PTPROt Inactivates the Oncogenic Fusion Protein BCR/ABL and Suppresses Transformation of K562 Cells*

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Chronic myelogenous leukemia (CML),3 a myeloproliferative disorder, accounts for more than half the cases of such disorders. The disease is characterized largely by the acquired genetic abnormality referred as Philadelphia chromosome (1). Philadelphia chromosome is a short chromosome 22 arising from reciprocal translocations between the long arms of chromosomes 9 and 22. In this translocation the c-abl gene located on 9q is translocated to the breakpoint cluster region (BCR) on 22q. Half of the CML patients that are negative for Philadelphia chromosome test positive for BCR/ABL fusion (1). The change in conformation of the abl-tyrosine kinase due to fusion with bcr as well as the transphosphorylation of bcr/abl facilitated by bcr-mediated dimerization confers constitutive activity to this kinase (2–4). The bcr/abl fusion protein is implicated in the etiology of CML (5, 6) and has become an attractive molecular target for therapeutic intervention of CML and other Philadelphia positive leukemia such as acute lymphoblastic leukemia and rare cases of acute myelogenous leukemia.

Therapeutic approaches to target this chimeric protein have focused on small molecule kinase inhibitors that led to the discovery of imatinib mesylate (also known as Gleevec, STI571, and CFP 57148), a potent and relatively selective kinase inhibitor (7). This molecule competes with ATP and, therefore, deprives bcr/abl of the phosphate source required for its kinase activity (1, 8). Treatment with Gleevec reduces proliferation and increases apoptosis of bcr/abl+ cells (9, 10). There are, however, drawbacks associated with Gleevec that include persistence of bcr/abl+ cells (residual disease) requiring continuous exposure to Gleevec and resistance due to mutations in bcr/abl that prevent drug binding (9, 11–13). In addition to binding ATP, the transforming activity of bcr/abl requires phosphorylation of key tyrosine residues (14), which could be targeted to control its activity and transformation potential. In this context it would be important to identify the protein-tyrosine phosphatase(s) that could potentially dephosphorylate and inactivate the bcr/abl-associated kinase. Furthermore, it is critical to determine whether the levels of these tyrosine phosphatases are reduced in CML, which could explain the constitutive activity of bcr/abl. Subsequently, understanding the mechanism of their suppression in bcr/abl-positive leukemia will facilitate the development of novel therapeutic strategies to treat this disease.

To date only two tyrosine phosphatases, PTP1B and SHP1, are known to dephosphorylate and moderately inhibit the transformation potential of bcr/abl (15, 16). The expression of PTP1B is initially up-regulated in chronic phase of CML as a defense mechanism against the fusion protein. It is, however, presumed that the suppressive effect of PTP1B on bcr/abl is lost
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due to secondary mutations associated with blast crisis (16). Similarly, the expression of SHP1 is down-regulated in CML (17). Lack of knowledge of the mechanism of their inactivation prevents their application as potential therapeutic targets. Although an escort/phosphatase approach has been used to enhance the anti-transformation potential of SHP1 by increasing its affinity for bcr/abl (18), clinical application of this technique does not appear to be feasible. It is interesting that a Ser/Thr phosphatase PP2A whose activity is negatively regulated by bcr/abl-induced SET protein can also dephosphorylate bcr/abl by recruiting the tyrosine phosphatase SHP1 (19). Targeting bcr/abl by inhibiting SET to activate PP2A will also require concurrent expression of SHP1 for its tyrosine dephosphorylation function. It was, therefore, important to identify a bcr/abl-targeting tyrosine phosphatase that could be used as a clinical target for the existing drugs against CML or for the development of new clinically applicable strategies to treat CML.

Protein-tyrosine phosphatase receptor-type O (PTPRO) is a transmembrane phosphatase with a large extracellular domain of fibronectin repeats and one catalytic domain. We have previously identified this tyrosine phosphatase as a candidate tumor suppressor in a screen for genes hypermethylated in cancer (20–22). Subsequently, we demonstrated its tumor suppressor characteristics in lung cancer (23). The same gene encodes a truncated variant (PTPROt), a transmembrane protein lacking the large extracellular fibronectin domains (22, 24) which also exhibits tumor suppressor characteristics (25, 26). Here, we demonstrate that the truncated isoform that is specifically expressed in hematopoietic cells can dephosphorylate bcr/abl and inhibit its downstream signaling. Such inhibition is reflected in the reversal of transformed phenotype of bcr/abl+ K562 cells expressing the catalytically active PTPROt and their increased susceptibility to drug-induced apoptosis. Interestingly, the catalytically inactive mutant of PTPROt also exhibits some anti-transformation potential, probably by functioning as a trapping mutant. Furthermore, we show that PTPROt is suppressed by promoter methylation in these cells, and the DNA hypomethylating agent 5-azacytidine, used for treating myeloproliferative disorders, can relieve this suppression.

