Src Family Protein-tyrosine Kinases Alter the Function of PTEN to Regulate Phosphatidylinositol 3-Kinase/AKT Cascades*

Received for publication, April 8, 2003, and in revised form, July 4, 2003
Published, JBC Papers in Press, July 17, 2003, DOI 10.1074/jbc.M303621200

Yiling Lu‡, Qinghua Yu‡, Jue Hui Liu‡, Jinyi Zhang‡, Hongwei Wang‡, Dimpy Koul¶, John S. McMurray‡, Xianjun Fang‡, W.K. Alfred Yung¶, Kathy A. Siminovitch**,**, and Gordon B. Mills‡‡

From the ‡Department of Molecular Therapeutics, Division of Cancer Medicine, M. D. Anderson Cancer Center, Houston, Texas 77030, the §Mount Sinai Research Institute, Department of Medicine and Immunology, University of Toronto, Toronto, Ontario M5G 1X5, Canada, and the ¶Department of Neuro-oncology, Division of Cancer Medicine, M. D. Anderson Cancer Center, Houston, Texas 77030

Src family protein-tyrosine kinases, which play an important role in signal integration, have been implicated in tumorigenesis in multiple lineages, including breast cancer. We demonstrate, herein, that Src kinases regulate the phosphatidylinositol 3-kinase (PI3K) signaling cascade via altering the function of the PTEN tumor suppressor. Overexpression of activated Src protein-tyrosine kinases in PTEN-deficient breast cancer cells does not alter AKT phosphorylation, an indicator of signal transduction through the PI3K pathway. However, in the presence of functional PTEN, Src reverses the activity of PTEN, resulting in an increase in AKT phosphorylation. Activated Src reduces the ability of PTEN to dephosphorylate phosphatidylinositol 3-phosphate in micelles and promotes AKT translocation to cellular plasma membranes but does not alter PTEN activity toward water-soluble phosphatidylinositols. Thus, Src may alter the capacity of the PTEN C2 domain to bind cellular membranes rather than directly interfering with PTEN enzymatic activity. Tyrosine phosphorylation of PTEN is increased in breast cancer cells treated with pervanadate, suggesting that PTEN contains sites for tyrosine phosphorylation. Src kinase inhibitors markedly decreased pervanadate-mediated tyrosine phosphorylation of PTEN. Further, expression of activated Src results in marked tyrosine phosphorylation of PTEN. SHP-1, a SH2 domain-containing protein-tyrosine phosphatase, selectively binds and dephosphorylates PTEN in Src transfected cells. Both Src inhibitors and SHP-1 overexpression reverse Src-induced loss of PTEN function. Coexpression of PTEN with activated Src reduces the stability of PTEN. Taken together, the data indicate that activated Src inhibits PTEN function leading to alterations in signaling through the PI3K/AKT pathway.

Nonreceptor Src family protein-tyrosine kinases transduce signals controlling a variety of cellular processes including proliferation, differentiation, motility, and adhesion (1–3). Src kinases are activated following engagement of many different classes of cellular receptors and participate as a convergence point in different signaling pathways (1, 3, 4). In this regard, Src is a critical component of the signaling cascades initiated by tyrosine kinase-linked receptors, such as the epidermal growth factor receptor, G protein-coupled receptors including the lysophosphatidic acid receptors and steroid receptors including the estrogen receptor (1, 5–8). Src has been found to be overexpressed or highly activated in a number of human neoplasms, including ovary, breast, lung, colon, esophagus, skin, parotid, cervix, and gastric carcinomas, as well as neuroblastomas and myeloproliferative disorders (9–14). An activated mutation of Src may be present in a number of neoplasms, contributing to their development or progression (15, 16). Transgenic mice expressing the polyomavirus middle T oncogene in the mammary epithelium develop multifocal mammary tumors that metastasize with high frequency because of an ability to associate with and activate Src family kinases, phosphatidylinositol 3-kinase (PI3K)† and the Shc adapter protein (17–20). Mice expressing polyomavirus middle T antigen in the absence of functional c-Src rarely develop mammary tumors, indicating that c-Src tyrosine kinase activity is required for polyomavirus middle T antigen-induced mammary tumorigenesis (21). Indeed, the c-Src protein level and activity are increased 30–40-fold in human breast cancers compared with normal breast tissue (14). The activation of c-Src activity in mammary tumors has been linked to its association and interaction with the HER2/Neu epidermal growth factor receptor family member (22, 23). Activation of Src kinase is an indicator of poor clinical prognosis in colorectal carcinomas, implicating Src in tumor progression (12). Together, the Src protein-tyrosine kinase appears to be an important mediator of tumor initiation and progression.

Src family protein-tyrosine kinases consist of nine members including Src, Fyn, and Yes, which are expressed in most tissues, and Blk, York Fgr, Hck, Lck, and Lyn, which are selectively expressed in particular cell lineages (9). Each member of the Src family members shares a series of common structure features (1, 2, 9). An amino-terminal myristylation site is required for association with cytoplasmic membranes

* This work was supported by National Institutes of Health Grants R01 CA82716 (to G. B. M. and K. A. S.), Center of Excellence Grants P01CA099031 and P50CA83639 (to G. B. M.) as well as by funds from the National Cancer Institute of Canada, the Canadian Institutes for Health Research, and the Canadian Arthritis Network (to K. A. S.). This work is also supported in part by National Institutes of Health Cancer Center Support Grant S-P30 CA16672. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by a Canadian Arthritis Network postdoctoral fellowship award.

