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Protein Tyrosine Phosphatase 1B Antagonizes Signalling by Oncoprotein Tyrosine Kinase p210 bcr-abl In Vivo

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The p210 bcr-abl protein tyrosine kinase (PTK) appears to be directly responsible for the initial manifestations of chronic myelogenous leukemia (CML). In contrast to the extensive characterization of the PTK and its effects on cell function, relatively little is known about the nature of the protein tyrosine phosphatases (PTPs) that may modulate p210 bcr-abl-induced signalling. In this study, we have demonstrated that expression of PTP1B is enhanced specifically in various cells expressing p210 bcr-abl, including a cell line derived from a patient with CML. This effect on expression of PTP1B required the kinase activity of p210 bcr-abl and occurred rapidly, concomitant with maximal activation of a temperature-sensitive mutant of the PTK. The effect is apparently specific for PTP1B since, among several PTPs tested, we detected no change in the levels of TCPTP, the closest relative of PTP1B. We have developed a strategy for identification of physiological substrates of individual PTPs which utilizes substrate-trapping mutant forms of the enzymes that retain the ability to bind to substrate but fail to catalyze efficient dephosphorylation. We have observed association between a substrate-trapping mutant of PTP1B (PTP1B-D181A) and p210 bcr-abl, but not v-Abl, in a cellular context. Consistent with the trapping data, we observed dephosphorylation of p210 bcr-abl, but not v-Abl, by PTP1B in vivo. We have demonstrated that PTP1B inhibited binding of the adapter protein Grb2 to p210 bcr-abl and suppressed p210 bcr-abl-induced transcriptional activation that is dependent on Ras. These results illustrate selectivity in the effects of PTPs in a cellular context and suggest that PTP1B may function as a specific, negative regulator of p210 bcr-abl signalling in vivo.

Chronic myelogenous leukemia (CML) is a clonal myeloproliferative disorder of the pluripotential hematopoietic stem cell characterized by the Philadelphia (Ph) chromosome. The Ph chromosome is the result of a reciprocal translocation in which the c-*abl* proto-oncogene on chromosome 9, encoding a protein tyrosine kinase (PTK), transposes to a new position on chromosome 22, in proximity to the breakpoint cluster region (bcr). The juxtaposition of *bcr* and *abl* creates a novel fusion gene that results in production of a chimeric protein termed p210 bcr-abl. This hybrid bcr-abl oncoprotein has enhanced PTK activity relative to c-Abl, which correlates with abnormal patterns of tyrosine phosphorylation in cells from patients with CML (reviewed in reference 29). bcr-abl can transform growth factor-dependent lymphoid (9) and myeloid (23) cells in culture into factor-independent and tumorigenic cells. When the *bcr-abl* gene is expressed in bone marrow cells through retroviral gene transfer in vitro followed by bone marrow transplantation into sublethally irradiated mice, a myeloproliferative CML-like syndrome occurs (10, 14, 27). These results strongly suggest that p210 bcr-abl plays a fundamental role in the pathogenesis of CML.

The state of tyrosine phosphorylation of proteins in vivo is governed by the coordinated and competing actions of PTKs and protein tyrosine phosphatases (PTPs). Current data suggest that the Ph chromosome translocation that generates the aberrantly activated p210 bcr-abl PTK fusion protein is the initiating event in CML (29). Therefore an understanding of the PTPs that have the ability to antagonize p210 bcr-abl function will provide a complementary perspective from which to study and intervene in the disease.

The PTPs represent a large (~75 members identified to date) and structurally diverse family of enzymes (reviewed in reference 57) that have been implicated in the regulation of cell growth and proliferation, differentiation, the cell cycle, and cytoskeletal integrity, as well as the etiology and pathogenesis of certain diseases. They have been identified in eukaryotes, prokaryotes, viruses, and plants. It is now apparent that the family of PTPs rivals that of the PTKs in structural diversity and complexity. In addition, through the process of dephosphorylation, PTPs can either antagonize or potentiate PTK-induced signaling events in vivo (57). Therefore, it is expected that control over reversible phosphorylation in vivo will be exerted at the level of both PTKs and PTPs.

The PTP domain is a 240-amino-acid segment that contains the invariant residues necessary for phosphatase activity. Within the PTP domain lies the signature motif (I/V)HCX AGXXR(S/T)G that uniquely defines this enzyme family. The cysteine residue within this sequence forms a thiophosphoenzyme intermediate necessary for catalysis. The members of the PTP family may be distinguished on the basis of the noncatalytic segments that are fused to either the N or C terminus of the catalytic domain. Like the PTK family, the family of PTPs can be divided into two large classes, the transmembrane, receptor-like PTPs and the nontransmembrane, intracellular species. The receptor-like PTPs have diverse extracellular segments, a single membrane-spanning region, and (with a few

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exceptions) two tandemly repeated cytoplasmic PTP domains. The nontransmembrane, intracellular PTPs contain a single catalytic PTP domain flanked by various N- and C-terminal motifs. These motifs are believed to have regulatory functions including targeting to defined subcellular locations. For example, PTP1B and the closely related, 48-kDa form of TCPTP (74% identity in the catalytic domain) are targeted to the cytoplasmic face of membranes of the endoplasmic reticulum via a hydrophobic segment at their extreme C termini (16, 31); TCPTP also exists as a spliced variant of 45 kDa that lacks the hydrophobic segment and localizes to the nucleus (36, 53). A second example is illustrated by the two SH2 (Src homology 2) domain-containing PTPs, SHP-1 and SHP-2, which have been shown to bind to specific phosphotyrosine residues in growth factor and cytokine receptors and therefore are targeted to signalling complexes at the plasma membrane (38).

Much research effort in the context of cellular signalling events in CML has focused on p210 bcr-abl and the role of aberrant tyrosine phosphorylation. Little is known about the role of PTPs in CML. There have been preliminary reports of uncharacterized PTPs with the ability to dephosphorylate p210 bcr-abl (39). In addition, the SH2 domain containing PTP, SHP-2, which has been shown to function positively in mediating tyrosine phosphorylation-dependent signalling events, is phosphorylated on tyrosine residue(s), and forms a complex with p210 bcr-abl in cells overexpressing the PTK (51); however, the significance of this association is unclear. To provide further insight into the function of PTPs in the disease, we initiated this study to ascertain which members of the PTP family had the potential to regulate p210 bcr-abl function in vivo. We have observed that PTP1B is upregulated specifically in response to the expression of the p210 bcr-abl PTK in model cell systems and in cell lines derived from a CML patient. We present evidence that PTP1B recognizes p210 bcr-abl as a substrate in a cellular context. Through the use of a mutant form of PTP1B (PTP1B-D181A) that is catalytically impaired but still binds to, and forms stable complexes with, its substrates, we have captured p210 bcr-abl and PTP1B-D181A in a physical complex in COS cells. This association, as well as dephosphorylation of p210 bcr-abl by wild-type PTP1B, inhibited binding of the Grb2 adapter molecule to the oncoprotein PTK. Furthermore PTP1B and PTP1B-D181A, but not its closest relative TCPTP or the cytoplasmic enzyme PTP-PEST, suppressed p210 bcr-abl-induced transcriptional activation of an AP-1/ets reporter that was dependent on the Ras pathway. Our results suggest that PTP1B functions as a specific antagonist of signalling induced by p210 bcr-abl in vivo.

MATERIALS AND METHODS

Cell culture and preparation of lysates. Mo7 and Mo7p210 (p210 bcr-abl-expressing Mo7) cells (34) were cultured in RPMI 1640 medium supplemented with 20% fetal bovine serum. For Mo7 cells, the medium was further supplemented with granulocyte/macrophage colony-stimulating factor at 20 ng/ml. Rat-1 fibroblasts, NIH 3T3 cells, and COS-1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. BN1 and BT1 cell pellets were kindly provided by B. Clarkson (Memorial Sloan Kettering Cancer Center) (40). BaF3 cells expressing a temperature-sensitive (*ts*) mutant of p210 bcr-abl (*ts*-p210 bcr-abl) were cultured as described by Jain et al. (25). Stable Rat-1 fibroblasts expressing p210 bcr-abl, v-Abl, and v-Myc (Rat-1p210, Rat-1v-Abl, and Rat-1v-Myc cells) were created as described elsewhere (41). In all cases, cells were lysed in buffer comprising 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% (vol/vol) Triton X-100, 10% glycerol, 1 mM EDTA, 10 μg of leupeptin per ml, 10 μg of aprotinin per ml, 1 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride.