EXPERIMENTAL PROCEDURES

Reagents—The antibodies used in this study are as follows: α-FLAG-M2 (Sigma), α-Tyr(P) mixture (4G10 from Millipore, Tyr(P)20 and Tyr(P)99 from Santa Cruz Biotechnology), α-c-abl (Santa Cruz Biotechnology), α-p27 (Abcam), Pathscan bcr/abl activity assay mixture (Cell signaling Technology), α-c-fos (Santa Cruz Biotechnology).

Cell Culture and 5-Azacytidine Treatment—K562 cells purchased from ATCC were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum. Cells were treated with 7.5 μM 5-azacytidine (AzA) (Sigma) for 24 h (for RT-PCR) or 5 μM for 96 h (for bisulfite sequencing). 6.15 (32D-BCR/ABL) cells (27) were a generous gift from Dr. Danilo Perrotti (The Ohio State University). These cells were maintained in Iscove’s modified Dulbecco’s medium supplemented with 10% fetal bovine serum.

Generation of K562 Cell Line Stably Expressing PTPROt—The coding region of PTPROt amplified from PTPU2L wild type (WT) and catalytic site mutant (CS; a generous gift of Dr. Hirohiko Seimiya, (28)) using the primers PTPt-EcoRI (5′-ATGAAATTC- AATGGTACAGAGATGA-3′) and PTP-R-Bam (5′-CTGGAGATCCCTTGCTACATTCTGC-3′ (restriction sites underlined)) was cloned into the EcoRI/BamHI sites of p3XFLAG-CMV-14 (Sigma). Plasmid DNA of p3XFLAG-CMV-14 (Vector) or p3XFLAG-PTPROt (WT or CS) was transfected into K562 cells using Lipofectamine 2000 (Invitrogen). After 48 h cells were selected with 500 μg/ml G418 for 7 days. The expression of PTPROt was monitored by Western blot using anti-FLAG M2 antibody.

In Vitro Phosphatase Assay—Whole cell extract of K562 cells treated with 100 μM pervanadate for 30 min was prepared in lysis buffer B (20 mM Tris, pH 7.5, 100 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM iodoacetic acid, 1 mM sodium orthovanadate, and protease inhibitors). After incubation for 30 min on ice, dithiothreitol and EDTA were added to a final concentration of 10 and 1 mM, respectively, to inactivate the iodoacetic acid and vanadate followed by another 15 min of incubation on ice. The extract was then centrifuged at maximum speed to remove any cell debris. Phosphorylated bcr/abl was immunoprecipitated from this extract using anti-c-Abl antibody (Santa Cruz Biotechnology). After washing the immunoprecipitate with lysis buffer A (20 mM Tris, pH 7.5, 100 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM iodoacetic acid, 1 mM EDTA, and protease inhibitors), the protein A-agarose beads (with pulled-down bcr/abl) were equilibrated and suspended in assay buffer (9.375 mM HEPES, pH 7.4, 18.75 mM NaCl, 0.9375 mM EDTA, 1.875 mM dithiothreitol, and 125 μg/ml bovine serum albumin). The suspension was then divided into three equal parts to which purified GST proteins were added (GST alone as control or GST-PTPROt-WT or GST-PTPROt-CS). The assay mix was rocked for 30 min at 37 °C before separation of proteins on 8% SDS-PAGE. The proteins were transferred to nitrocellulose membrane and probed with anti-Tyr(P) mixture. The same blot was washed and re-probed with anti-c-abl antibody to normalize protein.

DNA Replication Assay—K562 cells (vector control or PTPROt expressing) were serum-starved (0% fetal bovine serum) for 18 h. Equal numbers of serum-starved cells were allowed to grow in complete media containing [3H]thymidine (5 μCi) for 2 h followed by precipitation of DNA with 10% trichloroacetic acid. [3H]Thymidine incorporated into the DNA was measured in a scintillation counter.

Cell Cycle Analysis—PTPROt-WT and CS-expressing and vector-transfected K562 cells were treated with nocodazole (1 μM) for 18 h to synchronize the cells at G2/M phase. After 18 h the cell cycle block was released, and the cells were allowed to grow in complete medium devoid of nocodazole. The cells harvested at the indicated time points were stained with propidium iodide and subjected to flow cytometric analysis.

Soft Agar Assay—This assay was performed as described (23).