‡‡ To whom correspondence should be addressed: Dept. of Molecular Therapeutics, Div. of Cancer Medicine, Box 317, M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030. Tel.: 713-792-4857; Fax: 713-745-1184; E-mail: Gmills@mdanderson.org.

1 The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; PtdIns, phosphatidylinositol; PH, pleckstrin homology; HRP, horseradish peroxidase; HA, hemagglutinin; GFP, green fluorescent protein; PVDF, polyvinylidene difluoride; WT, wild type.
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and is essential for oncogenic transformation. Autophosphorylation or transphosphorylation by activating kinases of Src at tyrosine 416 is required for optimal activity. Displacement of the interaction between phosphorylated carboxyl-proximal tyrosine-527 and the SH2 domain of Src or the binding of SH3 domain to the SH2-kinase linker region converts Src from a relatively inactive form to an active form able to phosphorylate its downstream effector molecules.

Src regulates cell cycle progression and mediates cell survival through the activation of the PI3K/AKT pathway (2–4, 9). The PI3K signaling cascade is implicated in a broad range of cancer-related cellular processes (24). PI3K catalyzes phosphatidylinositol 3-kinase directed by the PH domain of PI3K, which possess two sites for PI3K activation: the PH domain and the C2 domain. The resulting PI3K products, PtdIns 3,4,5-trisphosphate and PtdIns 3,4-bisphosphate, bind to pleckstrin homology (PH) and other domains of intracellular signaling molecules recruiting them to the cell membrane. Phosphorylation and activation of the PH domain containing c-Akt contributes to cell proliferation, survival, and other cellular processes (24, 25). Growth factor responsive Class IA PI3Ks consist of heterodimers of a p110-kDa catalytic subunit associated with a p85-kDa noncatalytic regulatory subunit. We have previously shown that phosphorylation of Tyr^408 on p85 by Src relieves the inhibitory effect of p85 on p110 catalytic activity (26). We demonstrate herein that Src kinases also regulate PI3K signaling cascades via altering the function of PTEN, a negative regulator of the PI3K pathway.

PTEN, a tumor suppressor, dephosphorylates membrane PtdIns and cytosolic phosphatidylinositols as well as highly acidic tyrosine, serine, and threonine phosphorylated peptides (27–29). The protein phosphatase activity of PTEN has been proposed to decrease cyclin D1 expression, thereby contributing to cell cycle arrest and also to dephosphorylate focal adhesion kinase, consequently reducing cell migration and focal adhesion formation (30, 31). However, the tumor suppressor role of PTEN is thought to derive primarily from the capacity of PTEN to counteract the activity of PI3K by dephosphorylating the D3 position of membrane PtdIns 3,4,5-trisphosphate and PtdIns 3,4-bisphosphate (32, 33). The lipid phosphatase activity of PTEN is associated with down-regulated activities of multiple downstream components of the PI3K pathway, including most notably AKT and its downstream targets, BAD, p70S6K, GSK3, p21, p27, MDM2, TSC1/2, and forkhead transcription factors and altered expression of p21 (31, 34–36). Because PI3K/AKT activation has been implicated in cell proliferation, differentiation, survival, metastasis, angiogenesis, and cytoskeletal rearrangement, the inhibition of this pathway is consistent with a major role for PTEN in tumor suppression. In addition, the association of its lipid phosphatase activity with PTEN tumor suppressor activity has been corroborated by data revealing the ability of PTEN to regulate cell growth to be severely diminished in a PTEN protein carrying a mutation (G129E), which abrogates lipid but not protein phosphatase activity (33).

Although loss-of-function PTEN gene mutations have been detected in many types of tumors, reduced PTEN expression in at least some transformed cells appears to arise independently of gene mutation and in some instances has been linked to methylation of the promoter (37–39). Little is known about the regulation of PTEN function. PTEN is phosphorylated on serine and threonine residues in its tail by CK2 (40, 41). This phosphorylation regulates its stability, activity, and potentially its association with other proteins through its PDZ-binding motif (42–44). PTEN contains 23 tyrosine residues, several of which are in consensus phosphorylation sites and protein-protein interaction motifs, such as SH2-binding sites. Thus, tyrosine phosphorylation represents one potential mechanism whereby activity and/or expression of this phosphatase might be modified post-translationally. This possibility is supported by previous data from our group indicating that mutation of Tyr^420 or Tyr^415 abrogated the ability of PTEN to alter cell growth rate, saturation density, and colony formation in vitro. Mutation of Tyr^420 or Tyr^415 markedly decreased the ability of PTEN to limit tumor growth in nude mice (45). These data raise the possibility that tyrosine phosphorylation of PTEN could regulate the PI3K/AKT signaling pathway. However, either basal or induced tyrosine phosphorylation of PTEN has not been detected in previous studies (40–42). To evaluate the potential interactions between Src tyrosine kinases and PTEN in breast cancer, we have now investigated the effects of Src tyrosine kinases on PTEN function and on PI3K-mediated AKT activation. The results of our studies demonstrate that Src protein-tyrosine kinases regulate the PI3K/AKT pathway by altering PTEN function, which is associated with increased tyrosine phosphorylation of PTEN, leading to a decreased ability of PTEN to hydrolyze PtdIns and reduced PTEN stability.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**