Immunoblotting and antibodies. Protein concentrations were determined by the Bradford method (3), using bovine serum albumin as the standard. Monoclonal antibodies FG6, against PTP1B, and CF4, against TCPTP, were provided by David Hill (Calbiochem Oncogene Research Products, Cambridge, Mass.). Anti-Abl antibodies (21-63 and Pex 5) are described in reference 41. Anti-PTP-PEST polyclonal antibody is described in reference 17. The antiphosphotyrosine (anti-pTyr) monoclonal antibody 4G10 was from Upstate Biotechnology Inc. (Lake Placid, N.Y.). Monoclonal antibodies to SHP-2 and Grb2 were from Transduction Laboratories (Lexington, Ky.). Polyclonal anti-PTP1B was kindly provided by Ben Neel (Harvard Medical School). For immunoblotting, antigen-bound antibody was detected with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody and then visualized with enhanced chemiluminescence (ECL; Amersham).

Immunoprecipitation and substrate trapping. COS-1 cells were transfected by standard calcium phosphate precipitation. Vectors pSRαMSVtkneo and pSRα, expressing the various PTKs and PTK mutants, are described in reference 41. The generation of the PTP1B substrate-trapping mutant is described in reference 15. For immunoprecipitation, antibodies (Pex 5 and preimmune antiserum) were coupled to protein A-Sepharose and incubated at 4°C for 90 min with lysates that were precleared with IgGSorb (Enzyme Centre), followed by five washes of the immunoprecipitates with lysis buffer. For substrate trapping in vivo, following transient expression of mutant PTPs, COS-1 cells were lysed in lysis buffer lacking sodium vanadate and the lysates were precleared by addition of IgG Sorb reconstituted in lysis buffer, rocked at 4°C for 1 h, and then centrifuged at 15,000 × *g* (4°C) for 5 min. Precleared lysate was added to a fresh tube containing 30 μl of glutathione-Sepharose beads (resuspended 1:1 in phosphate-buffered saline [PBS]) or 30 μl of protein A beads (resuspended 1:1 in PBS). After rocking at 4°C for 90 min, complexes were collected by centrifugation for 10 s at 1,000 × *g*, the supernatant was discarded, and the beads were washed five times in lysis buffer. For substrate trapping in vitro, glutathione *S*-transferase (GST) fusion proteins (GST-PTP1B and GST–PTP1B-D181A, described by Flint et al. [15]) were expressed in *Escherichia coli*, purified to homogeneity, and bound to glutathione-Sepharose. Mo7p210 cells were lysed in the presence or absence of the PTP inhibitor sodium vanadate (1 mM), and 200 μg of lysate was incubated with the affinity matrix (GST–PTP1B-D181A) for 90 min at 4°C. Beads were collected by low-speed (1,000 × *g*) centrifugation for 10 s and washed five times with wash buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% [vol/vol] Triton X-100, 10% glycerol, 1 mM EDTA, 10 μg of leupeptin per ml, 10 μg of aprotinin per ml, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride).

Dephosphorylation in vivo. COS-1 cells were cotransfected with 10 μg of pSRαp210 together with 15 μg of pMT2, pMT2PTP1B, or pMT2TCPTP. In another set of experiments, 10 μg of either pSRαp210, pSRαp210Y177F, or pSRαv-abl was cotransfected with 15 μg of pMT2PTP1B or pMT2 vector control. After 48 h, cells were washed twice with PBS and then immediately lysed in Laemmli sample buffer and heated to 90°C for 5 min. Equal volumes of lysate were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. The membrane was incubated with an anti-Abl antibody, to control for equal protein loading, and then stripped and reblotted with anti-pTyr to determine the extent of dephosphorylation.

Northern analysis. Total RNA (20 μg) was extracted from Mo7 and Mo7/p210 cells lines by the RNAzol method (Tel-Test, Inc.). After electrophoresis on a formaldehyde-agarose (1%) gel, the RNA was transferred to GeneScreen membrane. After UV cross-linking, the blot was hybridized with a full-length cDNA fragment of PTP1B in hybridization solution (0.5 M Na_2HPO_4, 7% SDS, and 1 mM EDTA) at 65°C for 12 h. The PTP1B and TCPTP probe were labeled with [α-^{32}P]dCTP to a specific activity of 10^8 cpm/μg of DNA and used at 10^6 cpm/ml following heat denaturation. Finally, the blot was washed four times in 1 mM EDTA–1% SDS–40 mM Na_2HPO_4 at 65°C and subjected to autoradiography. The blot was stripped by boiling the membrane in hybridization solution, washed extensively at 65°C with the same solution, and then reprobed with full-length TCPTP cDNA, which was used as an internal control. The agarose gel was stained using ethidium bromide, prior to transfer, to visualize RNA integrity.

PTP assay. Precleared lysates (60 μg) from Rat-1 and Rat-1p210 cells were incubated with either monoclonal antibody FG6 (anti-PTP1B) or a nonspecific monoclonal antibody IgG coupled to protein A-Sepharose. After rocking at 4°C for 2 h, immune complexes were collected by centrifugation for 15 s at 1,000 × *g* and the supernatants were assayed for PTP activity (54). Immunodepleted supernatants (2 or 8 μg) were incubated with ^{32}P-labeled pTyr reduced, carboxamidomethylated, and maleylated (RCM) lysozyme in a total volume of 60 μl of assay buffer (25 mM imidazole HCl [pH 7.2], 1 mg of bovine serum albumin per ml, 0.1% β-mercaptoethanol). The reaction was terminated by addition of 290 μl of 10% suspension of Norit A 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 was counted in scintillant to measure release of $^{32}P_i$. Immunoblot analysis of the immunodepleted lysates were carried out with an anti-PTP1B antibody (FG6).

Transcriptional activation assay (CAT assay). NIH 3T3 cells were transfected by standard calcium phosphate precipitation with 1 μg of pB4X-CAT reporter plasmid and 2 μg of pC3LACZ reporter plasmid together with 0.5 μg of pSRαMSVtkneo expressing either v-Abl or p210 bcr-abl in the presence or absence of 5 μg of pMT2 expressing PTP1B, PTP1B-D181A, PTP-PEST, PTP-PEST-D191A, or TCPTP. After 48 h, cells were harvested, washed twice with PBS, and lysed in 400 μl of 1× reporter lysis buffer (Promega). Cell debris was removed by centrifugation at 12,000 × *g* for 5 min, and the resulting supernatants were assayed for chloramphenicol acetyltransferase (CAT) and β-galactosidase activities. β-Galactosidase activity was determined by incubating 0.15 ml of cell

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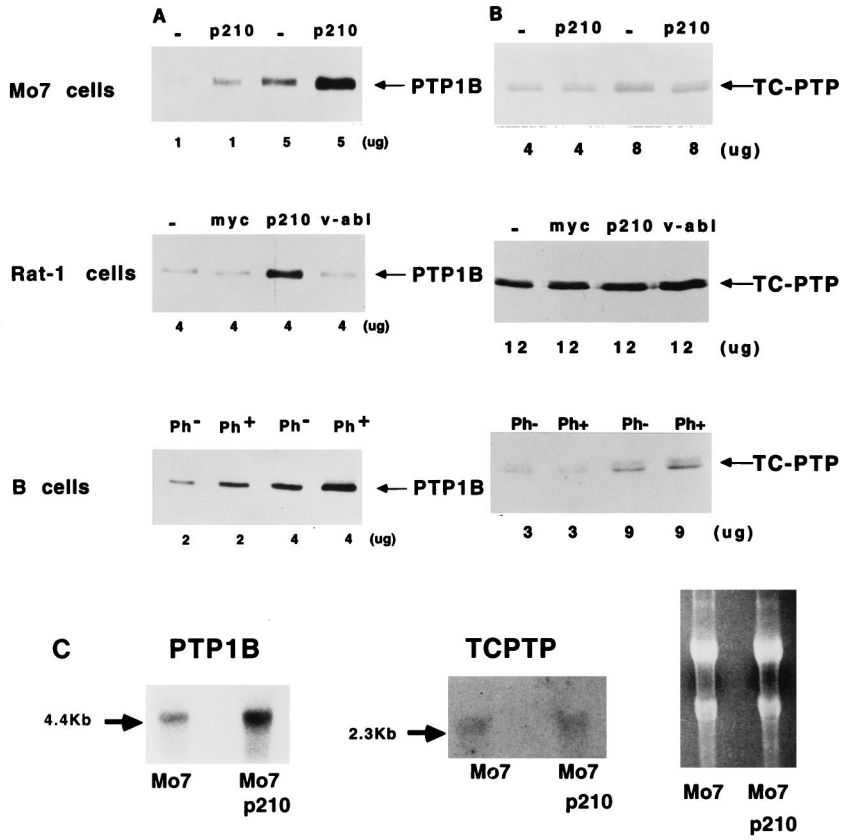