Tumor Growth in Nude Mice—Equal numbers (0.5 × 106) of K562 cells (Vector or PTPROt-WT) were injected subcutaneously into anterior and posterior sites on the back of athymic nude mice. These mice were then followed for tumor growth.
The cells were left untreated or treated with 10,000 cells were seeded in each well of the 96-well plate. For growth kinetics, the cells were seeded in previously (26). For growth kinetics, the cells were seeded in 96-well plate with random hexamers and murine Moloney leukemia virus reverse transcriptase (Applied Biosystems) according to the GeneAmp RNA PCR kit (PerkinElmer Life Sciences) instructions. RT-PCR primers for PTPROt and details of the procedure are as described earlier (26). The absence of DNA contamination was confirmed by negative RT-PCR for 18S rRNA performed on RNA samples subjected to cDNA synthesis in the absence of reverse transcriptase. Expression of c-fos was measured by RT-PCR as well as real-time RT-PCR with SYBR Green chemistry using the primers human hFos-RT-F (5′-GGGCAAGGTTGAACAGTTATC-3′) and hFos-RT-R (5′-TTCAGCGTTGCAATCTCGGTTC-3′). Relative expression was calculated using the ΔCt method (32).

**RESULTS**

**Bcr/abl Is a Substrate of PTPROt in K562 Cells—**K562 is a well studied chronic myelogenous leukemia cell line characterized by Philadelphia chromosome. These cells express the constitutively active tyrosine-phosphorylated 210-kDa fusion protein bcr/abl, which is thought to confer growth advantage. Identification of the protein-tyrosine phosphatase that dephosphorylates bcr/abl should, therefore, provide an important molecular target for therapy of bcr/abl positive CML or other bcr/abl+ leukemia such as acute lymphocytic leukemia. To
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![Graphs and Images]

**FIGURE 2. Ectopic expression of PTPROt in K562 cells results in inhibition of cell growth and anchorage independence.** a, equal numbers of vector transfected and PTPROt (WT and CS) expressing K562 cells seeded in 35-mm dishes were monitored for growth by manual counting under microscope. Cell number was plotted against time. b, K562 cells (vector and PTPROt-WT and CS expressing) were serum-starved for 18 h followed by [3H]thymidine incorporation for 2 h in 10% serum-containing medium. The incorporated [3H]thymidine in the 10% trichloroacetic acid precipitate was measured in a scintillation counter. c, K562 cells (vector (V) and PTPROt-expressing) were synchronized at G2/M phase with 1 μM nocodazole. Cells harvested immediately after release and 14 h after release into complete growth medium were washed, fixed, and stained with propidium iodide. DNA content was measured using a BD-Caliber Flow cytometer. These data are representative of three independent experiments. d, equal number of vector-transfected and PTPROt (WT and CS)-expressing K562 cells were seeded in 0.35% agarose over a layer of 0.5% agarose in complete growth medium. Colonies formed after release and 14 h after release into complete growth medium were washed, fixed, and stained with crystal violet. The data are representative of three independent experiments performed in triplicate.

determine whether PTPROt dephosphorylates this fusion protein, we generated K562 cells ectopically expressing both wild type (WT) PTPROt and its CS mutant as FLAG-tagged fusion proteins. Ectopic expression of PTPROt was monitored by Western blot analysis with anti-FLAG M2 antibody (Fig. 1a). The phosphorylation status of bcr/abl was assessed by immunoprecipitation of bcr/abl from vector control as well as PTPROt (WT and CS)-expressing K562 cells followed by Western blotting with anti-phosphotyrosine antibody. The same blot was re-probed with anti-c-abl antibody to demonstrate comparable level of the protein in all lanes (Fig. 1d). The data demonstrate an ~50% reduction in phosphorylation of bcr/abl upon incubation with PTPROt-WT but not when incubated with PTPROt-CS or GST alone (Fig. 1e). This observation confirms that PTPROt can indeed dephosphorylate bcr/abl in the absence of any other cellular proteins.

