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The Tumor Suppressor, PTEN/MMAC1, Dephosphorylates the Lipid Second Messenger, Phosphatidylinositol 3,4,5-Trisphosphate*

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Phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3) is a key molecule involved in cell growth signaling. We demonstrated that overexpression of PTEN, a putative tumor suppressor, reduced insulin-induced PtdIns(3,4,5)P_3 production in human 293 cells without effecting insulin-induced phosphoinositide 3-kinase activation. Further, transfection of the catalytically inactive mutant of PTEN (C124S) caused PtdIns(3,4,5)P_3 accumulation in the absence of insulin stimulation. Purified recombinant PTEN catalyzed dephosphorylation of PtdIns(3,4,5)P_3, specifically at position 3 on the inositol ring. PTEN also exhibited 3-phosphatase activity toward inositol 1,3,4,5-tetrakisphosphate. Our results raise the possibility that PTEN acts *in vivo* as a phosphoinositide 3-phosphatase by regulating PtdIns(3,4,5)P_3 levels. As expected, the C124S mutant of PTEN was incapable of catalyzing dephosphorylation of PtdIns(3,4,5)P_3 consistent with the mechanism observed in protein-tyrosine phosphatase-catalyzed reactions.

A recently identified candidate tumor suppressor gene, PTEN/MMAC1, shares sequence identity with the family of protein-tyrosine phosphatases (PTPases)[1] (1). Deletions and mutations within the PTEN gene have been observed in several cancer cell types and tumor cell lines (2, 3). Additional evidence that PTEN functions as a tumor suppressor was obtained by Furnari *et al*. (4), who showed that PTEN had a growth suppressor activity in glioma cells. PTEN encodes the active site consensus motif HC*XX*G*XX*R(S/T) found in all PTPases. In contrast, the recombinant protein is a poor catalyst toward both phosphoproteins and peptide substrates with the highest activity of PTEN observed toward the highly negatively charged, multiply phosphorylated polymer of $(Glu\text{-}Tyr)_n$ (5, 6). Based on these observations we thought it possible that PTEN could catalyze the dephosphorylation of acidic nonproteinaceous substrate. Identification of possible *in vivo* substrates would not only suggest a possible physiological function of PTEN, but they might also provide insight into how PTEN functions as a tumor suppressor.

PtdIns(3,4,5)P_3 is an important second messenger involved in cell growth signaling (7). PtdIns(3,4,5)P_3 is specifically produced from PtdIns(4,5)P_2 by PI 3-kinase upon stimulation by a variety of ligands (7). Recent studies have identified that PtdIns(3,4,5)P_3 can directly activate Akt, which in turn activates p70 S6 kinase and inhibits glycogen synthase kinase-3 (8, 9). Although there are several phosphoinositide 5-phosphatases, the mechanism of regulation and particularly the degradation pathway of PtdIns(3,4,5)P_3 *in vivo* is still unclear (10, 11). In the present study we demonstrate that recombinant PTEN has PtdIns(3,4,5)P_3 3-phosphatase activity. In addition, we provide evidence that PTEN may act *in vivo* as a regulator of PtdIns(3,4,5)P_3, which produces a substrate that can be recycled by PI 3-kinase.

EXPERIMENTAL PROCEDURES

Determination of PtdIns(3,4,5)P_3 in Vivo—The coding sequence of human PTEN and the C124S mutant of PTEN (gift from Yi Zhao) were amplified by polymerase chain reaction using 5′ primer (5′-CCGGTACCGCCACCATGGACTACAAGGACGACGATGACAAGACAGCCATCATCAAAGAG-3′) and 3′ primer (5′-CCGTCGACTCAGACTTTTGTAATTTGTG-3′). The product was cleaved with *Kpn*I and *Sal*I and ligated into the *Kpn*I/*Sal*I sites of pCMV5 (gift from David W. Russell) to produce FLAG-tagged PTEN/pCMV5 and PTEN(CS)/pCMV5. Human 293 cells were cultured on a 6-well plate, and transfection of the cells were performed as described (12) using 1 μg of the constructs. The efficiency of transfection was about 80% in this condition. 48 h after the transfection, the cells were serum-starved and labeled with [^{32}P]P_i (100 μCi/ml) for 4 h. The cells were stimulated by incubation with insulin (0.1 μg/ml) for 2.5 min at 37 °C, and the stimulation was quenched by the addition of 0.93 ml of $CH_3OH/CHCl_3$/1% $HClO_4$ (50/25/18, v/v/v). The lipids were extracted and separated on a TLC plate as described (13) to determine the amount of [^{32}P]PtdIns(3,4,5)P_3. To analyze the expression of FLAG-tagged PTEN protein, the transfected cells were lysed in Laemmli sample buffer and subjected to SDS-polyacrylamide gel electrophoresis. The samples were transferred to Immobilon filter (Millipore) and immunoblotted with anti-FLAG M2 antibody (Kodak), and the signal was visualized by Enhanced Chemiluminescence (Amersham Pharmacia Biotech) using the manufacturer's recommended protocols.

PI 3-Kinase Assay—Transfection, starvation and stimulation of human 293 cells were carried out as described above in the absence of radiolabel. After the stimulation, the cells were lysed, followed by immunoprecipitation as described (14) using 4G10 anti-phosphotyrosine antibody (Upstate Biotechnology, Inc.). To analyze the PI 3-kinase activity of the immunoprecipitants, the sample was incubated for 10 min at 37 °C in 33 mM Tris-HCl (pH 7.4), 2.5 mM EGTA, 5 mM $MgCl_2$, 30 mM NaCl, 0.1 mM [γ-^{32}P]ATP (20 μCi), 0.1 mg/ml PtdIns(4,5)P_2, and 0.15 mg/ml phosphatidylserine. The reaction was terminated by the addition of 0.47 ml of $CH_3OH/CHCl_3$/6% $HClO_4$ (30/15/2, v/v/v), and the phospholipids were extracted and separated on a TLC plate as described (13).