FIG. 1. Expression of PTP1B but not TCPTP is enhanced in Mo7, Rat-1, and B-lymphoid cells expressing p210 bcr-abl. Shown are results of immunoblot analyses of PTP1B (A), using monoclonal antibody FG6, and TCPTP (B), using monoclonal antibody CF4, visualized by ECL. (C) Enhancement of PTP1B mRNA following expression of p210 bcr-abl in Mo7 cells. A Northern blot containing 20 μg of total RNA from Mo7 and Mo7p210 cells was probed with PTP1B cDNA and then stripped and reprobed with TCPTP cDNA. Equal loading and integrity of the RNA samples were confirmed by ethidium bromide staining of the 28S and 18S rRNAs in the gel before transfer to nitrocellulose.

lysate with 0.15 ml of β-galactosidase reaction buffer (120 mM Na_2HPO_4, 80 mM NaH_2PO_4, 2 mM $MgCl_2$, 100 mM β-mercaptoethanol, 1.33 mg of *o*-β-D-thiogalactopyranoside per ml) at 37°C until a faint yellow color appeared. Reactions were stopped by the addition of 1.0 M sodium carbonate, and spectrophotometric readings were taken at 420 nm. CAT assays were normalized for transfection efficiency by reference to β-galactosidase activity measurements. CAT activity was determined by incubating 0.1 μCi of [^{14}C]chloramphenicol (NEN) and 0.44 mM acetyl coenzyme A in 250 mM Tris-HCl (pH 7.8) in a final reaction volume of 100 μl for 45 min at 37°C. The reaction was then terminated by extraction with ethyl acetate and subjected to thin-layer chromatography on silica gel plates, using 95% chloroform–5% methanol (vol/vol) as the solvent.

RESULTS

The level of PTP1B is enhanced in p210 bcr-abl-expressing cell lines. To examine whether expression of p210 bcr-abl exerted an effect on the expression of members of the PTP family, we examined, by immunoblotting, the levels of various intracellular PTPs in the human myeloid cell line Mo7 and in Mo7p210 cells (49). Strikingly, expression of only one of the enzymes tested, PTP1B, was enhanced two- to threefold in Mo7p210 compared to Mo7 cell lysates (Fig. 1A, top). In contrast, no change was observed in the levels of TCPTP, the phosphatase most closely related to PTP1B (74% identity in the catalytic domain) (Fig. 1B, top). We also observed a corresponding increase in PTP1B mRNA levels in Mo7p210 compared to Mo7 cells (Fig. 1C). The increased steady-state level of PTP1B appears to be attributable to the increase in mRNA levels since the protein half-life appeared unchanged. The increase in PTP1B was also induced in Rat-1 fibroblasts expressing p210 bcr-abl. In these cells, we observed a three- to fourfold increase in PTP1B levels that was not detected in cells transformed by v-Abl or v-Myc (Fig. 1A, middle). As in the Mo7 cells, the level of TCPTP was not altered as a consequence of p210 bcr-abl expression in Rat-1 cells (Fig. 1B, middle). To test whether the increase in PTP1B is observed clinically, we blotted lysates from Ph^+ and Ph^- B-lymphoid cell lines derived from a CML patient. Consistent with other p210 bcr-abl-expressing cell lines, there was an increase in PTP1B protein in the Ph^+ (BT1) compared to the Ph^- (BN1) cell line, in this case a difference of ~2-fold (Fig. 1A, bottom). Once again, there was no change in TCPTP protein expression between the two cell lines (Fig. 1B, bottom).

Increased PTP activity in Rat-1p210 cells correlates with the increase in PTP1B protein. Comparison of total PTP activities in Rat-1 and Rat-1p210 cells revealed an increase of ~2.5-fold in activity in the Rat-1p210 cells (Fig. 2A). The activity was measured using [^{32}P]pTyr RCM lysozyme as the substrate and therefore reflects the combined activity of a variety of PTPs present in the lysate. In view of the enhancement of PTP1B protein levels observed in Rat-1p210 cells, we examined the contribution of PTP1B to the increased activity by quantitative immunodepletion of the enzyme from lysates. The activity attributable to PTP1B was three- to fourfold higher in lysates of Rat-1p210 than in lysates of control Rat-1 cells (Fig. 2B); this correlated with the increase in PTP1B protein in Rat-1p210 cells (Fig. 1A, middle), suggesting that the intrinsic phospha-

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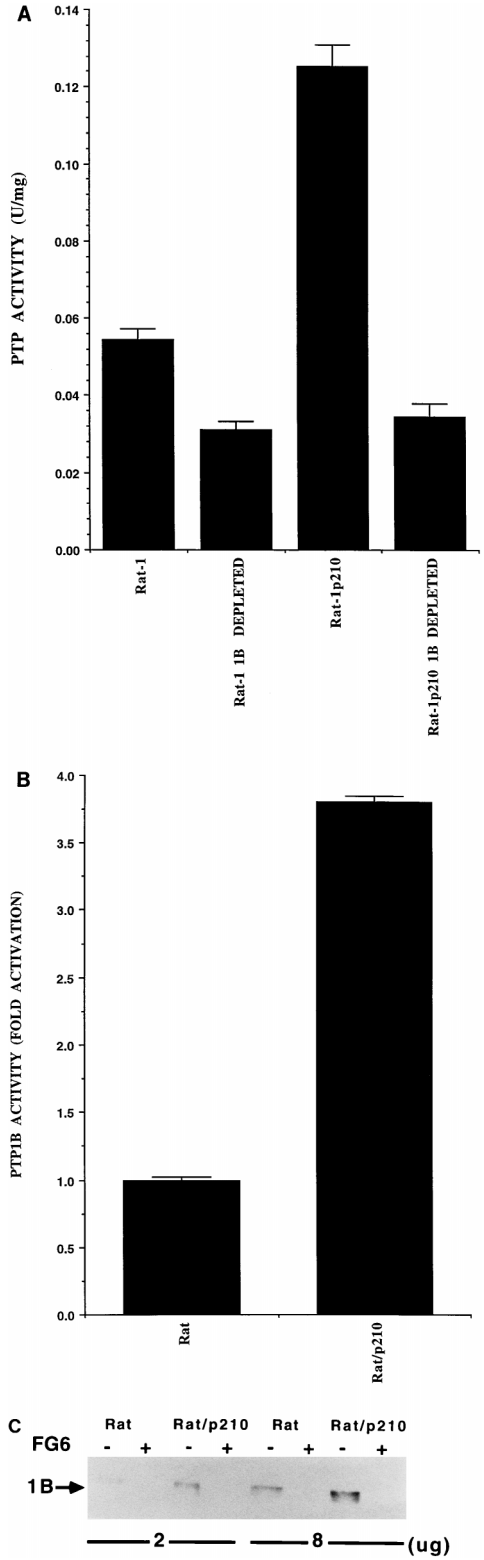

FIG. 2. Increased PTP activity in Rat-1p210 compared to Rat-1 cells correlates with the increase in PTP1B protein. (A) PTP activity from equal quantities of lysate protein (2 and 8 μg) from Rat-1 and Rat-1p210 cells was determined by using RCM lysozyme as the substrate, before and after quantitative immunodepletion of PTP1B. (B) Activity due specifically to PTP1B was determined by subtracting the activity in lysates following immunodepletion by antibodies to PTP1B from the total activity. The PTP activity is presented as the mean ± standard error of four independent assays. PTP1B activity in Rat-1p210 cells is expressed as fold increase in the activity observed in Rat-1 cells, with the activity in Rat-1 cells was set at a value of 1. (C) Immunoblot analysis of the quantitative immunodepletion of PTP1B from lysates of Rat-1 and Rat-1p210 cells used in the PTP activity assay. Total lysate (2 or 8 μg) was immunodepleted with anti-PTP1B antibody or control nonspecific mouse IgG; the depleted lysate was then subjected to immunoblot analysis using a monoclonal antibody (FG6) to PTP1B and visualized by ECL.