Ectopic Expression of PTPROt Inhibits Growth and Clonogenic Survival of K562 Cells—To study whether dephosphorylation and potential inactivation of bcr/abl by PTPROt could reverse the transformation potential of K562 cells, we first studied its effect on cell growth by manual counting. There was a significant (\(p < 0.04\)) decrease in proliferation rate of the PTPROt-WT-expressing cells compared with the PTPROt-CS and vector-transfected cells (Fig. 2a). We also examined the effect of ectopic PTPROt on the replication potential of K562 cells by thymidine incorporation, which showed a 40% decrease in the PTPROt-WT-expressing cells relative to PTPROt-CS-expressing and vector-transfected cells (Fig. 2b). We next monitored the cell cycle profile of PTPROt-expressing cells to examine whether the delayed growth was due to cell cycle arrest. For this purpose, K562 cells were synchronized at G2/M phase with the microtubule-stabilizing agent nocodazole for 18 h. Cell cycle distribution was then analyzed using propidium iodide staining immediately after release (0 h) and after 14 h. As observed previously for DLBCL cells (24), immediately after release from nocodazole block a greater number of PTPROt-WT-expressing cells were in G0/G1 phase with the microtubule-stabilizing agent nocodazole for 18 h. Cell cycle distribution was then analyzed using propidium iodide staining immediately after release (0 h) and after 14 h. As observed previously for DLBCL cells (24), immediately after release from nocodazole block a greater number of PTPROt-WT-expressing cells were in G0/G1 phase compared with PTPROt-CS-expressing and vector-transfected cells (Fig. 2c, 0 h). Additionally, our data also showed that 14 h after release from cell cycle block a large population of PTPROt-WT cells were in G0/G1 phase with the microtubule-stabilizing agent nocodazole for 18 h. Cell cycle distribution was then analyzed using propidium iodide staining immediately after release (0 h) and after 14 h. As observed previously for DLBCL cells (24), immediately after release from nocodazole block a greater number of PTPROt-WT-expressing cells were in G0/G1 phase compared with PTPROt-CS-expressing and vector-transfected cells (Fig. 2c, 0 h). Thus, reduced replication potential and prolonged G0/G1 phase of K562 cells expressing PTPROt-WT explains the reduced growth rate of these cells.

CML is a disease of immature blast cell expansion within the bone marrow, which enter circulation and further proliferate. This behavior reflects in vitro anchorage-independent growth (33). We, therefore, investigated whether ectopic expression of...
PTPROt affects anchorage-independent growth of the K562 cells in soft agar. As expected, although vector-transfected K562 cells were able to form colonies in soft agar, K562 cells expressing PTPROT-WT were unable to survive and form colonies under the same conditions (Fig. 2d). Surprisingly, K562 cells expressing PTPROT-CS were also unable to form colonies in soft agar (Fig. 2d). These observations suggest that although inhibition of growth/proliferation by PTPROT requires phosphatase activity, reversal of anchorage independence could be independent of phosphatase activity or that PTPROT-WT and PTPROT-CS may be functioning differently to inhibit growth in soft agar. It was, therefore, imperative to study the mechanism of this differential regulation of growth and anchorage independence by PTPROT.

PTPROt-WT and PTPROT-CS Probably Regulate Growth and Anchorage Independence of K562 Cells by Different Mechanisms—Bcr/abl oncogene can activate several signaling pathways that include Ras, mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase/Akt, and JAK/STAT pathways (4). Although each of these pathways plays important roles in the transformation of K562 cells by bcr/abl (34), they appear to function either independently or in cooperation with another signaling pathway to regulate different aspects of transformation, e.g. proliferation, apoptosis, and anchorage independence. It is, therefore, possible that two independent pathways are involved in the regulation of cell cycle and anchorage-independent growth of K562 cells by bcr/abl, and each of these is distinctly regulated by PTPROT. To explore this possibility we analyzed the potential regulatory mechanisms for these phenotypes. It appears that the Ras/Raf pathway is predominantly involved in cell cycle control by bcr/abl. Activated Raf-1 can activate Cdc25, which in turn can activate cyclin E/cyclin-dependent kinase 2 that favors G1 \rightarrow S transition (35) (see Fig. 3a). The kinase activity of CDK2 is also required for phosphorylation and proteosomal degradation of p27, a cyclin-dependent kinase (CDK) inhibitor involved in negative regulation of cyclin E/CDK2 complex itself (36). We, therefore, anticipate that inactivation of Raf-1 by PTPROT-WT as a result of dephosphorylation of bcr/abl will result in an inactive cyclin E/cyclin-dependent kinase 2 complex thereby accounting for the accumulation of these cells in G0/G1 phase. Furthermore, the inactive cyclin E/cyclin-dependent kinase 2 unable to target p27 for proteosomal degradation will result in accumulation of p27 that can add to the delay in transition of the cells to S-phase. To test this hypothesis, we performed Western blot analysis of whole cell extracts from K562 cells with anti-p27 antibody. The data indeed demonstrated higher p27 levels in PTPROT-WT-expressing cells compared with vector-transfected and PTPROT-CS expressing K562 cells (Fig. 3b).

