PTEN, mutated in a variety of human cancers, is a dual specificity protein phosphatase and also possesses D3-phosphoinositide phosphatase activity on phosphatidylinositol 3,4,5-tris-phosphate (PIP₃), a product of phosphatidylinositol 3-kinase. This PIP₂ phosphatase activity of PTEN contributes to its tumor suppressor function by inhibition of Akt kinase, a direct target of PI₃. We have recently shown that Akt regulates PDGF-induced DNA synthesis in mesangial cells. In this study, we demonstrate that expression of PTEN in mesangial cells inhibits PDGF-induced Akt activation leading to reduction in PDGF-induced DNA synthesis. As a potential mechanism, we show that PTEN inhibits PDGF-induced protein tyrosine phosphorylation with concomitant dephosphorylation and inactivation of tyrosine phosphorylated and activated PDGF receptor. Recombinant dephosphorylated PDGF receptor in vitro. Expression of phosphatase deficient mutant of PTEN does not dephosphorylate PDGF-induced tyrosine phosphorylated PDGF receptor. Rather its expression increases tyrosine phosphorylation of PDGF receptor. Furthermore, expression of PTEN attenuated PDGF-induced signal transduction including phosphatidylinositol 3-kinase and Erk1/2 MAPK activities. Our data provide the first evidence that PTEN is physically associated with platelet-derived growth factor (PDGF) receptor and that PDGF causes its dissociation from the receptor. Finally, we show that both the C2 and tail domains of PTEN contribute to binding to the PDGF receptor. These data demonstrate a novel aspect of PTEN function where it acts as an effector for the PDGF receptor function and negatively regulates PDGF receptor activation.

The tumor suppressor protein, PTEN (phosphatase and tensin homolog deleted on chromosome ten) is frequently inactivated in advanced cancer including endometrial carcinoma, sporadic glioblastoma, melanoma, meningioma, and breast, prostate, renal, and small cell lung cancer (1–3). Germ line mutations are often recognized in familial cancer predisposition syndromes, such as Cowden disease, Lhermitte-Duclos disease, and Bannayan-Zonana syndrome (2, 3). Disruption of the mouse PTEN gene demonstrates strong correlation between loss of PTEN function and tumorigenesis (4–7). Homozygous mutation of the PTEN locus is embryonically lethal in mice in all genetic backgrounds (4–8). PTEN heterozygous mice are viable but susceptible to different types of cancer; atypical endometrial hyperplasia occurs in females with 100% penetrance (6, 7).

Structurally, PTEN resembles the dual specificity phosphatases, which dephosphorylate serine, threonine, and tyrosine residues in protein (1). However, extensive studies revealed that PTEN is a poor phosphatase for proteins (9–11). Crystal structure of PTEN showed that the active site pocket in the phosphatase domain is very deep and wide unlike the catalytic sites of tyrosine and dual specificity phosphatases that indispensably recognize protein substrates (12). Another feature of the PTEN catalytic pocket is that it is highly basic, indicating that it may accommodate highly acidic substrates. This observation led to the discovery of phosphatidylinositol 3,4,5-trisphosphate (PIP₃), the product of PI 3-kinase, as the primary physiologic substrate of PTEN (13, 14). This observation is also supported by the presence of an increased level of PIP₃ in the embryonic fibroblasts isolated from PTEN null mice (7, 8). Restoration of wild type PTEN in mutated tumor cells has established that the lipid phosphatase activity of the protein is sufficient to repress the tumor cell growth (14). This ability of PTEN to antagonize PI 3-kinase signaling by downregulating its immediate target, Akt serine threonine kinase, is consistent in different cells, although the downstream consequences of this lipid phosphatase activity vary widely and include cell cycle arrest and apoptosis (15–17).

Whereas tumor suppressor activity of PTEN is elicited by its lipid phosphatase activity, its detectable protein phosphatase activity toward multiply phosphorylated proteins suggested that PTEN may also act on protein substrates in vitro (9). In fact, PTEN can dephosphorylate focal adhesion kinase and adaptor protein Shc in vitro. When overexpressed in cells, as a result of integrin ligation, PTEN dephosphorylates both of these signaling proteins (18–20).

Platelet-derived growth factor (PDGF) plays an important role in many proliferative disorders. It stimulates proliferation
of a variety of cells, including fibroblast, smooth muscle, glial, and glomerular mesangial cells (21–24). Four different polypeptide subunits (A, B, C, and D) have been identified, which can give rise to five different isoforms of PDGF (24). Most extensively studied PDGF is formed by dimerization of the B-chain to form BB homodimer or AB heterodimer. Although these dimers bind both PDGF receptors, the affinity for PDGF receptor β (PDGFR) is higher (23–25). Mice with a targeted homozygous mutation of PDGF B-chain or PDGFR exhibit a common phenotype of lacking glomerular mesangial cells (26, 27). Indeed, PDGF is required for the proliferation, survival, and development of mesangial cells (25–27). Expression of both PDGF B-chain and PDGFR is increased in mesangioproliferative glomerulonephritis in which proliferation of mesangial cells is the major pathologic feature (28). Introduction of antibody against PDGF B-chain ameliorates this disease (29). These results suggest that PDGFR signal transduction plays a key role in the development and survival of these cells during embryogenesis as well as contributes to their proliferation in glomerular disease.