Water-soluble PtdIns 3,4,5-trisphosphate (diC8-PtdIns 3,4,5-trisphosphate, n^+s-1,2-di-O-octanoylglycerol,3,4-D-phospho-linked phosphatidylinositol 3,4,5-trisphosphate) was from Echelon (Salt Lake City, UT). Phosphatidylinositol 4-monophosphate was from Sigma. Goat anti-PTEN polyclonal antibody, rabbit anti-SHP-1 antibody (used for immunoprecipitation), and rabbit anti-Src antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-PTEN antibody, rabbit anti-SHP-1 antibody (used for immunoblotting), mouse anti-phosphotyrosine monoclonal antibody (Clone 4G10), and rabbit anti-LEK antibody were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-phospho-AKT and anti-AKT polyclonal antibodies and anti-phospho-PTEN (Ser^380, Thr^382/383) polyclonal polyclonal antibody were from Cell Signaling Inc. (Beverly, MA). Anti-p-actin monoclonal antibody and anti-FLAG M2 monoclonal antibody were from Sigma. HRP-conjugated goat anti-rabbit IgG and HRP-conjugated goat antiserum IgG were from Bio-Rad. HRP-conjugated protein A and protein G-conjugated Sepharose 4B were from Amersham Biosciences. Monoclonal anti-HA antibody was purified from the mouse hybridoma 12CA5 (a kind gift from Dr. Bing Su, Houston, TX). Src inhibitors PP1 and herbimycin were from Calbiochem (San Diego, CA). [35S]Methionine was purchased from Amersham Biosciences.

**Expression Vectors**

FLAG epitope-tagged full-length wild type PTEN and catalytic inactive PTEN (CT124S) were from Drs. Dixon and Maehama (Ann Arbor, MI). Lipid-phosphatase inactive mutant of PTEN G129E was generated by site-directed mutagenesis (Stratagene, La Jolla, CA). The construct was sequenced in its entirety to confirm the mutagenesis. GFP fusion protein of full-length wild type PTEN and CK2 phosphorylation defective mutant PTEN A4, alanine substitutes at Ser^380, Thr^382/383, and Ser^383 from Dr. William R. Sellers (Boston, MA). HA epitope-tagged wild type AKT was from Dr. Downward. The AKT-PH domain fused to GFP was provided by Dr. Tobias Myer (Stanford University) and Dr. S. Grinstein (Toronto, Canada). The cDNA plasmid for activated Lck Y505F was a generous gift of Dr. A. Veillette (Montreal, Canada). The cDNA plasmids for activated Src Y527F, wild type SHP-1, and inactive SHP-1 (C463S) were described previously (5). Adenovirus containing the transgene of wild type PTEN was described previously (46).

**Cell Lines and Transient Transfection**

Tumor cell lines, MDA-MB-468, BT-549, MDA-MB-231, SKBr3, MCF-7, and Cos-7 cells were cultured in complete medium (RPMI 1640 supplemented with 10% fetal bovine serum). The cells were transiently transfected with different cDNA plasmids by FuGENE 6 transfection reagent (Roche Applied Science) or by Nucleofector Reagent (Amaxa Biosystems, Koeln, Germany) as suggested by the manufacturer. In some experiments, the cells were infected with adenovirus containing wild type PTEN as previously described (46). After transfection or infection, the cells were cultured for 30 h in complete medium and then serum-starved for 16 h prior to cell stimulation or cell lysis.
The cells were stimulated or treated with various inhibitors, as indicated, in serum-free medium. Pervanadate was prepared freshly by incubating 10 mM vanadate with 10 mM H2O2 for 15 min at room temperature. Catalase (final concentration, 0.2 mg/ml) was added to remove residual H2O2. Where indicated, the cells were stimulated by 30 μM of pervanadate for 10 min. After stimulation, the cells were washed twice with phosphate-buffered saline and lysed in ice-cold lysis buffer (1% Triton X-100, 50 mM Hepes, pH 7.4, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 100 mM NaF, 10 mM sodium pyrophosphate, 1 mM Na2VO4, 10% glycerol, 1 mM phenylmethylsulfonfluor fluoride, and 10 μg/ml apro- tinin). For assessment of tyrosine phosphorylation of PTEN, protease inhibitor mixture tablet (Roche Applied Science), 25 mM β-glycerol phosphate, 10 mM Na2VO4, and 1 mM dithiothreitol were included in the lysis buffer. Cellular protein concentration was determined by BCA reaction (Pierce). For immunoprecipitation, detergent lysates were pre- cleared by protein A- or protein G-conjugated Sepharose beads followed by incubation with anti-HA monoclonal antibody or anti-FLAG M2 monoclonal antibody or goat anti-AKT polyclonal antibody for 90 min. Immunocomplexes were captured by protein A-conjugated (for anti- HA) or protein G-conjugated (for anti-FLAG M2 and goat anti-PTEN) Sepharose beads. Immunoprecipitates were washed with 0.5% Triton X-100, 0.5% Nonidet P-40, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM Na2VO4, and 1 mM phenylmethylsulfonfluor fluoride. To detect tyrosine phosphorylation of endogenous PTEN, the immunoprecipitates were washed with lysis buffer containing PTEN inhibitors and 25 mM β-glycerol phosphate, 10 mM Na2VO4, and 1 mM dithiothreitol. The proteins were separated by SDS-PAGE and trans- ferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% bovine serum albumin and then incubated at 4°C for overnight with anti-phosphotyrosine (4G10) (0.01 μg/ml), anti-SHP-1 antibody (1 μg/ml), anti-PTEN rabbit polyclonal antibody (1 μg/ml), anti-C-terminal Src (0.5 μg/ml), anti-Lck (1:1000 dilution), anti-FLAG epitope-tagged wild type PTEN with HA-AKT in these PTEN- deficient cells were transfected with or without activated Lck (Y505F) into BT-549 cells (which lack endogenous PTEN). 48 h after transfection, the cells were lysed in lysis buffer without NaF and Na3VO4. PTEN was immunoprecipitated by goat anti-PTEN antibody as described above. The proteins were resolved by SDS-PAGE and transferred to PVDF membranes. The membranes were blotted with 5% bovine serum albumin and then incubated at 4°C for overnight with anti-phosphotyrosine (4G10) (0.01 μg/ml), anti-PTEN rabbit polyclonal antibody (1 μg/ml), anti-FLAG epitope-tagged wild type PTEN with HA-AKT in these PTEN-deficient cells were transfected with or without activated Lck (Y505F) into BT-549 cells (which lack endogenous PTEN). 48 h after transfection, the cells were lysed in lysis buffer without NaF and Na3VO4. PTEN was immunoprecipitated by anti-FLAG M2 monoclonal antibody as described above. The immunoprecipitates were washed and incubated with [32P]-labeled phosphatidylinositol 4-monophosphate in a PI3K reaction. The lipid phosphatase reaction was terminated by the addition of 0.1 × HCl, and the lipids were extracted by methanol:chloroform (1:1). The lipids were resolved by TLC and visualized by autoradiography.