Unlike proliferation, anchorage-independent growth of K562 cells requires the activity of STAT5, suggesting involvement of the JAK/STAT pathway. Based on growth characteristics in soft agar, it was logical to expect that the JAK/STAT pathway would be inactivated in both PTPROT-WT- and PTPROT-CS-expressing cells compared with vector-transfected cells. To test this possibility, we performed Western blot analysis with an antibody mixture consisting of anti-phospho-bcr/abl, anti-phosphoStat5, anti-phospho-CrkL, and anti-eIF4E (as internal normalizing control) (Pathscan bcr/abl activity assay mixture). The results indeed demonstrated that although bcr/abl was hypophosphorylated only in PTPROT-WT cells, the phosphorylation of both downstream targets (CrkL and Stat5) was significantly reduced in PTPROT-WT as well as PTPROT-CS-expressing K562 cells (Fig. 3, c and d). To confirm that Stat5 was dephosphorylated and inactivated in both PTPROT-expressing cells, we examined the expression of its transcriptional target c-fos (37–39) by RT-PCR (Fig. 4a), real-time RT-PCR (Fig. 4b), and Western blot analysis (Fig. 4, c and d), which showed reduced c-fos in K562 cells expressing PTPROT-WT and PTPROT-CS. The important question now is why the downstream targets of bcr/abl are inactive despite phosphorylation of bcr/abl in K562 cells expressing PTPROT-CS. Studies on CrkL in bcr/abl + cells have demonstrated that bcr/abl activates the function of CrkL as an adaptor protein that in turn activates the transcriptional activity of...
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Stat5 (40). Furthermore, the first SH3 domain of Crkl can bind to the proline rich region at the C terminus of bcr/abl (41). We, therefore, propose that although PTPROt-WT inhibits CrkL activation by dephosphorylating bcr/abl, PTPROt-CS could possibly exhibit the same phenomenon by trapping the bcr/abl in a complex, thereby inhibiting its ability to interact and phosphorylate CrkL. Furthermore, the localization of active bcr/abl to f-actin filaments via its actin binding domain is necessary for the anchorage-independent growth of K562 cells (42). It is likely that trapping of bcr/abl by membrane-bound PTPROt-CS prevents its interaction with f-actin, thereby altering the ability of the cell to grow in soft agar.

PTPROt Expression Increases Susceptibility of K562 Cells to Apoptosis—Treatment of K562 cells with Gleevec, which inhibits bcr/abl function, induces apoptosis of K562 cells (43). It is noteworthy that although PTPROt also inhibits bcr/abl function, the population of PTPROt-overexpressing cells that have survived and grown through drug selection pressure will not demonstrate high level of apoptosis. To explore whether the expression of PTPROt would render K562 cells prone to apoptosis, we studied the effect of an apoptosis-inducing agent CPT on these cells because CPT can act in synergy with Gleevec (44). The cells were treated with CPT (10 μg/ml) for 18 and 36 h followed by annexin/propidium iodide staining. The results showed a gradual increase in percentage of apoptotic cells in all cells, i.e. vector-transfected and PTPROt-WT and CS-expressing cells with increasing time of treatment with CPT (Fig. 5a). The apoptotic population in untreated (0 h) PTPROt-WT-expressing cells was slightly higher (6.2%) than in untreated vector-transfected (3%) or PTPROt-CS-expressing (2.5%) cells. Furthermore, the increase in CPT-induced apoptosis was higher in PTPROt-WT (2.5- and 6.7-fold)-ex-

FIGURE 4. Reduced expression of c-fos in K562 cells expressing PTPROt: mRNA expression. DNase I-treated total RNA isolated from K562 cells (vector and PTPROt-WT and CS) was used for RT-PCR (a) and real time RT-PCR (b) with primers specific for c-fos and β-actin using SYBR Green chemistry. The data from three independent experiments calculated using comparative Ct method is presented. c, protein expression. Whole cell extracts of K562 cells were separated on SDS-PAGE and subjected to Western blot analysis with anti-c-fos and eIF4E antibodies (as protein loading control). d, quantification of c-fos protein expression. Scanned images from three independent experiments were quantified using Kodak Digital Science 1D. Signal intensity of c-fos normalized to that of eIF4E is represented as fold change relative to vector-transfected cells.

