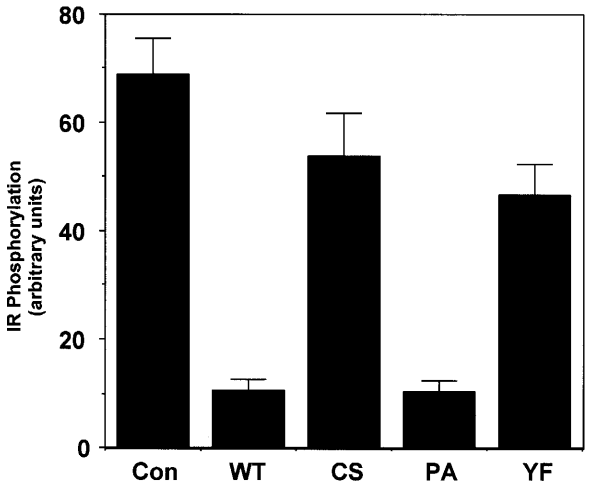

Figure 4 IR dephosphorylation by WT and mutant PTP1Bs

Various 3Y1 stable cell lines were stimulated with 100 nM insulin for 10 min. IR was immunoprecipitated with anti-IR antibody and immunoblotted with anti-phosphotyrosine antibody, and quantification of the phosphotyrosine content was assessed by densitometry of the immunoblot. Bars represent the means ± S.D. from three independent experiments. Con, control.

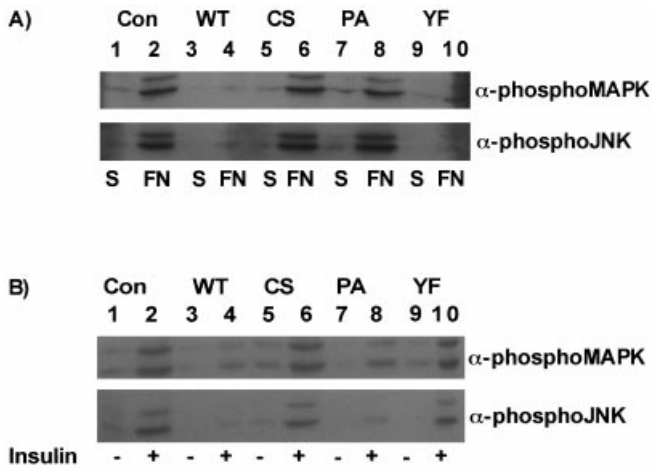

Figure 5 Effects of WT and mutant PTP1Bs on downstream integrin or insulin signalling

(**A**) 3Y1 stable cell lines were either maintained in suspension (S) or plated on fibronectin (FN), lysed and immunoblotted with the indicated phospho-specific antibodies. (**B**) 3Y1 stable cell lines were serum-starved (−) or stimulated with insulin (100 nM, 10 min; +), lysed and immunoblotted with the indicated phospho-form-specific antibodies for different proteins. Con, control.

PTP1B–IR interactions. Because the binding of PTP1B to the IR is stabilized when substrate-trap forms of the phosphatase are used [14,15], we carried out transient transfection of rat 3Y1 cells with substrate-trap mutants of PTP1B, to which the PA or YF mutations were added (Figure 2B). HA-PTP1B was immunoprecipitated with anti-HA antibody, followed by immunoblotting with anti-IR antibody. The results of this experiment show that the proline-rich domain of PTP1B is not required for interaction with the IR, whereas the tyrosine residues in the N-terminus of the phosphatase are required for efficient interaction. These results also suggest that PTP1B targets particular substrates using different and experimentally separable binding elements.

Effect of PTP1B on tyrosine phosphorylation

We examined the effects of WT and different mutants of PTP1B on the tyrosine-phosphorylation levels of cellular proteins following either integrin engagement or insulin stimulation (Figure 3). When control cells were plated on fibronectin, a number of proteins became tyrosine-phosphorylated, as assessed by probing the immunoblots with anti-phosphotyrosine antibody. The most prominent of these proteins migrated between 70 and 130 kDa, which may represent the different proteins involved in integrin signalling, such as Fak, paxillin, cortactin, p130^{Cas} and related proteins [31,32]. In cells expressing either WT or YF PTP1B, the overall level of tyrosine phosphorylation decreased dramatically, whereas the CS and PA mutants of PTP1B had no and very little effect on overall tyrosine-phosphorylation levels, respectively (Figure 3A). Similar phosphorylation patterns were observed in three independent clones for each mutant (results not shown). These data indicate that effects of PTP1B on integrin signalling are likely to involve SH3-dependent interactions.