**PSEN Lipid Phosphatase Assay**

*Micelle PtdIns Assay—FLAG epitope-tagged wild type PTEN was transiently transfected with or without activated Lck (Y505F) into BT-549 cells. 48 h after transfection, the cells were lysed in lysis buffer without NaF and Na3VO4. PTEN was immunoprecipitated by anti-FLAG M2 monoclonal antibody as described above. The immunoprecipitates were washed and incubated with [32P]-labeled phosphatidylinositol 4,5-bisphosphate in 100 mM Tris-HCl, pH 8.0, 10 mM dithiothreitol at 37°C for 1 h. [32P]-labeled phosphatidylinositol 4,5-bisphosphate was generated by phosphorylation of phosphatidylinositol 4-monophosphate in a PI3K reaction. The lipid phosphatase reaction was terminated by the addition of the C2 domain in proximity to PtdIns sub- strates at the membrane (48). To determine whether PTEN- deficient cells engendered a significant down-regulation in the phosphorylation of AKT (lane 3). As a control, AKT phosphorylation was not affected by the phosphatase-inactive mutant (G129E) of PTEN (lane 4), indicating that in this assay system, AKT phosphorylation is a sensitive surrogate of the lipid phosphatase activity of PTEN. Although activated Lck (Y505F) had no significant effect on AKT phosphorylation in the absence of functional PTEN (lane 5), overexpression of this PTK in con- junction with PTEN almost completely abrogated PTEN effects on AKT phosphorylation (compare lane 6 with lane 3). The effects of Lck (Y505F) on AKT phosphorylation were absolutely dependent on the presence of PTEN in BT-549 cells. Similar results were obtained when an active form of Src kinase, Src(Y527F), replaced Lck(Y505F) in PTEN-deficient cells (data not shown). Therefore, the effects of Src family kinases on PI3K/AKT signaling appears to be due, at least in part, to an alteration of PTEN activity (compare lane 6 with lane 3 and with lane 5).

**Active Src Kinase Reduces the Ability of PTEN to Dephosphorylate PtdIns**—The ability of PTEN to efficiently dephosphorylate PtdIns has been linked to both its catalytic activity and its positioning via the C2 domain in proximity to PtdIns sub- strates at the membrane (48). To determine whether PTEN-
mediated dephosphorylation of membrane PtdIns is also influenced by Src protein-tyrosine kinases. PTEN was introduced into BT-549 cells in the presence and absence of activated Lck, and the capacity of immunoprecipitated PTEN to dephosphorylate PtdIns 3,4-bisphosphates and PtdIns 3,4,5-trisphosphates was then evaluated. The results of this analysis revealed that PTEN can efficiently dephosphorylate by wild type PTEN but to be unaffected by lipid-phosphatase incompetent G129E PTEN (Fig. 2, A and B). The lipid phosphatase activity of PTEN immunoprecipitates was found to be reduced by about 40% in the context of PTEN coexpression with activated Lck (Fig. 2, A and B), a result suggesting that Src kinases suppress the capacity of PTEN to dephosphorylate PtdIns (p = 0.033 by Student's t test, PTEN (WT) versus PTEN (WT) + Lck). Because phosphatase inhibitors could not be used in this assay, PTEN may have undergone some degree of autodephosphorylation, and accordingly, the effects of Src family kinases on PTEN activity may have been underestimated. However, in contrast to the effects of activated Lck on PTEN-directed dephosphorylation of micellar PtdIns, activated Lck or activated Src had no effect on PTEN-mediated dephosphorylation of water-soluble diC₈PtdIns 3,4,5-trisphosphate in both Cos-7 (Fig. 2C) and BT-549 cells (Fig. 2D) (p = 0.27 and 0.463 in Cos and BT-549 cells, respectively, by Student’s t test, PTEN (WT) versus PTEN (WT) + Lck or Src). These observations suggest that inhibitory effects of Src family kinases on PTEN lipid phosphatase activity reflect interference with the ability of PTEN to associate productively with membrane lipids rather than a direct inhibition of its enzymatic activity.