Binding of PDGF to its cognate receptor induces a conformational change relieving an inhibitory effect of the juxtamembrane domain on the intrinsic tyrosine kinase activity (22, 25, 30). Activated PDGFR then undergoes autophosphorylation to create binding sites for signaling proteins containing Src homology 2 domains (22, 23). One of the Src homology 2 domain-containing proteins, PI 3-kinase, physically binds to the specific phosphotyrosines of the PDGFR, resulting in its activation to produce PIP3. This D3-phosphoinositide then binds to the pleckstrin homology domain of Akt (also known as PKB) serine threonine kinase to induce translocation to the plasma membrane, where it is phosphorylated and activated by PDK1 and PDK2 kinases (31, 32). We and others have shown that PI 3-kinase/Akt signal transduction is necessary for PDGFR-induced proliferation of various cells including mesangial cells (33–36).

The lipid phosphatase activity of PTEN has been extensively studied in the context of attenuating the tumor cell growth and apoptosis (37–41). However, its role in normal cell function is largely unknown. PTEN dephosphorylates highly negatively charged artificial substrate in vitro (9). Upon binding to PDGF, PDGFR is autophosphorylated in at least 16 tyrosine residues, thus becoming a negatively charged protein. We hypothesized that tyrosine-phosphorylated PDGFR may be a substrate for PTEN and that PTEN may regulate PDGF-induced biological activity by inhibiting the receptor phosphorylation at the membrane.

We report here that expression of PTEN in mesangial cells significantly abolished PDGF-induced Akt activation, resulting in inhibition of PDGFR-induced DNA synthesis without inducing apoptosis. This action of PTEN was due to inhibition of PDGFr-induced tyrosine phosphorylation of proteins. PTEN blocked tyrosine phosphorylation of PDGFR in vitro, resulting in inhibition of its tyrosine kinase activity. PTEN also dephosphorylated PDGFR in vitro. Furthermore, we provide evidence that PTEN is associated with PDGFR, and the addition of PDGF causes dissociation of this phosphatase from the receptor. We conclude that PTEN is a PDGFR phosphatase and may act to negatively regulate the receptor tyrosine kinase activity.

MATERIALS AND METHODS

Plasmids and Antibodies—Adenovirus vectors encoding PTEN (Ad PTEN) and phosphatase-dead PTEN CS (Ad PTEN CS) were provided by Dr. Ramon Parsons (Department of Pathology and Medicine, Columbia University, New York) and Dr. Christopher D. Kontos (Department of Medicine, Duke University Medical Center, Durham, NC), respectively. pGEX2T-PTEN expressing wild type PTEN and pSG5 HA PTEN were kind gifts from Dr. William R. Sellers (Dana-Farber Cancer Institute, Boston, MA). Human PDGFR was subcloned into pGEX for making [32P]methionine-labeled receptor protein using TNT transcription coupled translation system (Promega). The coding sequences for catalytic plus C2 domains (amin acids 1–353), C2 plus tail domains (amin acids 177–403) and tail domain (amin acids 354–403) were amplified from wild type PTEN template (pSG5 HA PTEN) by PCR and cloned in frame with glutathione S-transferase in pGEXTag bacterial expression vector (42). The sequence identity of the DNA fragments was confirmed by sequencing both strands. The recombinant proteins were purified by GSH-Sepharose. Cat-C2 (catalytic plus C2 domains) and tail domains were cloned into a 3× FLAG CMV expression vector. This vector and FLAG antibody were purchased from Sigma. PTEN, pGEX HA PTEN, pGEX MK, and pGEX HA MK were grown in DEEM with high glucose supplemented with 10% fetal bovine serum. C6 glioma cells were grown in DMEM with 1% glucose-containing 10% fetal bovine serum. All cells were kept at 37 °C in a humidified atmosphere of 5% CO2. Mesangial cells were made quiescent by serum deprivation for 48 h, whereas the NIH 3T3 and C6 cells were serum-deprived for 24 h. Cells were infected in PBS with Ad PTEN or Ad PTEN CS at a multiplicity of infection of 50 for 1 h at room temperature followed by the addition of serum-free medium. Experiments were carried out at 24 h postinfection (36, 44).

Transfection—HepG2 and mesangial cells were transfected with indicated plasmid constructs using LipofectAMINE Plus reagent as described (36, 43, 44).