Similarly, when the control cells were stimulated with insulin, a number of cellular proteins became tyrosine-phosphorylated. Cells expressing either WT or PA PTP1B showed a marked decrease in the level of tyrosine phosphorylation of most of these cellular proteins, whereas expression of the CS or YF mutants had little effect (Figure 3B). These results mean that these effects of PTP1B are mediated independently of SH3–proline interactions. Thus, as with binding, the negative effects of PTP1B on insulin versus integrin-stimulated tyrosine phosphorylation are entirely separable.

Dephosphorylation of IR by PTP1B

PTP1B is known to target the IR. Several SH3-domain-containing proteins dock at IRS-1, and it is conceivable that binding to such proteins might be important in placing PTP1B in proximity to the IR. *In vitro*, PTP1B associates with the SH3 domains of Crk and Grb2 [18,22] and therefore these proteins might link PTP1B to IRS-1 and the IR. To determine whether binding to SH3-containing proteins affects the ability of PTP1B to dephosphorylate the IR, we assessed IR tyrosine phosphorylation levels in cells expressing WT or mutant forms of PTP1B. The various cell lines were treated with insulin for 10 min and lysed, and the IR was immunoprecipitated and analysed for autophosphorylation. Quantification of IR β-subunit phosphotyrosine content from immunoblots was performed by densitometry. Expression of WT PTP1B as well as PA PTP1B reduced IR autophosphorylation by 85 %. In contrast, expression of the binding-defective YF PTP1B mutant reduced IR autophosphorylation by only 32 % (Figure 4). The results of this experiment show that the effect of PTP1B on IR autophosphorylation correlates with its ability to bind to this RPTK and that the tyrosine residues in the N-terminus, but not the proline-rich domain in the C-terminus of PTP1B, are required for IR dephosphorylation.

Differential effects of PTP1B mutants on signalling

We next examined the effects of WT and mutant forms of PTP1B on signalling events downstream of both the IR and integrin engagement, including activation of MAPK and JNK. For integrin-signalling assays, cells were either maintained in suspension or plated on fibronectin-coated tissue-culture plates for the indicated time periods (Figure 5A), whereas for insulin-

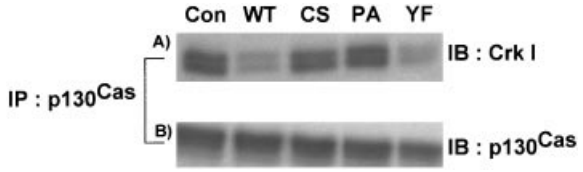

Figure 6 Expression of WT and YF PTP1B, but not PA PTP1B, decreases the amount of v-Crk associated with p130^{Cas}

Lysates derived from representative 3Y1 stable lines expressing vector alone (Con), WT, CS, PA and YF PTP1B were immunoprecipitated (IP) with polyclonal p130^{Cas} antisera. Immunocomplexes were separated by SDS/PAGE (10% gel) and immunoblotted (IB) with (**A**) monoclonal anti-Crk antibody or (**B**) monoclonal anti-p130^{Cas} antibody.

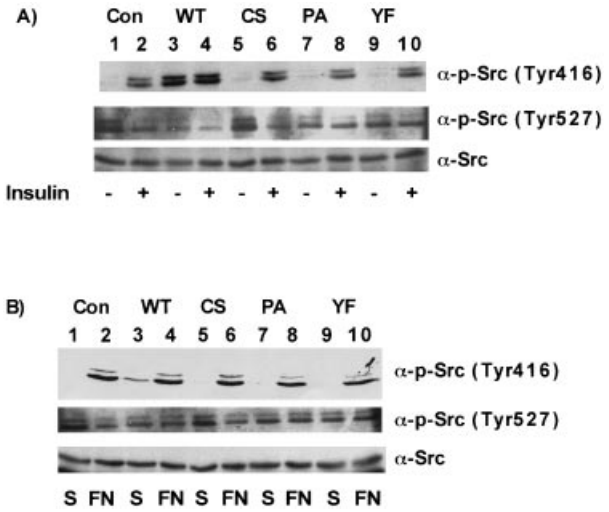

Figure 7 Activation of c-Src by PTP1B requires both the N-terminal tyrosines and C-terminal proline-rich regions