FIGURE 5. PTPROt expression facilitates CPT-mediated apoptosis and reduces tumorigenic potential of K562 cells. a, vector control and PTPROt-WT- and CS-expressing K562 cells were either left untreated (0 h) or exposed to 10 μg/ml CPT for 18 and 36 h. At the end of the treatment, cells were harvested and stained with annexin V/propidium iodide (BD Biosciences) for 15 min at room temperature according to the manufacturer’s protocol. The cells were analyzed in BD-Aria Flow Cytometer. The data, representative of three independent experiments, are plotted as the percent of apoptotic cells at each time point (a) or -fold increase in apoptosis after CPT treatment (b) compared with apoptosis without treatment (0 h). c, equal number of K562 cells (vector or PTPROt-WT) were subcutaneously injected in nude mice (n = 7) as described under "Experimental Procedures." The dimensions of visible tumors formed at week 2 were measured every 7 days until sacrifice at week 4. The rate of tumor growth was studied by plotting the volume of tumor against time after injection. d, ex vivo imaging of tumors formed by vector control (top) and PTPROt expressing (bottom) K562 cells harvested 28 days after injection. A summary of weight (gm) and volume (mm3) of each group of tumors is presented on the right.
Expression of PTPROt in K562 Cells Suppresses Tumor Growth in Nude Mice—Although the tumor suppressor property of PTPROt has been established using in vitro techniques, there have been no studies demonstrating the same under in vivo conditions. We, therefore, investigated the effect of PTPROt expression in K562 cells on tumor growth in vivo. For this purpose, vector control and PTPROt-expressing K562 cells were subcutaneously injected into the dorsal anterior and posterior sites, respectively, of immuno-compromised mice. In half the animals the site of injection was switched to avoid any position bias. Measurement of tumor volume each week showed that the tumors formed with PTPROt-expressing cells grew at a slower rate compared with the vector-transfected cells (Fig. 5c). At the end of 4 weeks of tumor growth the mice were sacrificed, and tumors were removed surgically to determine their weight and size. We observed visibly and measurably significant differences in tumor volume (p = 0.05) and weight (p = 0.04) between the two populations. The tumors expressing PTPROt were significantly smaller (~50%) than those that do not express the tyrosine phosphatase (Fig. 5d). This data demonstrate that PTPROt not only inhibits cell growth in culture but also acts as the tumor growth suppressor in vivo.

PTPROt Suppresses Growth and Enhances Apoptosis of Myeloid Cells Over-expressing bcr/abl—To test whether PTPROt functions similarly in another cell line expressing bcr/abl, we used 6.15 cells, a murine myeloid 32D cell line overexpressing bcr/abl (27). Retroviral infection was used to transiently express PTPROt (WT and CS) in these cells (Fig. 6a). We first determined whether PTPROt was able to dephosphorylate bcr/abl in these cells. As in the K562 cells, bcr/abl was immunoprecipitated from vector control and PTPROt (WT and CS)-expressing 6.15 cells. The immunoprecipitated protein was subjected to Western blot analysis with anti-phosphotyrosine antibody. The same blot was washed and probed with anti-c-abl antibody (vector and PTPROt) using anti-FLAG M2 antibody. Protein loading was normalized with anti-Ku-70 antibody. The phosphorylation signal normalized to signal corresponding to total bcr/abl is represented as % apoptosis between 0 and 7 h is used as a measure of the apoptotic cells.

The data demonstrate that phosphorylation of bcr/abl was indeed reduced by ~50% in the presence of PTPROt-WT (Fig. 6c). To determine the effect of PTPROt-mediated dephosphorylation of bcr/abl on cell growth, an MTT assay was performed on 6.15 cells (vector and PTPROt) were either left untreated or treated with 10 μg/ml CPT for 7 h followed by MTT assay as an indicator of cell metabolism. The percent loss of metabolism between 0 and 7 h was used as a measure of the apoptotic cells.

FIGURE 6. PTPROt suppresses growth and facilitates apoptosis in murine myeloid cell line expressing bcr/abl. a, ectopic expression of PTPROt in 6.15 cells. Western blot analysis of whole cell extract of 6.15 cells (vector and PTPROt) using anti-FLAG M2 antibody. Protein loading was normalized with anti-Ku-70 antibody. b, in vivo dephosphorylation of bcr/abl by PTPROt. Bcr/abl immunoprecipitated from 6.15 cells (vector and PTPROt expressing) using anti-c-abl antibody was separated by SDS-PAGE and used for Western blot analysis with anti-phosphotyrosine antibody. The phosphorylation signal normalized to signal corresponding to total bcr/abl is represented as % apoptosis between 0 and 7 h is used as a measure of the apoptotic cells.