Immunoprecipitation and Immunoblotting—Cells were lysed in radiolabeled precure buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40) at 4 °C for 45 min. Lysates were cleared of cell debris by centrifugation, and protein was estimated as described before (43, 44). Equal amounts of cell lysates were immunoblotted or immunoprecipitated with specific antibodies (36, 43, 44). For PDGFR immunocomplex assay, the immunoprecipitates were incubated with 20 μCi of [γ-32P]ATP at 30 °C for 15 min, and the phosphorylated receptor was analyzed by 7.5% SDS-gel electrophoresis followed by alkali treatment as described (43, 45). The PI 3-kinase assay was performed in PDGFR immunoprecipitates using PI as substrate in the presence of [γ-32P]ATP essentially as described (44, 48).

DNA Synthesis—[3H]Thymidine incorporation into trichloroacetic acid insoluble material was determined as a measure of DNA synthesis as described before (36, 43).

Annexin V Binding and Hoechst Staining for Examining Apoptotic Cells—Mesangial cells were cultured in 6-well plates and infected with Ad GFP or Ad PTEN for 24 h. Apoptosis was measured using an apoptosis detection kit, which utilizes Annexin V-FITC and propidium iodide fluorescence, as described by the vendor. The cells were analyzed by flow cytometry with the excitation wavelength at 488 nm. The FITC signal was detected at 518 nm. The propidium iodide fluorescence was measured at 620 nm. For Hoechst staining, cells in chamber slides were infected with a multiplicity of infection of 50 Ad PTEN or Ad GFP for 24 h at room temperature followed by fixation with 4% paraformaldehyde and stained with Hoechst 33258 essentially as described (47).

Protein-Protein Interaction—Protein association was analyzed by incubating [32P]methionine-labeled PTEN with PDGFR immunoprecipitates. After incubation, the beads were washed three times with radioimmunoprecipitation buffer, twice with 0.5 M LiCl plus 0.1 M Tris-HCl, pH 7.5, and once with PBS before analyzing by SDS-gel electrophoresis and autoradiography. In some experiments, [32P]methionine-labeled PDGFR was incubated with full-length PTEN or domains of PTEN fused to GST immobilized on GSH-Sepharose beads. After incubation, the beads were washed as described above and analyzed by gel electrophoresis and autoradiography.

Fluorescence Microscopy—Confocal cells in chamber slides were serum starved for 48 h, washed with PBS, fixed, and double-stained with rabbit anti-PDGFR and Cy3-conjugated donkey anti-rabbit antibodies and with anti-PTEN monoclonal antibody and FITC-tagged donkey anti-mouse secondary antibodies. PDGFR and PTEN
were visualized with a confocal laser microscopy system (Olympus Fluoview 500). The confocal images were analyzed by FluoView software to determine the colocalization of PDGFR and PTEN.

**PTEN Lipid Phosphatase Assay**—Radiolabeled PI 3-32P was prepared using a PI 3-kinase assay of PDGFR immunoprecipitates from PDGF-stimulated mesangial cells. Equal amounts of radioactive PI 3-32P were incubated with PTEN immunoprecipitates. The reaction products were extracted with chloroform/methanol/12 N HCl (50:100:1) followed by methanol/1 N HCl (1:1). The organic layer was separated by TLC as described (44, 46).