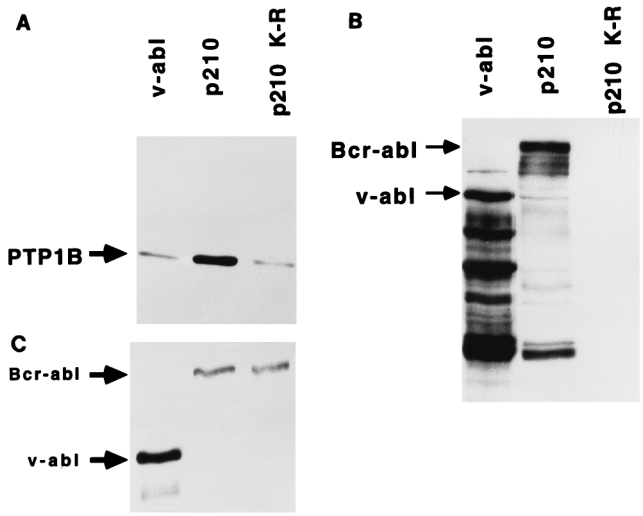

FIG. 3. The intrinsic tyrosine kinase activity of p210 bcr-abl is required for the overexpression of PTP1B. Equal quantities of lysate protein from Rat-1v-Abl, Rat-1p210, and Rat-1p210-K1172R cells were prepared and immunoblotted with antibodies to PTP1B (A), pTyr (B), and Abl (C). Immunoblots were visualized by ECL.

tase activity of PTP1B was not altered in p210 bcr-abl-expressing cells. Following immunodepletion of PTP1B, there was no apparent difference in activity in lysates of cells in the presence or absence of p210 bcr-abl expression, suggesting that the increase in total PTP activity in lysates from Rat-1p210 cells can be attributed almost exclusively to the increased expression of PTP1B. Immunoblot analysis revealed that PTP1B protein had been quantitatively immunodepleted from the cell lysates (Fig. 2C). Similar results were observed in a comparison of PTP activity in lysates of Mo7 and Mo7p210 cells (data not shown).

The tyrosine kinase activity of p210 bcr-abl is essential for the overexpression of PTP1B. To investigate whether the PTK activity of p210 bcr-abl plays a role in the enhancement of PTP1B levels, we generated stable Rat-1 fibroblasts expressing a catalytically inactive mutant of p210 bcr-abl in which the lysine residue in the ATP binding site was mutated to arginine (K1172R). The kinase-deficient p210 bcr-abl protein was expressed at levels similar to those of the wild-type protein (Fig. 3C). Immunoblotting with an anti-pTyr antibody confirmed that the p210 bcr-abl-K1172R mutant is inactive as a PTK (Fig. 3B). Immunoblot analyses of PTP1B from lysates of Rat-1p210 and Rat-1p210 K1172R cells revealed that PTP1B was increased only in the cells expressing the catalytically active PTK (Fig. 3A). These data implicate the PTK activity of p210 bcr-abl as essential for the upregulation of PTP1B levels.

The increase in PTP1B levels is induced rapidly following activation of a *ts* mutant of p210 bcr-abl. To investigate whether the increase in PTP1B expression that occurs following activation of the PTK is a rapid or a long-term, adaptive response, we have used BaF3 cells containing the *ts*-p210 bcr-

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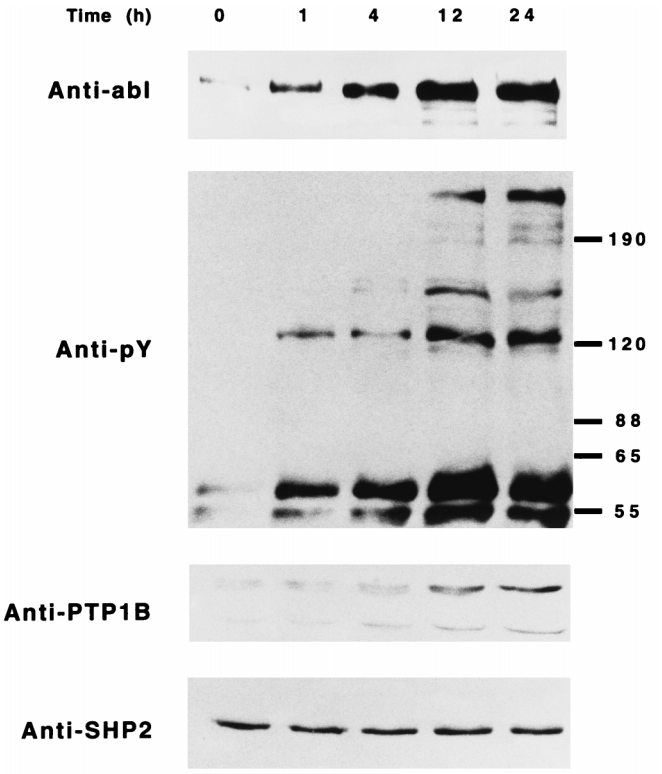

FIG. 4. PTP1B levels are enhanced rapidly following induction of p210 bcr-abl. BaF3 cells expressing a *ts* mutant of p210 bcr-abl were maintained at the nonpermissive temperature (39°C) for 18 h prior to a shift to the permissive temperature (33°C). At various time points (1, 4, 12, and 24 h), the levels of expression of p210 bcr-abl, PTP1B, and SHP-2 and the pTyr content of the cells were assessed by immunoblotting. For each time point, ~10^6 cells were centrifuged, washed with PBS, and then quick-frozen in an ethanol-dry ice bath. The cell pellets were lysed, and equal quantities of lysate protein was subjected to SDS-PAGE. Immunoblotting was performed with monoclonal antibodies to Abl, pTyr (pY), and PTP1B, as indicated. The PTP1B blot was stripped and reprobed with an antibody to SHP-2 (bottom). The zero time point represents lysate from cells grown at the nonpermissive temperature. Molecular size standards (in kilodaltons) are indicated to the right.

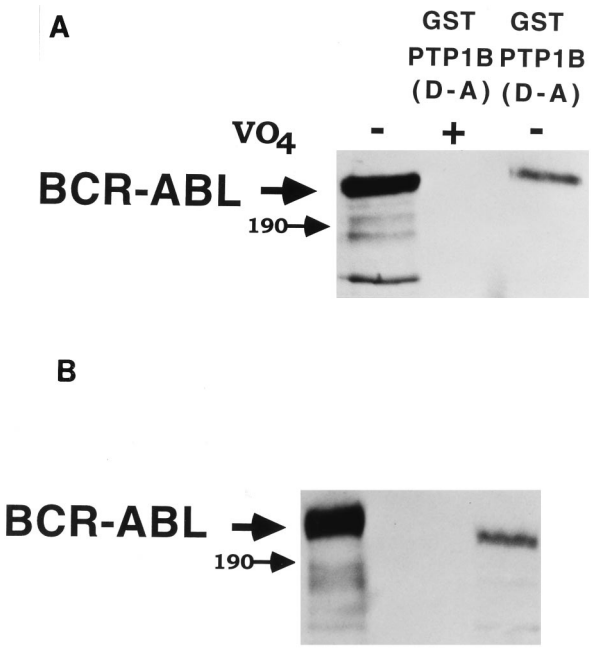

FIG. 5. Association of p210 bcr-abl with a substrate-trapping mutant of PTP1B in vitro. Mo7p210 cells were lysed in the presence or absence of the PTP inhibitor sodium vanadate (1 mM), and 100 μg of lysate was incubated with an affinity matrix comprising GST–PTP1B-D181A immobilized on glutathionine-Sepharose for 90 min at 4°C. The affinity matrix was pretreated with 1 mM sodium vanadate before incubation. Beads were collected by low-speed (1,000 × *g*) centrifugation for 10 s and washed five times with wash buffer. Samples were analyzed by immunoblotting with anti-Abl (A) and anti-pTyr (B) antibodies. Immunoblots were visualized by ECL. The first lane in each panel illustrates an immunoblot of 25 μg of Mo7p210 lysate. Molecular size standards (in kilodaltons) are indicated to the left.

abl gene (25). Cells growing at the nonpermissive temperature (39°C) were switched to the permissive temperature (33°C), and lysates were prepared at various times up to 24 h. We observed a gradual increase in both p210 bcr-abl expression and overall tyrosine phosphorylation, reaching a maximum at 12 to 24 h after the shift in temperature. We also observed that the increase in expression of PTP1B coincided with the period of maximal activation of p210 bcr-abl (Fig. 4). There was no detectable difference in SHP-2 expression following p210 bcr-abl activation (Fig. 4, bottom). These data make the important point that the increase in PTP1B levels is a relatively rapid response to the activation of p210 bcr-abl, suggesting that this may reflect a compensatory change to the presence of the oncoprotein PTK rather than a long-term adaptive response of the cell. This finding suggests either that PTP1B may play a positive, permissive role in p210 bcr-abl induced signalling or that the induction of PTP1B may reflect a feedback mechanism to curtail the effects of the PTK.