Upon growth factor stimulation, AKT is recruited to the activated compartments of cellular plasma membrane through the binding of its PH domain to PtdIns products, PtdIns 3,4,5-trisphosphates and PtdIns 3,4-bisphosphates. Effect of activated Src kinases on the ability of PTEN to dephosphorylate PtdIns was further investigated in intact BT-549 cells expressing GFP fusion protein of AKT-PH domain. Cellular membrane localization of AKT-PH-GFP fusion protein in the presence or absence of activated Src and/or PTEN would represent the amount of PtdIns products PtdIns in cytoplasmic membrane. As shown in Fig. 3, the GFP-AKT-PH fusion protein generally localizes in cytoplasm under resting conditions (Fig. 3A). Although activated Src does not significantly alter the localization of the fusion protein in the absence of functional PTEN (Fig. 3B), wild type PTEN itself effectively constrains AKT-PH in cytosol (Fig. 3C). In contrast, coexpression of activated Src with wild type PTEN markedly promotes the translocation of the AKT-PH fusion protein to the leading edge of cytoplasmic membrane (data not shown), indicating the loss of PTEN function to dephosphorylate PtdIns. Introducing wild type SHP-1, a protein-tyrosine phosphatase, into the cells expressing both activated Src and wild type PTEN reconstitutes the localization of AKT-PH in cytosol (Fig. 3E). These data, consistent with in vitro PTPase assays (Fig. 2, A and B), demonstrate that activated Src alters the ability of PTEN to dephosphorylate PtdIns.

Src Kinases Induce PTEN Tyrosine Phosphorylation and SHP-1 Phosphatase Down-regulates the Tyrosine Phosphorylation—The PTEN carboxyl-terminal C2 domain, a region required for PTEN binding to membrane PtdIns, contains three tyrosine residues located in phosphorylation and protein-protein binding consensus motifs. Tyr²⁴⁰ and Tyr²³⁶ map within consensus SH2 domain-binding motifs for SHP-1. Tyr²¹⁵ locates within a consensus Shc-binding site. Tyr²³⁶ is also located within a consensus phosphorylation site for the Abelson (Abl), epidermal growth factor receptor, and platelet-derived growth factor receptor kinases. Accordingly, the capacity of PTEN to undergo inducible tyrosine phosphorylation and its correlation with the ability to reduce AKT phosphorylation were examined. Compatible with previous reports (40–42), PTEN is not tyrosine-phosphorylated in resting cells, and ligand stimulation did not cause significant tyrosine phosphorylation of PTEN in intact cells (data not shown). We assessed the possibility that PTEN could be tyrosine-phosphorylated in vivo but susceptible to rapid dephosphorylation. Pervanadate inhibits tyrosine phosphatases in intact cells, resulting in an increase in the level of tyrosine phosphorylation of many signaling proteins triggering pathways normally regulated by growth factors, such as those stimulated by insulin (49, 50). As indicated by the anti-tyrosine phosphatase immunoblotting shown in Figs. 4 and 5, treatment of breast cancer cells with pervanadate causes a marked increase in the tyrosine phosphorylation of endogenous (Fig. 4) or ectopically expressed PTEN (Fig. 5). Incubation of the cells with Src kinase inhibitors, PP1, or herbimycin prior to pervanadate treatment significantly reduces the tyrosine phosphorylation of PTEN (Fig. 5A). In contrast, the Abl inhibitor STI571 does not affect pervanadate-induced phosphorylation of PTEN (Fig. 5A), demonstrating that Src protein-tyrosine kinases are involved in pervanadate-induced tyrosine phosphorylation of PTEN. Consistent with this effect, pervanadate, similar to activated Src kinases, abrogates the ability of PTEN to reduce AKT phosphorylation (Fig. 5B). Inhibition of PTPase tyrosine phosphorylation by PP1 or herbimycin reconstitutes the function of PTEN as assessed by Akt phosphorylation (Fig. 5B).