**RESULTS**

**PTEN Inhibits PDGF-induced DNA Synthesis in the Absence of Apoptosis**—Depending on the cell type, PTEN induces either cell cycle arrest or apoptosis of tumor cells by directly down-regulating the level of PI3, resulting in inhibition of the Akt kinase activity (37–40). However, its role in normal cells is poorly understood. We have recently shown that expression of dominant negative Akt in mesangial cells isolated from Sprague-Dawley rats blocked PDGF-induced DNA synthesis (36). To investigate the role of Akt in more detail, we used an adenovirus vector encoding PTEN. Infection of mesangial cells with Ad PTEN showed sustained expression of PTEN in these cells (Fig. 1A). In order to examine the functional consequences of PTEN expression, we examined its effect on PDGF-induced Akt activation using a phospho-Akt-specific antibody, which recognizes the activated form of Akt only. Expression of PTEN significantly blocked PDGF-induced activation of Akt, consequently preventing the DNA synthesis induced by PDGF (Fig. 1B and C, respectively). One mechanism by which PTEN induces cell cycle arrest in many tumor cells is by increasing the expression of p27kip1 cyclin kinase inhibitor (38, 48). Expression of PTEN significantly blocked PDGF-induced DNA synthesis in mesangial cells (Fig. 1D). To investigate the mechanism of its expression, mesangial cells were transfected with a reporter plasmid, in which the firefly luciferase cDNA is driven by p27kip1 promoter (36). Also, a Renilla luciferase plasmid was included in the transfection mix as an internal control. Dual luciferase activity was determined in the lysate. Data are expressed as the ratio of firefly and Renilla luciferase activity.
cell cycle arrest followed by apoptosis (39, 41). To assess this mode of action of PTEN in mesangial cells, plasma membrane expression of phosphatidylserine as a measure of early changes in apoptotic cells was analyzed. FITC-tagged Annexin V binding to mesangial cells after infection with Ad PTEN for 24 h was used to detect early phase of apoptosis. Additionally, propidium iodide was used to distinguish between viable and nonviable cells. Flow cytometric analysis showed nearly 90% Ad GFP or Ad PTEN-infected cells did not bind Annexin V or propidium iodide (Fig. 2, A and B). These data indicate that expression of PTEN did not induce apoptosis of these cells. To examine the late changes in apoptosis, mesangial cells were stained with Hoechst 33258, which only stains DNA in condensed chromatin and allows distinction between apoptotic cells and viable cells with normal chromatin structure. Expression of PTEN for 48 h did not show any apoptosis of mesangial cells (Fig. 2C). In contrast, incubation of mesangial cells with H2O2, which was used as a positive control for inducing apoptosis, resulted in the formation of condensed chromatin, indicative of apoptosis (Fig. 2C, dark blue spots indicated by the arrowhead). These data indicate that expression of PTEN inhibits DNA synthesis in the absence of apoptosis in mesangial cells.

PTEN Inhibits PDGF-induced Tyrosine Phosphorylation of Proteins—PTEN-mediated inhibition of PDGF-induced DNA synthesis can be explained as a consequence of the lipid phosphatase activity of PTEN, which dephosphorylates PIP3, resulting in inhibition of Akt activity. A corollary of this observation is that inhibition of PDGF-induced DNA synthesis by expression of PTEN might be elicited by inhibition of PDGF tyrosine kinase activity due to PTEN-mediated dephosphorylation of the receptor. This hypothesis is also supported by the observation that PTEN can dephosphorylate highly negatively charged peptide substrate (9). To examine this possibility, mesangial cells were infected with Ad-PTEN followed by incubation with PDGF. The lysates were immunoblotted with anti-phosphotyrosine antibody. PDGF increased protein tyrosine phosphorylation as expected (Fig. 3A). Expression of PTEN significantly blocked tyrosine phosphorylation of all proteins induced by PDGF (Fig. 3A, compare lane 4 with lane 2). To examine whether it is a cell-specific phenomenon, Ad PTEN-infected NIH 3T3 fibroblasts were stimulated with PDGF. Similar to the mesangial cells, expression of PTEN significantly reduced PDGF-induced tyrosine phosphorylation in these cells (Fig. 3B, compare lane 4 with lane 2).

PTEN was originally identified in glioblastoma (1). In many gliomas, PDGFR-mediated signal transduction is constitutively activated (49). Blocking PDGFR tyrosine kinase activity using small molecule inhibitor in many glioma cells reduced focus formation in soft agar (49). Furthermore, transduction of dominant negative PDGFR in C6 glioma cells significantly abrogated the tumorigenicity of the cells, indicating a dominant role of PDGFR-induced signaling in tumorigenesis (50). Therefore, to examine the effect of PTEN, we used C6 glioma cells. Antiphosphotyrosine immunoblot showed constitutive tyrosine phosphorylation of proteins (Fig. 3C, lane 1). The addition of PDGF to these cells increased tyrosine phosphorylation (Fig. 3C, compare lane 2 with lane 1). However, expression of PTEN significantly inhibited basal as well as PDGF-induced tyrosine phosphorylation of proteins (Fig. 3C, compare lanes 3 and 4 with lanes 1 and 2, respectively). Taken together, we conclude that PTEN may block PDGF-induced signal transduction by
inhibiting protein tyrosine phosphorylation.