Association of p210 bcr-abl with a substrate-trapping mutant of PTP1B. With the resolution of the structure of PTP1B, alone and in a complex with a phosphotyrosyl peptide substrate (2, 26), several residues that are important for substrate recognition and catalysis have been identified. Based on these observations, a series of mutations within the catalytic domain of PTP1B were created and tested for substrate recognition (15). In the past, we and others have shown that mutant PTPs in which the catalytically essential, nucleophilic cysteine, from the signature motif, has been mutated to serine or alanine retain the ability to bind substrates in vitro and in some cases in vivo (22, 26, 35, 48, 50). However, not all such mutants can bind to physiological substrates in vivo. We have generated an additional substrate-trapping mutant in which the catalytically essential, invariant aspartate (D181 in PTP1B), which functions as a general acid in protonating the tyrosyl leaving group of the substrate, has been changed to alanine. In this mutant, the affinity for substrate (K_m) is maintained but catalytic efficiency (V_{max}) is severely impaired. The properties of these substrate-trapping mutants are described elsewhere (15, 17).

Use of the PTP1B-D181A mutant has provided evidence suggesting that p210 bcr-abl is a physiological substrate of PTP1B. Using an affinity matrix that comprises an immobilized GST fusion protein of PTP1B-D181A, we were able to precipitate p210 bcr-abl from lysates of Mo7p210 cells (Fig. 5). To confirm that the interaction between p210 bcr-abl and PTP1B-D181A involved the catalytic center of the phosphatase, we tested the effects of the PTP inhibitor sodium vanadate. Sodium vanadate (a transition-state analog of phosphate) has been shown to inhibit PTP activity by covalently modifying the essential, nucleophilic cysteine residue at the active site (13, 24). We observed that the interaction between p210 bcr-abl and GST–PTP1B-D181A was inhibited in the presence of 1 mM sodium vanadate (Fig. 5).

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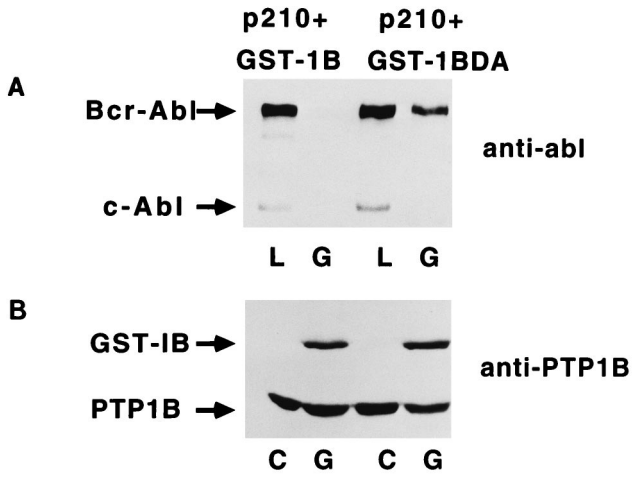

FIG. 6. PTP1B-D181A physically associates with p210 bcr-abl in vivo. COS-1 cells were cotransfected with 10 μg of pSRαMSVtkneop210 plasmid DNA together with 10 μg of pMT2 expression plasmid for GST-PTP1B, wild type or D181A mutant. (A) Lanes: L, immunoblot of cell lysate (25 μg) following cotransfection; G, immunoblot of the glutathione-Sepharose precipitates from 200 μg of lysate. (B) Lanes C and G, 10 μg of the lysates of untransfected COS cells (C) or COS cells coexpressing GST-PTP1B fusion proteins together with p210 bcr-abl (G). Samples were subjected to immunoblot analysis with anti-Abl (A) and anti-PTP1B (B) antibodies and visualized by ECL.

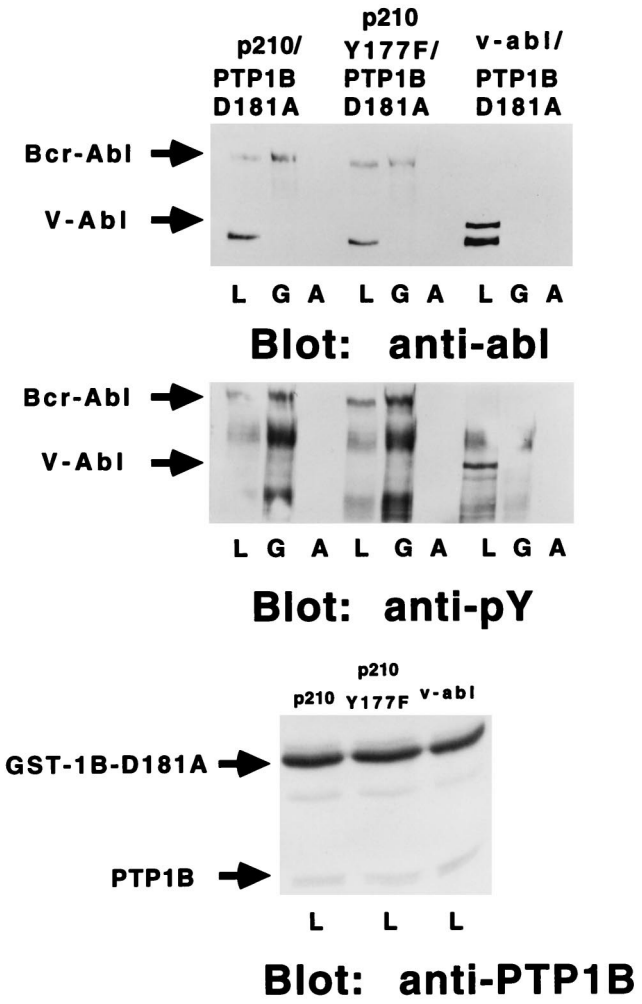

FIG. 7. Tyrosine-phosphorylated p210 bcr-abl, but not v-Abl, is selectively recognized by PTP1B-D181A in vivo. COS-1 cells were cotransfected with 10 μg of plasmid DNA pSRαMSVtkneop210, pSRαMSVtkneop210-Y177F, or pSRαMSVtkneov-abl together with 10 μg of plasmid pMT2 GST-PTP1B-D181A. Lanes: L, immunoblot of cell lysate (25 μg) following cotransfection; G, immunoblot of the glutathione-Sepharose precipitates from 250 μg of lysate; A, protein A precipitates from 250 μg of lysate. Samples were immunoblotted with anti-Abl, anti-pTyr (anti-pY), and anti-PTP1B antibodies and visualized by ECL.

To determine whether PTP1B-D181A forms a physical complex with p210 bcr-abl in intact cells, we examined whether the two proteins coprecipitated following transient cotransfection. We prepared lysates from COS cells coexpressing p210 bcr-abl and GST-PTP1B or GST–PTP1B-D181A and isolated the PTP fusion proteins on glutathione-Sepharose. We observed ~10% of p210 bcr-abl in association with PTP1B-D181A, whereas wild-type enzyme failed to precipitate p210 bcr-abl (Fig. 6).

PTP1B-D181A selectively binds tyrosine-phosphorylated p210 bcr-abl but not v-Abl in vivo. To characterize further the interaction between p210 bcr-abl and PTP1B-D181A, we again used a cotransfection assay in COS cells. GST–PTP1B-D181A was coexpressed with either p210 bcr-abl, v-Abl, or p210 bcr-abl-Y177F, a mutant in which the autophosphorylation site responsible for binding Grb2 was changed to Phe. The cells were lysed, and GST–PTP1B-D181A was precipitated by using glutathione-Sepharose. We observed that both p210 bcr-abl and p210 bcr-abl-Y177F were recovered in a stable complex with the trapping mutant form of PTP1B (Fig. 7). Specificity in the interaction between PTP1B and tyrosine-phosphorylated p210 bcr-abl is illustrated by the fact that when v-Abl and GST–PTP1B-D181A were coexpressed in the same system, no association between the PTK and the mutant PTP was observed, despite the fact that v-Abl was expressed abundantly and was phosphorylated on tyrosyl residues (Fig. 7). Unlike autophosphorylated p210 bcr-abl, endogenous c-Abl, which was not detectably tyrosine phosphorylated, was not recognized by the trapping mutant of PTP1B. The 190-kDa tyrosine-phosphorylated protein recognized by the mutant PTP is the epidermal growth factor receptor (EGF-R), as confirmed by immunoblotting with an anti-EGF-R antibody (15) (data not shown).