In light of our previous data showing that the association of the SHP-1 tyrosine phosphatase with the PI3K p85 subunit
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Fig. 2. Active Src kinase reduces the ability of PTEN to dephosphorylate membrane PtdIns. A, Src protein-tyrosine kinases reduce the ability of PTEN to dephosphorylate PtdIns in micelles. Wild type PTEN or PTEN (G129E) was transfected into BT-549 cells, with or without activated Lck (Y505F) (as described in the legend to Fig. 1). 30 h after transfection, the cells were serum-starved overnight prior to cell lysis. FLAG epitope-tagged PTEN was immunoprecipitated by anti-FLAG M2 monoclonal antibody. The immunoprecipitates were subjected to in vitro lipid phosphatase activity assay using 32P-labeled phosphatidylinositol 3,4-bisphosphate in micelles as substrate. After the reaction was terminated, the lipids were extracted, resolved by TLC, and visualized by autoradiograph. B, the results from A were quantified by phosphorimaging. PTEN phosphatase activity was calculated (from arbitrary units) and presented as percentages of wild type — (Mock — wild type PTEN expressed with Lck or PTEN G129E)/(Mock — wild type PTEN expressed by itself) × 100%. The data were shown as the means ± S.D. from two similar experiments. Statistical significance was determined by Student’s t test (p = 0.033, PTEN (WT) versus PTEN (WT) + Lck). C and D, Src-protein-tyrosine kinases do not affect the ability of PTEN to dephosphorylate water-soluble diC8PtdIns. Wild type PTEN or PTEN (C124S) was transfected into Cos-7 cells (C) or BT-549 cells (D) with or without activated Lck (Y505F) (C) or activated Src (Y527F) (D). The cells were serum-starved overnight prior to cell lysis. FLAG epitope-tagged PTEN was immunoprecipitated by anti-FLAG M2 monoclonal antibody. The immunoprecipitates were subjected to in vitro lipid phosphatase activity assay using water-soluble diC8PtdIns 3,4,5-trisphosphate as substrate. PTEN activity was measured by colorimetric detection of phosphate release and quantitated by A650 nm and calculated for relative activity as described under “Experimental Procedures.” The data are presented as the means ± S.D. from three similar experiments. Anti-PTEN immunoblot confirmed that equal amount of PTEN was immunoprecipitated from each sample (data not shown). Statistical significance was determined by Student’s t test. C, in Cos-7 cells: Mock versus PTEN (WT) p = 0.05; PTEN (WT) versus PTEN (C124S) p = 0.06; PTEN (WT) versus PTEN (WT) + Lck p = 0.93; PTEN (WT) versus PTEN (C124S) + Lck p = 0.27. D, in BT549 cells: Mock versus PTEN (WT) p = 0.013; PTEN (WT) versus PTEN (C124 S) p = 0.011; PTEN (WT) versus PTEN (WT) + Src p = 0.463.

depends upon Src family PTK-mediated phosphorylation of p85 (47), the possibility that Src family kinases induce tyrosine phosphorylation of PTEN and its association with SHP-1 was further investigated. The ability of activated Src family kinases to induce association of SHP-1 with PTEN was assessed by coexpression of activated forms of Src (Y527F) or Lck (Y505F), catalytically inert PTEN (C124S), and either wild type or catalytically inactive SHP-1 (C453S). An enzymatically inactive PTEN was used here to prevent autodephosphorylation and/or dephosphorylation of PTEN-binding proteins. As shown in Fig. 6A, coexpression of either activated Src or Lck with PTEN induces a marked increase in the tyrosine phosphorylation of PTEN (lane 3 and 7). In the presence but not the absence of activated Src, PTEN associated with the catalytically inert form of SHP-1 (lane 5). By contrast, expression of wild type rather than inactive SHP-1 abrogated both the Src-induced tyrosine phosphorylation of PTEN and the association of PTEN with SHP-1 (lane 4). Thus, Src family kinases induce association of PTEN with SHP-1, likely through association of its SH2 domain with phosphorylated PTEN, which subsequently dephosphorylates PTEN. The capacity of SHP-1, and potentially other tyrosine phosphatases, to efficiently dephosphorylate PTEN may contribute to the difficulty in detecting PTEN phosphorylation in cells replete in SHP-1 and may contribute to the ability of pervanadate to increase PTEN phosphorylation in a Src-dependent manner.

To determine whether Src-induced PTEN tyrosine phosphorylation and the ability of SHP-1 to reverse the effect of Src could translate into alterations in signaling through PI3K cascade, HA-AKT was coexpressed with PTEN in the presence or absence of activated Src and/or wild type SHP1 and AKT phosphorylation evaluated. As shown in Fig. 6B, Src induces tyrosine phosphorylation of PTEN and alters the ability of PTEN to reduce AKT phosphorylation (lane 3). SHP-1 decreases Src-induced phosphorylation of PTEN reconstituting its ability to decrease AKT phosphorylation (lane 4). In contrast, SHP-1 itself does not influence AKT phosphorylation (lane 6). These studies further support the concept that Src kinases alter the
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P13K signaling pathway by inducing the phosphorylation of specific substrates including PTEN, a process that is reversed by tyrosine phosphatases such as SHP-1.

Cotransfection of Src PTKs with PTEN resulted in PTEN tyrosine phosphorylation in intact cells. Furthermore, pervanadate increases PTEN tyrosine phosphorylation in a Src-dependent manner. However, recombinant Src family kinases were unable to phosphorylate purified PTEN in in vitro kinase assays (data not shown). This observation suggests the involvement of an intermediary kinase coupling the Src PTK to PTEN tyrosine phosphorylation, a hypothesis supported by a failure of active Src to alter PTEN function as indicated by AKT phosphorylation (Fig. 9). Thus, Src regulates PTEN function independently of CK2-induced phosphorylation of PTEN.

To further assess whether alterations in CK2-mediated phosphorylation of PTEN could contribute to the ability of Src to alter signal transduction through the PI3K pathway, wild type PTEN and A4 PTEN were introduced into BT-549 cells, and AKT phosphorylation was assessed in the presence or absence of activated Src. The levels of A4-PTEN in intact cells were decreased compared with wild type PTEN compatible with the concept that CK2 phosphorylates and stabilizes PTEN (data not shown). When A4-PTEN levels were adjusted by introduction of higher amounts of the expression vector, A4-PTEN reduces AKT phosphorylation in BT-549 cells in a similar manner to wild type PTEN (Fig. 8). Furthermore, the mutation of the CK2 phosphorylation sites did not limit the ability of activated Src to alter PTEN function as indicated by AKT phosphorylation (Fig. 9). Thus, Src regulates PTEN function independently of CK2-induced phosphorylation of PTEN. Together these observations demonstrate that Src kinases regulate signaling through the PI3K pathway by altering PTEN function, which includes increased tyrosine phosphorylation of PTEN associated with its decreased ability to hydrolyze PtdIns and reduced PTEN stability.