PTEN Is a Target of PTEN—Since PDGF undergoes tyrosine phosphorylation to initiate signal transduction, we examined whether it is a target of PTEN. Lysates of PDGF-stimulated mesangial cells were immunoprecipitated with PDGFR antibody followed by immunoblot analysis with anti-phosphotyrosine antibody. PDGF increased tyrosine phosphorylation of PDGFR (Fig. 4A, compare lane 2 with lane 1). Expression of PTEN significantly inhibited PDGF-induced tyrosine phosphorylation of PDGFR (Fig. 4A, compare lane 4 with lane 2). Similar to all known receptor tyrosine kinases, binding of PDGF to the cognate receptor increases the intrinsic tyrosine kinase activity as determined by in vitro autophosphorylation of PDGFR immunoprecipitated from lysates of cells incubated with PDGF (Fig. 4B, compare lane 2 with lane 1). Expression of PTEN abolished the PDGF-induced tyrosine kinase activity of PDGFR (Fig. 4B, compare lane 4 with lane 2). These data provide the first evidence that PTEN blocks PDGFR-induced tyrosine kinase activity possibly by dephosphorylation of PDGFR in vitro. To directly examine this possibility, 32P-labeled tyrosine-phosphorylated PDGFR was incubated with bacterially produced GST-PTEN in vitro. Recombinant PTEN partially dephosphorylated tyrosine-phosphorylated PDGFR (Fig. 4C, compare lane 2 with lane 1). Similar results were obtained when PTEN produced by in vitro translation was used in a dephosphorylation reaction (data not shown). To confirm this observation, we immunoprecipitated PTEN from lysates of mesangial cells and incubated with tyrosine-phosphorylated PDGFR. Immunopurified PTEN dephosphorylated PDGFR significantly (Fig. 4D, compare lane 2 with lane 1). On the basis of these data, we conclude that PDGFR is a substrate for PTEN.

To examine the specificity of PTEN, we used the phosphatase-dead dominant negative mutant of this protein (PTEN C/S) (51). Lysates of mesangial cells infected with Ad PTEN C/S were immunoblotted with anti-phosphotyrosine antibody. Expression of PTEN C/S increased tyrosine phosphorylation of the 185-kDa protein (Fig. 5A). To confirm that this increased tyrosine phosphorylation may involve PDGFR, lysates of mesangial cells infected with Ad PTEN C/S were immunoprecipitated with PDGFR antibody followed by anti-phosphotyrosine immunoblotting. Expression of PTEN C/S increased tyrosine phosphorylation of PDGFR (Fig. 5B, compare lane 3 with lane 1). In contrast to the effect of wild type PTEN on dephosphorylation of PDGFR (Fig. 4A), phosphatase-dead PTEN did not inhibit PDGF-induced PDGFR tyrosine phosphorylation (Fig. 5B, compare lane 4 with lane 2). These data along with the data presented in Fig. 4 conclusively demonstrate that PTEN protein phosphatase activity targets PDGFR in mesangial cells.

PTEN Attenuates PDGFR-induced Signal Transduction—Increase in PDGFR tyrosine kinase activity upon ligand binding is the most proximal event in PDGFR-induced signal transduction. Since PTEN inhibits the tyrosine kinase activity of the PDGFR, it was expected that any downstream signaling pathway should be attenuated by expression of PTEN. Therefore, the Erk1/2 type of MAPK activation was assessed. As expected, PDGF increased abundance of phospho-Erk1/2 as determined by immunoblot analysis of mesangial cell lysate with an antibody which specifically recognizes the activated form of MAPK (Fig. 6A, compare lane 2 with lane 1). Expression of PTEN significantly inhibited PDGF-induced MAPK activation (Fig. 6A, compare lane 4 with lane 2). Similar results were obtained in NIH 3T3 fibroblasts (Fig. 6B). These data demonstrate that PTEN-dependent dephosphorylation and inactivation of PDGFR shown above results in inhibition of PDGFR-induced MAPK activity. PI 3-kinase activation is also initiated by PDGFR, which depends upon the direct interaction of PI 3-kinase with the tyrosine-phosphorylated PDGFR (33, 35). Therefore, change in PDGFR-associated PI 3-kinase activity would reflect a direct measure of tyrosine-phosphorylated PDGFR-dependent signaling. PDGFR was immunoprecipitated from lysates of PDGF-stimulated mesangial cells followed by immunocomplex kinase assay using PI as substrate. As expected, PDGF increased PDGFR-associated PI 3-kinase activity (Fig. 6C, compare lane 2 with lane 1). Expression of PTEN significantly attenuated PDGF-induced activation of PI 3-kinase (Fig. 6C, compare lane 4 with lane 2). These data along with the results described above indicate that PTEN inactivates PDGFR directly and suggest a novel mechanism by which PTEN negatively regulates PDGFR-mediated downstream mitogenic signaling.

PTEN Is Associated with PDGFR—We established that PDGFR is a direct substrate for PTEN in vivo as well as in vitro (Figs. 4 and 5). To examine whether PTEN interacts with PDGFR, PTEN immunoprecipitates from lysates of mesangial cells were analyzed by anti-PDGFR immunoblotting. Significant amounts of PDGFR were detected in the PTEN immunoprecipitates in quiescent cells (Fig. 7A, lane 1). Surprisingly, significantly less PDGFR was associated with PTEN when immunoprecipitated from cells incubated with PDGF (Fig. 7A, compare lane 2 with lane 1). Similarly, appreciable association of PTEN was detected in PDGFR immunoprecipitates in the absence of PDGF (Fig. 7B, lane 1), which was drastically reduced by incubation with PDGF (Fig. 7B, compare lane 2 with lane 1). Identical results were obtained when PDGFR immunoprecipitates from C6 glioma cells were analyzed by antiprotein phosphatase activity of the PTEN immunoprecipitation (Fig. 7C, compare lane 2 with lane 1).
Double immunostaining of PDGFR and PTEN in mesangial cells revealed colocalization of these proteins, confirming association of PTEN with PDGFR in vivo (Fig. 7D, panel c, arrowheads). These results indicate that PTEN and PDGFR form a signaling complex in the cell.