PTP1B dephosphorylates p210 bcr-abl in a cellular context. To determine whether p210 bcr-abl is a substrate of wild-type PTP1B in a cellular context, we used COS cells transiently expressing cDNA for p210 bcr-abl together with cDNA for either PTP1B or the 48-kDa form of TCPTP. Two days posttransfection, cells were lysed directly in Laemmli sample buffer, thus preventing any dephosphorylation postlysis in vitro. Immunoblotting the lysates with an anti-Abl monoclonal antibody revealed that p210 bcr-abl is expressed (Fig. 8A). PTP1B and TCPTP, which display comparable specific activities in assays in vitro (55, 62), were overexpressed in the p210 bcr-abl transfectants (Fig. 8C and D). When we compared phosphotyrosine levels in p210 bcr-abl, using an anti-pTyr monoclonal antibody, we found a significant reduction in the levels of phosphotyrosine in p210 bcr-abl when cotransfected with PTP1B, but the effects were much less pronounced with TCPTP (Fig. 8B). This result confirms that in a cellular context, p210 bcr-abl PTK is a better substrate for PTP1B than TCPTP. Interestingly, PTP1B efficiently dephosphorylated p210 bcr-abl as well as p210 bcr-abl-Y177F, but not v-Abl, even though v-Abl is tyrosine phosphorylated (Fig. 9). These results are consistent with the trapping mutant data, in which PTP1B-D181A associates with p210 bcr-abl and p210 bcr-abl-Y177F but not with v-Abl (Fig. 7).

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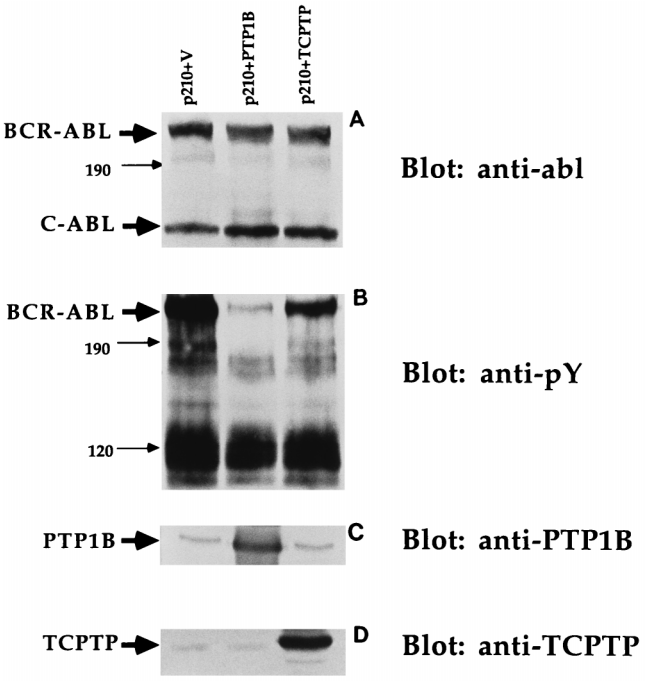

FIG. 8. Preferential dephosphorylation of p210 bcr-abl by PTP1B, compared to TCPTP, in a cellular context. COS-1 cells were transfected with 10 μg of pSRαp210 together with 15 μg of either pMT2 vector, pMT2-PTP1B, or pMT2-TCPTP. At 48 h posttransfection, 2×10^6 cells were washed once with PBS and then immediately lysed in Laemmli sample buffer and heated at 95°C for 5 min. Equal quantities of lysate protein were then separated by SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblotting with anti-Abl, anti-pTyr (anti-pY), anti-PTP1B, and anti-TCPTP antibodies and visualized by ECL. Molecular size standards (in kilodaltons) are indicated on the left.

Coexpression of p210 bcr-abl with either PTP1B or PTP1B-D181A disrupted the association of endogenous Grb2 with the PTK. These data strongly suggest that PTP1B recognizes p210 bcr-abl as a substrate. Therefore, we tested the effects of PTP1B on signalling events triggered by the PTK. Rat-1p210 cells grow in soft agar and form tumors in nude mice (32). Transformation of fibroblasts by p210 bcr-abl is dependent on phosphorylation of the tyrosine residue at position 177 (which is located in the consensus Grb2 binding site YXNX) within the bcr region. This residue becomes autophosphorylated, binds to the SH2 domain of the adapter protein Grb2, and links bcr-abl to the Ras signalling cascade. Mutation of tyrosine 177 to phenylalanine abolishes Grb2 binding and Ras activation and reduces the ability of p210 bcr-abl to transform Rat-1 fibroblasts (41). In addition, expression of Grb2 SH2 domain deletion mutants in p210 bcr-abl-transformed cells inhibits bcr-abl-induced activation of Ras and reverses the transformed phenotype (19). To address the effect of PTP1B on p210 bcr-abl signalling, we determined whether the interaction with the mutant PTP or dephosphorylation by wild-type enzyme affected the ability of the PTK to associate with endogenous Grb2. Using transient cotransfection in COS cells followed by immunoprecipitation from cell lysates with an anti-Abl antibody, we observed that in the presence of either PTP1B or PTP1B-D181A, the association of Grb2 and p210 bcr-abl was reduced by ~90% (Fig. 10A). Equal quantities of wild-type and mutant p210 bcr-abl were expressed in each of these assays (Fig. 10B).

PTP1B selectively antagonizes p210 bcr-abl-induced, Ras-dependent induction of expression of an AP-1/ets reporter

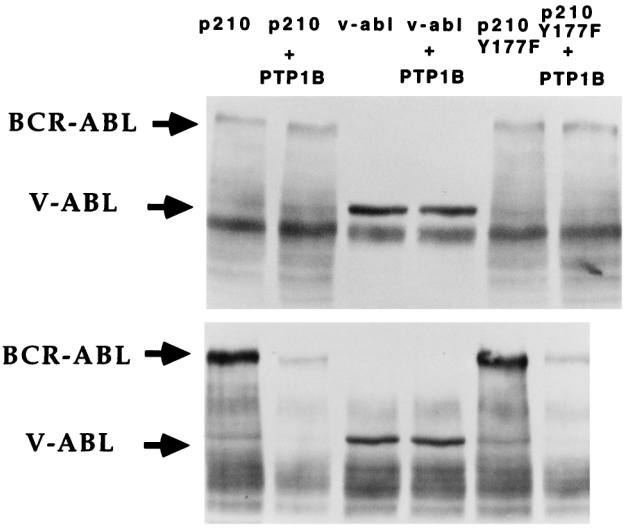

FIG. 9. PTP1B dephosphorylates tyrosine phosphorylated p210 bcr-abl but not v-Abl in vivo. COS-1 cells were cotransfected with 10 μg of plasmid DNA for pSRαMSVtkneop210, pSRαMSVtkneop210-Y177F, or pSRαMSVtkneov-abl together with 10 μg of plasmid pMT2 or pMT2PTP1B. At 48 h posttransfection, 2×10^6 cells were washed once with PBS and then immediately lysed in Laemmli sample buffer and heated at 95°C for 5 min. Equal quantities of lysate protein were then separated by SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblotting with an anti-pTyr antibody (bottom). The blot was stripped and reprobed with an anti-Abl antibody (top). Blots were visualized by ECL.

construct. A correlation exists between the ability of oncogenic PTKs to activate transcription from a Ras-responsive (AP-1/ets) element and their ability to transform cells (29, 41). To determine whether disruption of the Grb2–p210 bcr-abl interaction by PTP1B and PTP1B-D181A (Fig. 10A) inhibited the ability of p210 bcr-abl to activate a Ras-responsive element, we used a transcriptional activation assay. We utilized a CAT