DISCUSSION

In the current study, we have investigated the possibility that Src protein-tyrosine kinases regulate the P13K/AKT prosurvival pathway via altering PTEN function and stability. Src participates in the regulation of many signal transduction pathways. Src regulates the P13K/AKT cascade through multiple mechanisms. Tyrosine phosphorylation of cellular proteins induced by Src family kinases recruits P13K to activated membrane compartments wherein P13K phosphorylates membrane PtdIns and recruits its downstream effector molecules, thus fulfilling its biological activities (24, 25). Src also activates P13K via the binding of the Src-SH3 domain to the proline-rich region in the P13K p85 subunit (52). The regulatory p85 subunit of P13K stabilizes the catalytic p110 subunit and inhibits the P13K enzymatic activity (53). Tyrosine phosphorylation of p85, more specifically of Tyr405, by Src family kinases induces the formation of an intramolecular complex in p85, thereby relieving the inhibitory effect of p85 on p110 leading to the
activation of the PI3K/AKT cascade (26). The ability of Src to inactivate PTEN function provides an additional interaction site between Src and the PI3K pathway.

PTEN has been demonstrated to undergo phosphorylation at multiple serine and threonine sites in its carboxyl-terminal tail (40–43). Phosphorylation at these sites, which is at least in part mediated by the CK2 kinase, has been shown to alter both stability and catalytic activity of the PTEN protein (40, 42, 43). The identity of the CK2-mediated phosphorylated residues in the PTEN tail with mass spectrometry results in contradictory conclusions with mutagenesis studies (40, 41). It appears likely that the dominant phosphorylation sites in PTEN are Ser370 and Ser385. However, it is possible that additional Ser and Thr residues contribute to PTEN phosphorylation, particularly when the conformational structure of PTEN is altered by mutagenesis (41). Phosphorylation of serine/threonine residues within the PTEN tail acts as an inhibitory switch by preventing its recruitment into a protein complex (43, 44). Consequently, it interferes with PTEN binding to MAGI family proteins and may thereby impede PTEN association with cell membranes, particularly at points of focal contact (42, 54).

Src and CK2 both decrease the ability of PTEN to dephosphorylate PtdIns. In contrast Src decreases PTEN stability and CK2 increases PTEN stability (40, 42). Thus, it is unlikely that the effects of Src on PTEN are mediated by alterations in CK2-mediated PTEN phosphorylation. Furthermore, Src does not alter CK2 kinase-induced serine/threonine phosphorylation of PTEN. Mutation of putative serine and threonine CK2 phosphorylation sites in the tail of PTEN does not alter the ability of Src to inhibit PTEN function as indicated by changes in AKT phosphorylation. Although the identity of the CK2 phosphorylation sites of PTEN is controversial, the PTEN mutants utilized in mutagenesis studies may not present as a physiological substrate for CK2 either as a consequence of structural changes in the PTEN tail or serving as a pseudosubstrate with highly negative charged residues (40, 41).

Tyrosine phosphorylation of PTEN has not been detected in previous studies (40–42). Indeed, rather than cotransfection of activated Src family kinases or inhibition of tyrosine phosphatases, we are unable to reveal consistent high levels of PTEN tyrosine phosphorylation. However, it remains possible that specific phosphatase in particular cellular lineages may limit the detection of PTEN tyrosine phosphorylation. The ability of SHP-1 to bind and dephosphorylate PTEN suggests that phosphatase-mediated dephosphorylation of PTEN may mask PTEN tyrosine phosphorylation under certain physiological conditions.

*Fig. 5.* Src kinase inhibitors block pervanadate-induced tyrosine phosphorylation of PTEN and resume the ability of PTEN to reduce AKT phosphorylation. Breast cancer cells, MDA-MB-468 lacking functional PTEN, were infected with adenovirus containing wild type PTEN. 30 h after viral infection, the cells were serum-starved overnight. The cells were incubated with PP1 (10 μM), herbimycin (100 μg/ml), or STI571 (5 μM) for 30 min prior to the treatment of pervanadate for 15 min. The cells were lysed. A, PTEN was immunoprecipitated by goat anti-PTEN polyclonal antibody and protein G beads as described under “Experimental Procedures.” The immunoprecipitates were subjected to SDS-PAGE and transferred to PVDF membranes. Tyrosine phosphorylation of PTEN was detected by immunoblot with anti-phosphotyrosine antibody (pTyr, Clone 4G10). The membrane was stripped and reprobed with anti-PTEN antibody to verify equal efficiency of PTEN immunoprecipitation. B, aliquots of cell lysate (30 μg of cellular protein) from the same experiment were subjected to SDS-PAGE and immunoblotted by anti-phospho-AKT (Ser473) antibody and reprobed by anti-AKT antibody. Similar results were obtained in two independent experiments.