To confirm the interaction of PTEN with PDGFR, [35S]methionine-labeled PTEN protein was synthesized from PTEN cDNA as a template using in vitro transcription-coupled translation (Fig. 8A). Incubation of this labeled protein with PDGFR immunoprecipitates showed specific association of PTEN with the receptor (Fig. 8B, lane 2). These data provide evidence that PTEN interacts with PDGFR. To address whether PTEN interacts directly with PDGFR, [35S]methionine-labeled PDGFR was synthesized (Fig. 8C) and incubated with GST fusion protein containing full-length PTEN (Fig. 8E). Analysis of this complex showed binding of PTEN with PDGFR and thereby...
demonstrates a direct interaction between these two proteins (Fig. 8F). PTEN contains three distinct regions, an N-terminal phosphatase catalytic domain (residues 7–185), a lipid binding C2 domain (residues 186–351), and a tail domain (residues 354–403). To identify the specific domain that interacts with PDGFR, GST fusion proteins with N-terminal truncated PTEN containing C2 plus tail domain and tail domain only were generated (Fig. 8, D and E). Incubation of these fusion proteins with [35S]methionine-labeled PDGFR showed that both domains interact with the receptor (Fig. 8, E). Incubation of these fusion proteins with [35S]methionine-labeled PDGFR showed interaction between these proteins (Fig. 8J, lane 2). Taken together, these data provide the first evidence that PTEN physically interacts with PDGFR and that both the C2 domain and the tail domain of PTEN contribute to this binding.

C-terminally Truncated PTEN Regulates PDGFR Activation—We demonstrated that PDGFR is dephosphorylated in vivo and in vitro in the presence of PTEN (Fig. 4). Also, our data showed that PTEN-Cat-C2 containing the phosphatase domain and the tail domain is sufficient to associate with PDGFR. To examine the activity of this PTEN mutant, we transfected FLAG-tagged Cat-C2 into HepG2 cells, which do not express detectable PDGFR (33). Lysates of transiently transfected cells showed expression of Cat-C2 mutant (Fig. 9A). To test the effect of Cat-C2 on PDGF-induced tyrosine phosphorylation, HepG2 cells were transfected with PDGFR and Cat-C2. Lysates of transiently transfected cells incubated with PDGF were immunoblotted with anti-PDGF antibody. As expected, PDGF increased tyrosine phosphorylation of proteins in these cells (Fig. 9B, compare lane 2 with lane 1).
Cat-C2 significantly inhibited PDGF-induced tyrosine phosphorylation (Fig. 9B, compare lane 4 with lane 2). To examine the effect of Cat-C2 on PDGF activation, lysates of HepG2 cells transiently transfected with PDGFR and Cat-C2 were immunoprecipitated with PDGFR antibody followed by an immune complex kinase assay. Expression of Cat-C2 blocked PDGF-induced PDGFR tyrosine kinase activity (Fig. 9C, compare lane 4 with lane 2). These data indicate that the PTEN catalytic domain along with C2 is sufficient to inactivate the PDGFR tyrosine kinase activity in the absence of tail domain.

Since PTEN tail domain also associates with PDGFR in vitro (Fig. 8H), we examined the effect of this domain on PDGFR activation. FLAG-tagged PTEN tail was expressed in HepG2 cells (Fig. 10A). To test the effect of PTEN tail in vivo, lysates of transiently transfected HepG2 cells with PDGFR and PTEN-tail were immunoprecipitated with PDGFR antibody followed by anti-phosphotyrosine immunoblotting. Expression of PTEN-tail resulted in increased PDGFR tyrosine phosphorylation (Fig. 10B, compare lane 2 with lane 1). Furthermore, expression of PTEN-tail increased PDGFR tyrosine kinase activity (Fig. 10C, compare lane 2 with lane 1). These data indicate that PTEN-tail acts as a dominant negative PTEN, which blocks the negative regulatory effect of endogenous PTEN on PDGFR. However, expression of PTEN-tail did not have any effect on the PTEN lipid phosphatase activity (Fig. 10D, compare lanes 1 and 3 with lane 2).
Fig. 9. Expression of Cat-C2 regulates tyrosine phosphorylation induced by PDGF. A, upper panel represents the structure of Cat-C2 fused with FLAG epitope. The middle panel shows expression of Cat-C2 using anti-FLAG antibody. The bottom panel shows tubulin in the same lysates as loading control. B, expression of Cat-C2 inhibits PDGF-induced tyrosine phosphorylation. HepG2 cells were transfected with PDGFR along with vector or Cat-C2. Transiently transfected cells were incubated with PDGF. Lysates were immunoblotted with anti-phosphotyrosine antibody. The middle and bottom panels show Cat-C2 and PDGFR expression, respectively. C, effect of Cat-C2 on PDGFR tyrosine kinase activity. HepG2 cells were transfected with PDGFR along with vector only or Cat-C2 constructs. Lysates were immunoprecipitated with PDGFR antibody followed by immunocomplex kinase assay in the presence of [γ-32P]ATP. Note that along with PDGFR autophosphorylation (indicated by the arrows), associated proteins with PDGFR in the presence of PDGF were also phosphorylated in vitro (lane 2). The middle and bottom panels show Cat-C2 and PDGFR expression, respectively.