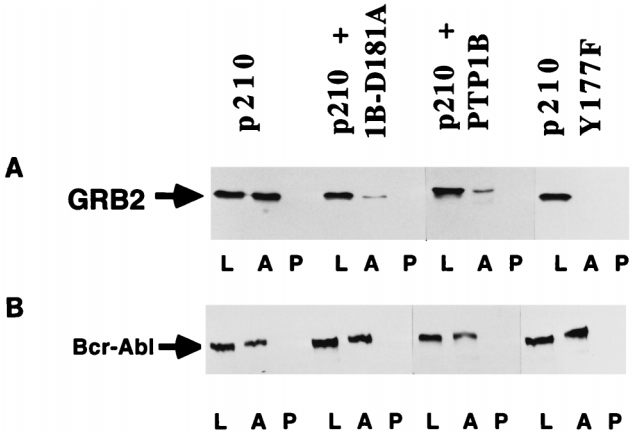

FIG. 10. Expression of PTP1B and PTP1B-D181A disrupts the association of endogenous Grb2 with p210 bcr-abl. COS-1 cells were cotransfected with 5 μg of plasmid DNA pSRαp210 or pSRαp210Y177F together with 15 μg of plasmid DNA pMT2, pMT2PTP1B, or pMT2PTP1B-D181A as indicated. Lanes L depict cell lysate (25 μg) following cotransfections. Cell lysates (250 μg) were prepared as described in the text, and p210 bcr-abl was immunoprecipitated with an anti-Abl antibody, Pex 5 (lanes A); a preimmune anti-serum (lanes P) was included as a control. Immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with an antibody to Grb2 (A). (B) Anti-Abl immunoblot of the immunoprecipitates from 50 μg of lysate, as a control to illustrate constant levels of p210 bcr-abl. Immunoblots were visualized by ECL.

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reporter gene construct under the control of a β-globin promoter containing four tandem Ras-responsive elements, pB4X-CAT (47, 58). We observed ~75% suppression of p210 bcr-abl-induced Ras activation in NIH 3T3 cells cotransfected with p210 bcr-abl and either wild-type or D181A mutant PTP1B compared to p210 bcr-abl alone (Fig. 11A). In the same assay, expression of PTP1B and PTP1B-D181A exerted minimal effect upon v-Abl-induced transcriptional activation. Although v-Abl has not been shown to bind directly to Grb2, it is known that v-Abl activates Ras through other mechanisms, most likely via phosphorylation of Shc and formation of Shc-Grb2 complexes (43, 52, 56, 64). Thus, although v-Abl utilizes the same signalling pathway downstream of Grb2, it is not inhibited by PTP1B. This important control illustrates that the effects of PTP1B are exerted upstream of Grb2, at the level of p210 bcr-abl. Interestingly, expression of neither the closest relative of PTP1B, TCPTP, nor wild-type PTP-PEST and the substrate-trapping mutant PTP-PEST-D199A exerted a significant effect on p210 bcr-abl-induced transactivation of the Ras-responsive element (Fig. 11A). The expression levels of the various proteins involved in the CAT assay are shown in Fig. 11B. These data illustrate selectivity in the effects of PTP1B on this p210 bcr-abl-induced signalling response.

DISCUSSION

One of the earliest events in the development of CML is the generation of the Ph chromosome, the translocation that results in production of the p210 bcr-abl fusion protein. The various aspects of the progression of CML suggest that the p210 bcr-abl PTK oncoprotein aberrantly regulates multiple signalling pathways involved in proliferation, differentiation, and apoptosis (7). Consequently, much research effort has focussed on characterization of the effects of p210 bcr-abl on cell signalling.

Expression of p210 bcr-abl leads to transformation of fibroblasts and hematopoietic cells and prevents apoptosis that is normally triggered by cytokine deprivation of factor-dependent cells (8). Various point mutant forms of p210 bcr-abl, including a mutant in the site of autophosphorylation that serves as a docking site for the adapter protein Grb2 (Y177F), a mutant in the SH2 domain that is defective in interaction with pTyr proteins (R552L), and a mutant in the major autophosphorylation site in the catalytic domain (Y793F), have been used to demonstrate that multiple signalling pathways emanate from p210 bcr-abl (8). Further sites of tyrosine phosphorylation have been identified in p210 bcr-abl. Y283 is phosphorylated in vitro, and Y360 is phosphorylated in vitro and in vivo; however, the implications for PTK function are unclear (30). Additional substrates have been identified, including the GTPase-activating protein (GAP)-associated p62 protein $p62^{dok}$ (5, 61), $p95^{vav}$, which contains SH2 and SH3 domains and may possess GDP-GTP exchange factor activity (33), and the proto-oncoprotein $p120^{cbl}$, which can serve as a docking protein (12, 44, 45). In addition to p210 bcr-abl, $p120^{cbl}$ coprecipitated the p85 subunit of phosphatidylinositol 3′-kinase, suggesting potential links to phospholipid-dependent signalling pathways (45), and certain focal adhesion proteins (44). There are data to suggest that members of the Src family of PTKs become activated in p210 bcr-abl-expressing cells (11), thus raising the possibility that additional PTKs contribute to the aberrant tyrosine phosphorylation patterns observed in CML. Clearly, the situation is complex and much remains to be discovered about the tyrosine phosphorylation-dependent signalling events that contribute to the CML phenotype.

Whereas much attention has focused on p210 bcr-abl and

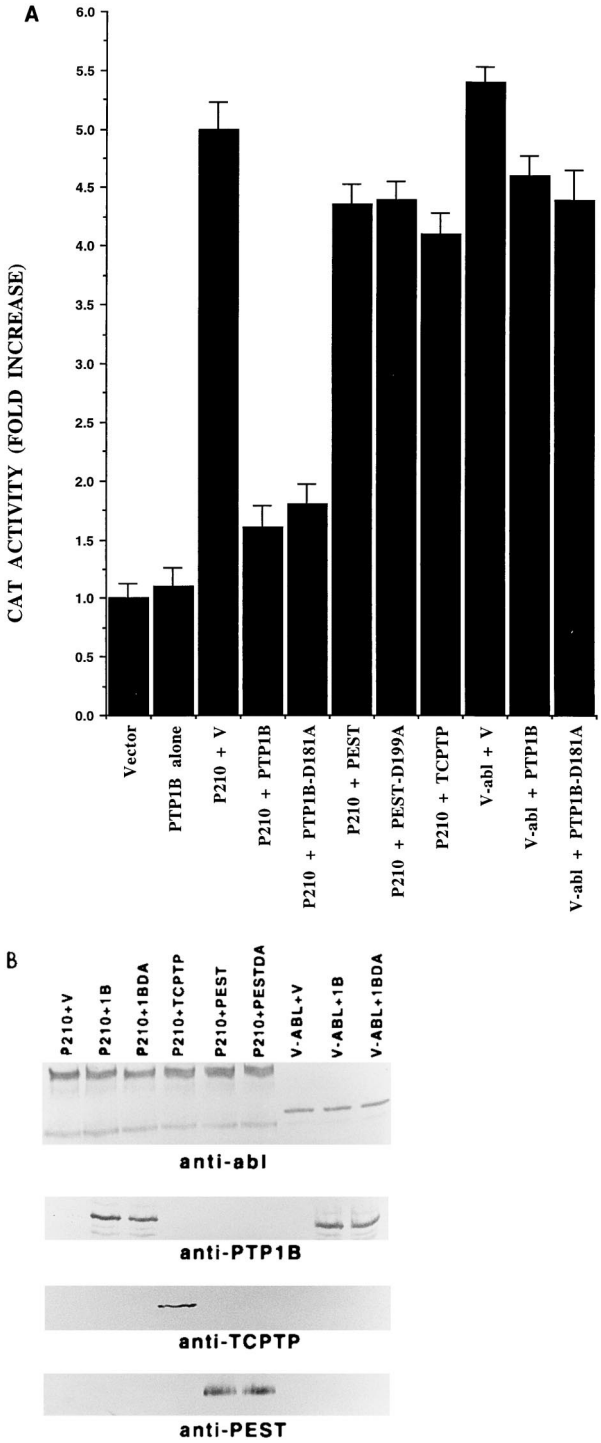

FIG. 11. PTP1B antagonizes a Ras-dependent activation of transcription by p210 bcr-abl but not v-Abl. (A) Transcriptional activation from a Ras-responsive (AP-1/ets) promoter was performed essentially as described previously (41). CAT activity was measured from NIH 3T3 cells transfected with plasmid pB4X-CAT together with either plasmid vector p210 bcr-abl or v-Abl expression plasmids, in the presence or absence of various PTP expression plasmids. Percentage conversion to acetylated forms was quantitated with a PhosphorImager. Data are means ± standard errors of four independent experiments; results from cells expressing the reporter gene alone (vector) were assigned a value of 1. The various PTPs alone did not alter basal CAT activity significantly. (B) Expression levels of the various proteins involved in the CAT assay. A 50-μg amount of total lysate was used to immunoblot for p210 bcr-abl and v-Abl. A 5-μg amount of total lysate was used to detect PTP1B, TCPTP, and PTP-PEST. Antibodies to the various proteins are described in the text.