(**A**) Lysates from serum-starved (−) or insulin-stimulated (100 nM, 10 min; +) 3Y1 stable lines were prepared, separated by SDS/PAGE (10% gel) and immunoblotted with anti-phospho-Src Tyr-416 antibody (upper panel), anti-phospho-Src Tyr-527 (middle panel) or anti-Src (lower panel) antibody. (**B**) 3Y1 stable cell lines were either maintained in suspension (S) or plated on fibronectin (FN), lysed and immunoblotted as in (**A**). Con, control.

signalling assays, they were stimulated with insulin (Figure 5B). Following cell lysis, the protein extracts were separated by SDS/PAGE and immunoblotted with phosphospecific antibodies as described in the Figure legends. Both insulin treatment and integrin engagement led to strong stimulation of both MAPK and JNK, as measured by phosphospecific antibodies (Figures 5A and 5B, lanes 2). The MAPK and JNK protein levels were similar in all the cell lines used (results not shown). As we and others have observed previously, expression of WT PTP1B strongly reduces this activation (Figures 5A and 5B, compare lanes 3 and 4), whereas the catalytically inactive CS mutant has little effect. Expression of PA PTP1B, which cannot bind SH3 proteins, had no effect on the integrin-signalling pathway, but acted like WT PTP1B in insulin signalling. On the other hand, YF PTP1B, which is defective in IR binding, negatively regulates the integrin-signalling pathway, but has little effect on insulin signalling. These findings again highlight the independent targeting mechanisms used by PTP1B to regulate growth-factor versus adhesive signal-transduction pathways.

Expression of PTP1B in 3Y1-v-crk cells induces loss of association between p130^{Cas} and Crk

In 3Y1-v-crk cells (3Y1 cells that have been transformed with v-Crk), the SH2 domain of v-Crk has been shown to mediate binding to tyrosine-phosphorylated p130^{Cas} [33,34]. To investigate the effect of expressing PTP1B on the association between v-Crk and p130^{Cas}, p130^{Cas} was immunoprecipitated from these cell lines and the amount of v-Crk in the immunocomplexes was determined by immunoblotting (Figure 6). In the PA PTP1B- and CS PTP1B-expressing cells, approximately the same amount of v-Crk was associated with p130^{Cas} as in control cells. In cells expressing WT PTP1B and YF PTP1B, much less v-Crk was associated with p130^{Cas}. The total amount of p130^{Cas} in each immunoprecipitate was approximately equal (Figure 6). These data show that the C-terminal proline-rich region of PTP1B, but not the N-terminal region required for efficient IR binding, is involved in mediating its interaction with p130^{Cas} and thereby inhibiting complex formation between p130^{Cas} and v-Crk.

Activation of a Src-family kinase by PTP1B requires both the N-terminal di-tyrosine motif and the C-terminal proline-rich region

c-Src is activated by both insulin and integrin signalling [27–29]. PTP1B has been reported to activate c-Src by dephosphorylating the inhibitory site at Tyr-527 [17,30]. c-Src has an SH3 domain, but the role of SH3–proline interactions, if any, for regulation of c-Src or other Src-like kinases by PTP1B has not been reported. We therefore asked if expression of PTP1B activates Src-family kinases in rat 3Y1 cells and what the structural requirements are for such activation. Using two phosphospecific antibodies (against Tyr-416, which is phosphorylated when Src is active, and Tyr-527, which is phosphorylated when Src is inactive) we found that, in control cells, insulin treatment activates a Src-family kinase (Figure 7A, upper panel, compare lanes 1 and 2). Expression of WT PTP1B induces constitutive activation of this Src-family kinase, consistent with published reports (Figure 7A, upper panel, lane 3). Interestingly, disruption of either the C-terminal proline-rich motif, which is important in SH3-binding, or the N-terminal di-tyrosine motif, which is important in IR binding, markedly reduces the ability of PTP1B to activate this Src-family kinase (Figure 7A, upper panel, compare lane 3 with lanes 7 and 9). Upon integrin engagement, this Src-family kinase is also activated (Figure 7B, upper panel, compare lanes 1 and 2). Expression of WT PTP1B partially activates the Src-family kinase in suspended cells (Figure 7B, upper panel, lane 3), and this activation, as with insulin, requires both the C-terminal proline-rich motif that is important in SH3-binding and the N-terminal di-tyrosine motif.