Fig. 10. PTEN tail regulates tyrosine phosphorylation. A, upper panel represents a the structure of PTEN tail domain fused with the FLAG epitope. The middle and bottom panels show expression of PTEN tail and actin, respectively. B, expression of PTEN tail increases tyrosine phosphorylation of PDGFR. HepG2 cells were transfected with PDGFR along with vector or PTEN tail. Lysates of transiently transfected cells were immunoprecipitated with PDGFR followed by immunoblotting with anti-phosphotyrosine antibody. The middle and bottom panels show expression of PDGFR and PTEN tail in these cells. C, expression of PTEN tail increases tyrosine kinase activity of PDGFR. HepG2 cells were transfected with PDGFR and vector or PTEN-tail expression construct. Lysates were immunoprecipitated with PDGFR antibody followed by immune complex kinase assay in the presence of [γ-32P]ATP. Note that along with PDGFR autophosphorylation (indicated by the arrows), associated proteins with PDGFR in the presence of PTEN tail were also phosphorylated in vitro (lane 2). The middle and bottom panels show PTEN tail and PDGFR expression, respectively. D, expression of PTEN tail does not have any effect on lipid phosphatase activity of PTEN. HepG2 cells were transfected with PDGFR and vector alone or PTEN-tail expression construct. Cell lysates were immunoprecipitated with PTEN antibody. The immunoprecipitates were assayed for PTEN activity in the presence of 32P-labeled phosphatidylinositol 3-phosphate as described under "Materials and Methods." The reaction products were separated by TLC. Lane 2 represents vector-transfected, and both lanes 1 and 3 represent PTEN-tail-transfected samples.
**DISCUSSION**

We conclude that PTEN inactivates PDGFR by directly dephosphorylating the activated tyrosine-phosphorylated receptor. This dephosphorylation of activated PDGFR inhibits PDGF-induced mitogenic signaling. Furthermore, we provide the first evidence that PDGFR and PTEN are associated and PDGFR causes dissociation of PTEN from the receptor. These data provide an alternative target of PTEN, the PDGFR, other than PIP₃, to regulate Akt activity and mitogenic signaling. PTEN null mouse embryo fibroblasts display increased PIP₃ level and are resistant to apoptotic stimuli (8). Similarly, tumor cells with mutation in the PTEN allele show resistance to apoptosis, and restoration of wild type PTEN with intact phosphatase activity induces increased sensitivity to apoptosis (14, 39, 40). Apart from regulating proliferation and apoptosis of cells, the normal function of PTEN has been shown to regulate the cell size in different organ. In Drosophila, it regulates cell size during eye development (52–54). In mice, deletion of PTEN in neuronal cells causes an increase in the size of neuronal stem cells (55). Glia-specific deletion of PTEN displayed increased soma size, resembling Lhermitte-Duclos syndrome, a rare autosomal dominant disorder in which germ line PTEN mutation is observed (56, 57). Interestingly, increased levels of activated Akt were detected without any effect on cell proliferation and apoptosis in these mice (57). Furthermore, all mice died within 29 weeks, but glioblastomas were excluded as the cause for death, although a high level of activated Akt was detected in glial cells (57). These data indicate that the tumor suppression effect of PTEN that was observed in glioblastoma cell lines may include targets other than only PIP₃, to regulate Akt activity. In fact, increased Ras activation has been observed in many glioblastomas (58). However, neither expression of constitutively active Akt nor Ras in neuronal precursor cells was sufficient alone to induce glioblastoma formation in mice. Coexpression of both, however, induced high grade gliomas (59). Ras is one of the major downstream targets of PDGFR (22, 25). Our data provide the first evidence that PDGFR is a substrate for PTEN in different cell backgrounds including in a glioma cell line. We demonstrate that PTEN-mediated dephosphorylation of PDGFR results in inhibition of signal transduction including MAPK, the direct downstream target of Ras, and PI 3-kinase. In addition, we demonstrated that PTEN inhibited PDGF-induced Akt activity similar to the observation in many tumor cells, where Akt is targeted by the lipid phosphatase activity of PTEN, which directly acts on PIP₃, the product of PI 3-kinase. Therefore, PTEN, by directly targeting PDGFR and PIP₃, may have an impact on both Ras and Akt. Furthermore, our finding, that expression of PTEN inhibited EGF-induced tyrosine phosphorylation of proteins (Supplementary Fig. S1A, compare lane 4 with lane 2), suggests that this phosphatase can target other receptor tyrosine kinases along with PDGFR. In fact, expression of PTEN significantly inhibited EGF-induced epidermal growth factor receptor tyrosine phosphorylation (Supplementary Fig. S1B). Therefore, the direct regulation of PTEN on tyrosine phosphorylation produced by different activated growth factor receptors and oncogenes may provide a synergistic inhibitory effect on cell proliferation.