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Expression of PTPROt in K562 Cells Suppresses Tumor Growth in Nude Mice—Although the tumor suppressor property of PTPROt has been established using in vitro techniques, there have been no studies demonstrating the same under in vivo conditions. We, therefore, investigated the effect of PTPROt expression in K562 cells on tumor growth ex vivo. For this purpose, vector control and PTPROt-expressing K562 cells were subcutaneously injected into the dorsal anterior and posterior sites, respectively, of immuno-compromised mice. In half the animals the site of injection was switched to avoid any position bias. Measurement of tumor volume each week showed that the tumors formed with PTPROt-expressing cells grew at a slower rate compared with the vector-transfected cells (Fig. 5c). At the end of 4 weeks of tumor growth the mice were sacrificed, and tumors were removed surgically to determine their weight and size. We observed visibly and measurably significant differences in tumor volume (p = 0.05) and weight (p = 0.04) between the two populations. The tumors expressing PTPROt were significantly smaller (~50%) than those that do not express the tyrosine phosphatase (Fig. 5d). This data demonstrate that PTPROt not only inhibits cell growth in culture but also acts as the tumor growth suppressor in vivo.

PTPROt Suppresses Growth and Enhances Apoptosis of Myeloid Cells Over-expressing bcr/abl—To test whether PTPROt functions similarly in another cell line expressing bcr/abl, we used 6.15 cells, a murine myeloid 32D cell line overexpressing bcr/abl (27). Retroviral infection was used to transiently express PTPROt (WT and CS) in these cells (Fig. 6a). We first determined whether PTPROt was able to dephosphorylate bcr/abl in these cells. As in the K562 cells, bcr/abl was immunoprecipitated from vector control and PTPROt (WT and CS)-expressing 6.15 cells. The immunoprecipitated protein was subjected to Western blot analysis with anti-phosphotyrosine antibody. The same blot was washed and probed with anti-c-abl antibody (vector and PTPROt) using anti-FLAG M2 antibody. Protein loading was normalized with anti-Ku-70 antibody. The phosphorylation signal normalized to signal corresponding to total bcr/abl is represented as % apoptosis between 0 and 7 h is used as a measure of the apoptotic cells.

The data demonstrate that phosphorylation of bcr/abl was indeed reduced by ~50% in the presence of PTPROt-WT (Fig. 6c). To determine the effect of PTPROt-mediated dephosphorylation of bcr/abl on cell growth, an MTT assay was performed on 6.15 cells (vector and PTPROt) were either left untreated or treated with 10 μg/ml CPT for 7 h followed by MTT assay as an indicator of cell metabolism. The percent loss of metabolism between 0 and 7 h was used as a measure of the apoptotic cells.

FIGURE 6. PTPROt suppresses growth and facilitates apoptosis in murine myeloid cell line expressing bcr/abl. a, ectopic expression of PTPROt in 6.15 cells. Western blot analysis of whole cell extract of 6.15 cells (vector and PTPROt) using anti-FLAG M2 antibody. Protein loading was normalized with anti-Ku-70 antibody. b, in vivo dephosphorylation of bcr/abl by PTPROt. Bcr/abl immunoprecipitated from 6.15 cells (vector and PTPROt expressing) using anti-c-abl antibody was separated by SDS-PAGE and used for Western blot analysis with anti-phosphotyrosine antibody. The phosphorylation signal normalized to signal corresponding to total bcr/abl is represented as % apoptosis between 0 and 7 h is used as a measure of the apoptotic cells.
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Hypomethylating Agent—To determine whether K562 cells express endogenous PTPROt, we performed RT-PCR with primers specific for PTPROt and 18S rRNA. The data showed that PTPROt was silenced in these cells (Fig. 7a, lane 2). Our earlier studies have shown that the PTPRO CGI is tumor-specifically methylated in hepatocellular carcinoma (21), primary human lung tumors (23), and chronic lymphocytic leukemia (26). To investigate whether suppression in K562 is also due to CGI methylation, we initially treated K562 cells with the DNA hypomethylating agent AzaC, which resulted in re-expression of PTPROt (Fig. 7a, lane 3). To confirm that the re-expression of PTPROt was indeed due to hypomethylation of the CGI, we performed bisulfite genomic sequencing on control and AzaC-treated cells. The data revealed dense methylation of the CGI in control cells, which was significantly (p = 0.002) reduced after AzaC treatment (Fig. 7b).

Although suppression of PTPROt in K562 cells was overcome by AzaC treatment with concurrent hypomethylation of the CGI, it was important to rule out the possibility that PTPROt silencing was due to non-availability of transcription factors in the control cells. To address this issue, a region encompassing −1049 to +261 with respect to transcription start site of the PTPROt isoform was cloned upstream of the luciferase reporter gene in pGL3-basic vector. The promoter reporter construct (PTP-P-luc) was transfected into K562 cells along with the internal control pRL-TK. The data clearly demonstrate that PTPROt promoter is active in K562 cells (Fig. 7c), suggesting that K562 cells express transcription factors for PTPROt promoter, and the lack of expression of endogenous have been successful in most cases, this approach has frequently led to drug resistance upon prolonged exposure. Although the activities of these kinases often rely on the phosphorylation state of Ser/Thr or Tyr residues that are effectively regulated by specific protein phosphatases, the strategy of targeting the function of an oncogenic kinase by altering its phosphorylation state has not been widely explored. The present study has made an effort to address this issue.