DISCUSSION

The intracellular enzyme PTP1B presents a particularly interesting example of the role of targeting motifs in substrate selection. It targets certain RPTKs, in particular the IR, as well as SH3-containing proteins, such as p130^{Cas}, Fak and Crk. The basis for IR dephosphorylation is 2-fold. First, the presence of tandem phosphotyrosines 1162 and 1163 on the IR forms a favourable substrate site for the catalytic domain of PTP1B [35,36]. Second, the N-terminal tyrosine residues 152 and 153 are involved in IR binding [15], as well as in PTP1B activation by insulin [14]. The combination of these elements may explain PTP1B's relative specificity for the IR versus other growth-factor

receptors under physiological conditions. On the other hand, PTP1B also targets SH3-containing proteins, such as p130^{Cas}, Fak and Crk, which play a prominent role in integrin signalling [16,18,19,37,38]. We show here that regulation of these two types of signalling pathway can be separated completely by means of strategically placed point mutations in PTP1B. These results suggest that it might be possible to inhibit PTP1B activity selectively in one pathway without disturbing its effects on the other.

The IR signalling apparatus includes several SH3-domain-containing proteins. IRS-1, which represents the major IRS, binds the adaptor proteins Crk, Grb2 and Nck-2. Of these proteins, PTP1B is known to bind Crk and Grb-2 [18,22] and may well bind additional SH3-containing adaptor proteins. It was therefore important to test whether PTP1B mutants lacking SH3-binding could efficiently down-regulate insulin signals. Our results, which include examinations of total phosphotyrosine levels, and assays for insulin-stimulated Erk and JNK phosphorylation, indicate that PTP1B does not require interactions with SH3 proteins to down-regulate insulin signals. These data are consistent with the view that PTP1B directly binds and dephosphorylates the IR, and that interactions with SH3 proteins do not impact on this association. On the other hand, the regulation of integrin signals depends on SH3–proline interactions, and RPTK binding is of no consequence to this regulation. As integrin signals can transactivate RPTKs such as the epidermal growth factor receptor [23–26], these results imply that such transactivation is not required for tyrosine phosphorylation of key integrin-signalling targets such as p130^{Cas}.

Both insulin and integrin activation up-regulate c-Src [27–29]. PTP1B has been shown to activate c-Src by dephosphorylating the negative regulatory Tyr-527 in the C-terminus of c-Src [17,30]. Here we show that c-Src, or a closely related kinase, is an unusual substrate for PTP1B, in that both the proline-rich and di-tyrosine motifs of PTP1B are required for efficient interaction with the c-Src-family kinase and its subsequent activation. Interestingly, not only the C-terminal proline-rich region of PTP1B, but also the N-terminal di-tyrosine motif, affects binding to the SH3 domain of the c-Src. Whereas the enzyme activity data shown in Figure 1(D), using artificial substrates, argue against the possibility of major conformational changes in the YF PTP1B mutant, it is possible that more-subtle alterations are present that affect interactions with proteins such as c-Src. Alternatively, Src-family kinases in the basal state might phosphorylate the di-tyrosine motif in PTP1B, leading to activation of the enzyme, which then further activates the PTK by dephosphorylating the negative regulatory site (in c-Src, Tyr-527).

It is interesting to note that insulin and insulin growth factor-1 induce tyrosine dephosphorylation of Fak, p130^{Cas} and paxillin [39–42]. PTP activity is required for this dephosphorylation, as it is blocked by PTP inhibitors such as phenylarsine oxide [41]. As insulin phosphorylates and activates PTP1B [14,43], it is possible that the effects of insulin on dephosphorylating cytoskeletal proteins such as Fak, p130^{Cas} and paxillin are mediated by PTP1B.

One implication of our findings is that it might be possible to impair selectively PTP1B's ability to regulate insulin versus integrin signalling. Unlike a catalytic inhibitor, an agent that could disrupt IR–PTP1B or p130^{Cas}–PTP1B interactions is expected to have less global effects on signalling. In addition, since the proline-rich region is unique to PTP1B, such agents might distinguish this enzyme from its close relative T-cell phosphatase (TC-PTP). Loss of TC-PTP function is lethal in mice [44], and catalytic inhibitors of PTP1B that affect TC-PTP function would therefore need to be viewed with caution. Our data provide evidence that the regulation of insulin versus integrin signalling by PTP1B is separable, that substrate specificity of PTP1B is imparted by unique regions outside the catalytic core of this enzyme and that manipulating this region might selectively impact either insulin or integrin signalling.

We thank Peter Adams and Erica Golemis for their comments on the manuscript.

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Received 26 September 2001/21 January 2002; accepted 19 March 2002