Tyrosine-specific protein phosphatase PTP1B has been shown to target PI 3-kinase in response to activation of tyrosine kinase insulin receptor by dephosphorylating directly the tyrosine-phosphorylated receptor (60). This observation is similar to the present study, where we demonstrate that PTEN targets tyrosine-phosphorylated PDGFR. PDGFR has been shown to be a substrate of PTP1B (61, 62), thus providing an alternative mechanism for how PDGFR-mediated signals may be attenuated. However, expression of PTEN in the presence or absence of PDGF did not have any effect on PTP1B expression (Supplementary Fig. S2A). Also, PTEN did not change the activity of PTP1B (64) (Supplementary Fig. S2B). Thus, the effect of PTEN on PDGFR dephosphorylation and signaling is not via PTP1B and may represent a direct effect on the PDGFR.

Binding of PDGF to the extracellular domain of receptor tyrosine kinase relieves an inhibitory effect of the juxtamembrane domain, allowing the receptor to dimerize for autophosphorylation and activation (30). We show that PTEN is associated with PDGFR via its C2 domain and C-terminal tail domain, similar to certain other signaling proteins (63). PDGFR causes dissociation of the lipid phosphatase from the receptor. Expression of dominant negative mutant, PTEN/C5, increased tyrosine phosphorylation of proteins as well as PDGFR. Thus, our data demonstrate that PTEN may negatively regulate the PDGFR in the absence of the ligand, thus keeping the receptor in its nonphosphorylated state. However, in the presence of PDGF, the conformational change of the receptor causes dissociation of PTEN from the receptor, resulting in tyrosine phosphorylation, signal transduction, and normal cell function (Fig. 11). When PTEN is overexpressed, it is constitutively associated with the receptor (data not shown), leading to inhibition of tyrosine phosphorylation and inactivation of signal transduction. Together, our data represent a potential mechanism for how the tumor suppressor PTEN directly regulates PDGF-induced signal transduction. Thus, PTEN may play an important role in proliferative disorders, such as glomerulonephritis and atherosclerosis, where PDGF-induced proliferation of a specific cell type is the major cause of the disease.

**Acknowledgments**—We thank Sergio Garcia for technical assistance and Drs. Dan Riley, Brent Wagner, and B. S. Kasinath for critically reading the manuscript.

**REFERENCES**

1. Li, J., Yen, C., Liaw, D., Podeypanina, K., Bose, S., Wang, S. I., Puc, J., Millianesias, C., Rodgers, L., McCombie, R., Bigner, S. H., Giovannella, B. C., Ittmann, M., Tycko, B., Hilsenroth, H., Wigler, M. H. & Parsons, R. (1997) *Science* **275**, 1943–1947.

2. Cantley, L. C. & Neel, B. G. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 4240–4245.

3. Besson, A., Robbins, S. M. & Yong, V. W. (1999) *Eur. J. Biochem.* **263**, 655–661.

4. Di Cristofano, A., Pesce, B., Cardon-Cordo, C. & Pandolfi, P. P. (1998) *Nature Genet.* **19**, 349–355.

5. Suzuki, A., de la Pompa, J. L., Stambolic, V., Elia, A. J., Sasaki, T., del Braco Barrantes, I., Ho, A., Wakeham, A., Itie, A., Khoo, W., Fukumoto, M. & Mak, T. W. (1998) *Curr. Biol.* **8**, 1169–1178.

6. Podeypanina, K., Elleson, L. H., Nemes, A., Gu, J., Tamura, M., Yamada, K. M., Cardon-Cardo, C., Catrrett, G., Fisher, P. E. & Parsons, R. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 1563–1568.

7. Stambolic, V., Tano, M. S., Marcherson, D., Suzuki, A., Chapman, W. B. & Mak, T. W. (2000) *Cancer Res.* **60**, 3605–3611.