Previous study in our laboratory has established the growth suppressive and pro-apoptotic potential of PTPRO in lung cancer (23). The same gene encodes a truncated isoform (PTPROt) that is specifically expressed in hematopoietic cells and also exhibits growth inhibitory characteristics (25, 26). The present study focused on exploring the potential of using this tyrosine phosphatase as a physiological antagonist for targeting oncogenic signal transduction pathways. Among all types of cancers, chronic myelogenous leukemia is one of the very few cancers characterized by a single signature oncogene bcr/abl, which plays a central role in its etiology. Because of the importance of the constitutive kinase activity of this fusion protein for all aspects of cellular transformation affecting proliferation, anchorage dependence, and apoptosis, bcr/abl has been the preferred target for CML therapy. To date the most successful therapy for CML has been the use of the small molecule inhibitor Gleevec that blocks binding of ATP, a source of phosphate group, to bcr/abl. The present study has demonstrated that PTPROt can inactivate bcr/abl by dephosphorylating one of its tyrosine residues (Tyr-1127), phosphorylation of which is required for complete activation and the resulting transforming.
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function of bcr/abl (4, 47). Furthermore, we showed that dephosphorylation of bcr/abl by PTPROt-WT and probably its trapping by PTPROt-CS inactivate the key signaling events downstream of bcr/abl. It is noteworthy that the expression of catalytically active PTPROt and its phosphatase-deprived mutant could reverse the transformed phenotype of K562 cells.

Analysis of the pathways downstream of bcr/abl revealed that the Ras/Raf pathway involved in cell cycle regulation was inhibited only in cells expressing PTPROt-WT. On the contrary, the JAK/STAT pathway that plays a critical role in anchorage-independent growth and inhibition of apoptosis was suppressed in both PTPROt-WT and –CS-expressing cells. These alterations explain the differential behavior of PTPROt-CS in regulating transformation. Based on the present findings, we propose that PTPROt interacts with bcr/abl at its C-terminal c-abl region to dephosphorylate Tyr-1127. Interestingly, the proline-rich region of bcr/abl required for CrkL recruitment, and Stat5 activation is located in close proximity to Tyr-1127 toward the C-terminal of the fusion protein. This region may, therefore, be masked by PTPROt-CS, preventing Stat5 activation (Fig. 8). On the contrary, Tyr-177 and the SH2 domain responsible for recruiting Grb2 and SHC adaptor proteins, respectively, and for the activation of Ras/Raf pathway are located toward the N-terminal region of bcr/abl. These are, therefore, probably not blocked by the interaction between bcr/abl and PTPROt-CS. It is noteworthy that the actin binding domain required for anchorage-independent growth is also present at the C-terminal end that may be unavailable in PTPROt-CS-expressing cells.

Although studies with other phosphatases have mainly focused on studying transformation phenotype by measuring growth and clonogenic survival of fibroblasts (15, 48), we have shown here that PTPROt can suppress growth, clonogenic survival, tumorigenic potential, and enhance drug-induced apoptosis of the CML cell line K562. It would be of interest to determine whether PTPROt is widely methylated in primary CML samples and whether its methylation correlates with specific stage of the disease. Such an extensive analysis using large cohort of primary human CML samples is not within the scope of the present study that focuses on the functional and mechanistic aspects of PTPROt in chronic myelogenous leukemia cells. Furthermore, our earlier observations of tumor-specific methylation of PTPROt in solid and liquid tumors suggest the possibility that PTPROt will be methylated at least in acute and blast crisis CML. The present study demonstrating re-activation of PTPROt by AzaC and the anti-transformation potential of PTPROt in bcr/abl+ cells underscores the clinical significance of PTPROt as a therapeutic target/marker for epigenetic therapy.

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FIGURE 8. Proposed model for the trapping function of PTPROt-CS. The predicted interaction between the c-abl region of bcr/abl and PTPROt is depicted. Only the key domains of bcr/abl and downstream signaling elements relevant to this study are shown. ABD, actin binding domain; PP, proline-rich region; Y-K, tyrosine kinase domain harboring Tyr-1294; S/T-K, Ser/Thr kinase domain harboring Tyr-177; Y-P, mutated tyrosine phosphatase domain.
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