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the role of aberrant tyrosine phosphorylation in CML, the PTPs represent an underutilized resource for the study of this oncoprotein PTK. It is clear that members of the PTP family have the potential to exert a considerable influence on p210 bcr-abl function, acting at the level of either the PTK itself or its downstream substrates. Our data indicate that a specific PTP, PTP1B, can antagonize the ability of p210 bcr-abl to signal in a cellular context.

PTP1B is the prototypical PTP. It comprises an N-terminal catalytic domain fused to a C-terminal, regulatory segment (4, 6, 21, 55, 56). The extreme C-terminal 35 residues comprise a hydrophobic segment that is both necessary and sufficient for targeting the enzyme to the cytoplasmic face of membranes of the endoplasmic reticulum (16), and as such, PTP1B will be exposed to a substantial array of cellular phosphotyrosyl proteins. However, information regarding the physiological function of PTP1B is limited at present. It has been shown to revert partially the phenotype of v-Src-transformed 3T3 cells when overexpressed up to 25-fold (60) and, again upon overexpression, to confer resistance to transformation by the Neu PTK (63) and to inhibit signalling in response to interleukin-3 (18). In addition, through the use of overexpression strategies and inhibitory antibodies, PTP1B has been implicated in negative regulation of signalling events triggered by the insulin receptor (1, 28, 37). Generally, these observations have relied on strategies involving overexpression of PTP1B, and thus it is unclear whether these results reflect a physiological function of the enzyme or whether there is specificity for PTP1B in these effects.

Our observation that the levels of PTP1B were enhanced rapidly and specifically as a consequence of expression of p210 bcr-abl suggests an important role for PTP1B in signalling events induced by p210 bcr-abl, potentially acting either positively in a permissive function or negatively as an antagonist of the PTK-induced signals. The increased expression of PTP1B was observed in a variety of model cell systems for CML as well as, more importantly, in Ph^+ cell lines derived from a patient with CML. Specificity is illustrated by the fact that although the levels of PTP1B were increased following expression of p210 bcr-abl, we did not detect an increase in the level of TCPTP, the closest relative of PTP1B. In addition, we did not detect changes in the expression of SHP-2, which has been shown to form a complex with p210 bcr-abl in cells overexpressing the PTK (51). Furthermore, specificity in the response was also evident from the perspective of the PTK, in that although PTP1B was upregulated in response to p210 bcr-abl, in a manner that was dependent on the catalytic activity of the PTK, there was no change in the level of the phosphatase in Rat-1 fibroblasts expressing v-Abl, which has the same catalytic domain as p210 bcr-abl. In addition, PTP1B levels were not altered in Rat-1 fibroblasts transformed by v-Myc, illustrating that the change in PTP1B levels was not a general response to cellular transformation.

Our study of PTP1B in the context of p210 bcr-abl-induced signalling was predicated upon the observation that the level of this PTP was increased specifically in response to expression of this PTK oncoprotein. This observation is consistent with a situation in which the cell may respond to expression of p210 bcr-abl by increasing the levels of a natural antagonist of the PTK in an attempt to maintain a normal status of tyrosine phosphorylation. This is reminiscent of compensatory changes to an initial stimulus that have been observed in other systems. For example, heterologous expression of the catalytic subunit of protein kinase A in NIH 3T3 cells results in a compensatory upregulation of expression of the endogenous regulatory subunit (58). Interestingly, this is also consistent with the observation that upregulation of mRNA for PTP1B, LAR, PTPH1, and PTPα was reported in response to heterologous overexpression of another PTK oncoprotein, Neu (59, 63). However, in this latter case the effects on PTP protein levels and the consequences for cellular signalling events have not been addressed.

In this study, we present data to illustrate that PTP1B can function in a cellular context as a specific inhibitor of p210 bcr-abl-induced signalling events. Ras has been implicated strongly in transformation by bcr-abl (46). It has been proposed that a site of autophosphorylation in the bcr portion of p210 bcr-abl (Y177) serves as a docking site for the adapter protein Grb2 and functions to assemble the multiprotein complex that triggers the Ras cascade (41, 42). In fact, dominant-negative mutant forms of Grb2 have been shown to suppress p210 bcr-abl-induced activation of Ras and to revert the transformed phenotype of K562 cells and Rat-1 cells expressing p210 bcr-abl, without inactivating its intrinsic PTK activity (19). For transformation of hematopoietic bone marrow cells or for tumor formation in vivo, alternative pathways to activation of Ras that involve another adapter protein, Shc, have also been proposed (20). Using the AP-1/ets reporter construct to measure the activation of the Ras cascade by p210 bcr-abl in a cellular context, we have shown that PTP1B was capable of antagonizing p210 bcr-abl-induced signalling. Specificity in the response is reflected in the fact that PTP1B did not inhibit transcriptional activation of the same reporter by v-Abl, suggesting that Y177 in p210 bcr-abl is one of the targets for the PTP. Furthermore, expression of TCPTP or PTP-PEST did not affect p210 bcr-abl-induced signalling, as measured by this transcriptional activation assay.

Signalling by p210 bcr-abl was also inhibited by a substrate-trapping mutant of PTP1B, suggesting that a critical substrate in the signalling pathway was being held in a complex with this mutant and being rendered nonfunctional. As observed for the active PTPs, this mutant of PTP1B did not affect induction of the reporter gene by v-Abl and an equivalent mutant of PTP-PEST did not inhibit signalling by p210 bcr-abl. This substrate-trapping mutant of PTP1B contains a substitution of alanine for the invariant aspartate (Asp181) that serves as a general acid in protonating the tyrosyl leaving group of the substrate. The catalytic activity of this mutant is severely impaired, but it maintains a high affinity for substrate and therefore can form complexes with its targets in the cell that are sufficiently stable to withstand isolation. It is known that the isolated catalytic domain of PTP1B is highly promiscuous in vitro, dephosphorylating a wide variety of phosphotyrosyl proteins. However, the application of substrate-trapping mutants has revealed that PTP1B displays an unexpected degree of substrate selectivity in a cellular context, recognizing primarily the EGF-R in COS cells (15). In these experiments, we have used the substrate-trapping mutant of PTP1B to investigate further elements of specificity in the action of this phosphatase, particularly with regard to its antagonism of p210 bcr-abl-induced signalling. Thus, upon coexpression in COS cells, PTP1B-D181A formed a stable complex with p210 bcr-abl but not v-Abl, despite the fact that the latter was expressed abundantly and was tyrosine phosphorylated. Furthermore, we have shown that the interaction between p210 bcr-abl and PTP1B-D181A blocked by >90% the association of the PTK with Grb2, suggesting that Y177 in p210 bcr-abl is one of the targets of PTP1B. Disruption of the interaction with Grb2 is the most likely mechanism of inhibition of p210 bcr-abl-induced activation of an AP-1/ets reporter by wild-type and substrate-trapping mutant forms of PTP1B. Therefore, the selectivity observed in the upregulation of PTP1B levels in response to expression of p210 bcr-abl is

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reflected in the selectivity of PTP1B in antagonizing p210 bcr-abl-induced signalling.

Although these data are consistent with a function of PTP1B as an antagonist of p210 bcr-abl signalling in vivo, it remains to be established whether the effects of the PTP are sufficient to abrogate the transforming potential of this oncoprotein PTK. In this respect, it is interesting that CML is characterized by a biphasic disease progression. The indolent chronic phase, where the only detectable abnormality is the Ph chromosome, is a semitransformed state that is difficult to distinguish from normal hematopoiesis and is manifested by an increase in mature myeloid cells. The disease then progresses to an aggressive phase termed blast crisis, where myeloid cells fail to differentiate. This phase is characterized by multiple chromosomal abnormalities resulting in a truly transformed state that is reminiscent of acute leukemia (7). Our data suggest the intriguing possibility that PTP1B may function to suppress some of the signalling effects of p210 bcr-abl in the chronic phase of the disease, and such suppression may be lost in the multiple secondary mutations that accompany blast crisis.

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