**A**

| Inhibitor | - | - | PP1 | Herbimycin | STI571 |
|-----------|---|---|-----|------------|--------|
| Pervanadate | - | - | + | + | + |
| Ad-PTEN Infection | - | + | + | + | + |

**Anti-pTyr Blot**

PTEN

**Anti-PTEN Blot**

**Anti-PTEN Immunoprecipitation**

**B**

| Inhibitor | - | - | PP1 | Herbimycin | STI571 |
|-----------|---|---|-----|------------|--------|
| Pervanadate | - | - | + | + | + |
| Ad-PTEN Infection | - | + | + | + | + |

**Anti-Phospho-AKT (Ser473) Blot**

Phospho-AKT (Ser473)

**Anti-AKT Blot**

AKT

**Cell Lysates**
conditions and especially in cells stimulated with ligands such as epidermal growth factor, platelet-derived growth factor, or insulin-like growth factor (40–42). Thus, the difficulty in detecting tyrosine-phosphorylated PTEN following growth factor stimulation may be due to rapid attenuation by SHP-1 or other tyrosine phosphatase-mediated dephosphorylation of PTEN and/or by tyrosine phosphorylation-induced degradation of PTEN. Nevertheless, because Src family kinases are important mediators in tumorigenesis in breast and other tissues, the ability of Src family kinases to decrease PTEN activity and stability may contribute to the effects of Src on tumorigenesis.

Despite the presence of functional PTEN in most cells, extracellular ligands are normally able to induce transient increases in intracellular D3 phosphorylated PtdIns and signaling through the PI3K cascade. Similarly, adenoviral-induced high level expression of PTEN does not abrogate PI3K signaling and the consequent induction of AKT, BAD, and p70S6K phosphorylation in PTEN replete cells (34, 46). These observations indicate that PTEN is either catalytically inefficient, recruited relatively slowly to the cell membrane, and/or post-translationally regulated. The data presented here favor the last possibility, suggesting that Src-mediated phosphorylation of PTEN may contribute to the inactivation of this phosphatase during ligand perturbation and thereby provide a "permissive" role in promoting the activation of the PI3K signaling pathway.

Although Src family kinases efficiently induced PTEN tyrosine phosphorylation when these proteins were overexpressed in cell lines, purified or recombinant Src or Lck did not phosphorylate purified PTEN in vitro assays. These data suggest the absence of required cofactors or, alternatively, an intermediary kinase whose activation dependent upon Src family kinases. Because the PTEN Tyr336 residue is located within a consensus site for phosphorylation by the Abl tyrosine kinase, the potential for this protein-tyrosine kinase to phosphorylate PTEN was examined by overexpression of PTEN and Abl in Cos-7 cells. However, no induction of PTEN tyrosine phosphorylation was observed in this assay (data not shown). Further, the Abl inhibitor STI571 does not block pervanadate-induced tyrosine phosphorylation of "Src Alters Ability of PTEN to Regulate PI3K/AKT Signaling"
PTEN, indicating that Abl is not required for pervanadate- and Src-induced increases in tyrosine phosphorylation of PTEN. The identity of a protein subserving an intermediary role in tyrosine phosphorylation of PTEN remains to be determined.

SHP-1 has been shown to limit signaling through multiple cell surface receptors and thereby to inhibit ligand-induced cell growth (55). Further, we have demonstrated that SHP-1 regulates Src family protein-tyrosine kinases (56) and dephosphorylates multiple different Src phosphorylated targets (47). Signaling through the PI3K/AKT pathway, which plays an important role in tumorigenesis in multiple lineages, is increased in homozygous SHP-1 negative mice (26), potentially contributing to the development of tumors in these mice. We have also demonstrated that SHP-1 modulates signaling through the PI3K pathway via its association with and dephosphorylation of the PI3K p85 regulatory subunit (47). The studies described herein reveal the additional possibility that SHP-1 could exhibit a tumor suppressor role by restoring the function of PTEN, potentially through dephosphorylating PTEN and allowing PTEN to productively associate with its substrates in the cell membranes.

Although the tyrosine phosphorylation of PTEN was associated with a reduction in PTEN-mediated dephosphorylation of membrane PtdIns in vitro and in PTEN-mediated down-regulation of AKT phosphorylation and membrane localization in intact cells, tyrosine phosphorylation did not affect PTEN-mediated dephosphorylation of water-soluble PtdIns. These observations suggest that phosphorylation of PTEN may alter the capacity of PTEN to productively associate with membrane PtdIns but does not result in direct inactivation of the PTEN catalytic domain. PTEN possesses a C2 domain, which has been previously described to be required for PTEN to bind membrane PtdIns, productively positioning the catalytic domain where it can fulfill its 3-phosphatidylinositol phosphatase enzymatic activity (48). Mutations of PTEN in the C2 domain found in human cancers suggest that an intact C2 domain may not only participate in membrane recruitment of PTEN but may also regulate PTEN protein stability (48, 57). Tyrosine 336 is located on the surface of the C2 domain, where it would be

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 Src Alters Ability of PTEN to Regulate PI3K/AKT Signaling

highly accessible for tyrosine phosphorylation. Alternatively, phosphorylation of PTEN may contribute in some manner of molecular switch to the decreased ability of PTEN to diphosphorylate membrane PtdIns and thereby regulate PI3K signaling. Although these possibilities require further investigation, the current data strongly suggest that PTEN lipid phosphatase activity is regulated by Src-induced tyrosine phosphorylation in intact cells.

Together, these data reveal that not only mutational inactivation observed in multiple lineages of tumor but also phosphorylation and potentially other post-translational modifications regulate PTEN catalytic activity, protein-protein interaction, localization, and substrate contact. Further elucidation of the mechanisms regulating PTEN may provide insight into the role of PTEN and Src-family kinases on PI3K/AKT signaling pathways and thus modulation of cell growth and tumorigenesis.

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