Bacterial Expression and Purification of PTEN—The expression vector for PTEN was constructed by ligating a blunted *Nde*I/*Sal*I fragment from PTEN/pT7–7 (12) into the *Sma*I site of pGEX-KG (15). This vector was used to transform *Escherichia coli* strain JM109. Protein expression in 4-liter culture was carried out as described (16). All of the following procedures were performed at 4 °C. Cells were harvested, resuspended in 80 ml of lysis buffer (20 mM Tris-HCl (pH 8), 2 mM EDTA, 2 mM DTT, 300 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride) and lysed by sonication. The crude lysate was diluted by the addition of 720 ml of the lysis buffer containing 1% (w/v) Triton X-100 and stirred for 30 min. Cell debris was removed by centrifugation at 27,000 × *g* for 20 min. A 5-ml slurry of glutathione-Sepharose 4B

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[1] The abbreviations used are: PTPase, protein-tyrosine phosphatase; PI, phosphoinositide; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; Ins(1,3,4,5)P_4, inositol 1,3,4,5-tetrakisphosphate; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; DTT, dithiothreitol; VHR, VH1-related.

(Amersham Pharmacia Biotech) was then added to the supernatant. After the incubation for 2 h, the resin was packed in a column and washed with 100 ml of the lysis buffer, and the glutathione *S*-transferase-fusioned PTEN was eluted with 10 ml of the lysis buffer containing 10 mM glutathione. After overnight incubation with thrombin, the eluate was dialyzed against the lysis buffer and passed through glutathione-Sepharose 4B column, followed by a *p*-aminobezamidine-agarose column for adsorption of glutathione *S*-transferase and thrombin, respectively. Then the eluate was diluted with equal volume of TED buffer (20 mM Tris-HCl (pH 8), 2 mM EDTA, 2 mM DTT) and applied to MonoQ HR5/5 (Amersham Pharmacia Biotech) column equilibrated with TED buffer containing 150 mM NaCl. PTEN was eluted with a linear gradient of NaCl (150–500 mM, 20 ml), followed by concentration using Centricon-30 (Amicon) and stored at −80 °C until use.

Phosphatase Assays—PtdIns(3,4,5)P_3 phosphatase assay was performed at 37 °C in a buffer (20 μl) consisting of 100 mM Tris-HCl (pH 8), 10 mM DTT, [^{32}P]PtdIns(3,4,5)P_3 and 1 μg of purified PTEN. The reaction was terminated by the addition of 0.47 ml of $CH_3OH/CHCl_3$/6% $HClO_4$ (30/15/2, v/v/v). Then the phospholipids were extracted and separated on a TLC plate as described (13). To prepare [^{32}P]PtdIns(3,4,5)P_3, the phosphorylation of PtdIns(4,5)P_2 by PI 3-kinase using [γ-^{32}P]ATP was carried out as described above. Then the phospholipids were extracted as described (13) and stored at −20 °C until use. The PI 3-kinase used was prepared by immunoprecipitation from the 293 cell lysate with anti-p85 antibody (Upstate Biotechnology, Inc.) as described (14). For identification of the dephosphorylation site (see Fig. 2*B*), dephosphorylation of PtdIns(3,4,5)P_3 by PTEN was carried out in a buffer (20 μl) consisting of 100 mM Tris-HCl (pH 8), 10 mM DTT, 0.1 mg/ml of PtdIns(3,4,5)P_3 (BIOMOL), 0.15 mg/ml of phosphatidylserine, and 1 μg of purified PTEN. The reaction was terminated by the addition of 0.47 ml of $CH_3OH/CHCl_3$/6% $HClO_4$ (30/15/2, v/v). The phospholipids were extracted as described (13), dried, and then used for PI 3-kinase-catalyzed reaction. Inositol phosphatase assays were performed using commercially available [^{3}H]inositol phosphate (NEN) as substrates. Assay was carried out at 37 °C in a buffer (20 μl) consisting of 100 mM Tris-HCl (pH 8), 10 mM DTT, 60 μM [^{3}H]inositol phosphate (0.01 μCi) and 1 μg of enzyme. After an incubation of 30 min, the reaction was terminated by the addition of 1 ml of stop solution. Then, to separate the dephosphorylated product from the substrate, the sample was applied to AG1-X8 column (0.5 ml) equilibrated with the stop solution. Dephosphorylated [^{3}H]inositol phosphate was eluted with 5 ml of the stop solution, whereas the substrate remained in the column, and the radioactivity of the eluate was measured. The stop solutions used were 0.2 M $HCOONH_4$/0.1 M HCOOH, 0.4 M $HCOONH_4$/0.1 M HCOOH, and 0.7 M $HCOONH_4$/0.1 M HCOOH for [^{3}H]inositol 1, 4-bisphosphate, [^{3}H]Ins(1,4,5)P_3, and [^{3}H]Ins(1,3,4,5)P_4, respectively. Recombinant human VHR, Cdc25B, and PTP1D were kindly gifts from Harris Vikis, Elizabeth Gottlin, and Jin Zhou, respectively. For kinetic analysis (see Fig. 3*B*), inositol phosphatase activity was assayed in the same buffer as described above using 10–150 μM Ins(1,3,4,5)P_4 instead of [^{3}H]inositol phosphate. After an incubation of 1 min at 37 °C, the reaction was terminated by the addition of 1 ml of ice-cold water, and the amount of Ins(1,4,5)P_3 produced was estimated using BIOTRAK Ins(1,4,5)P_3 detection kit (Amersham Pharmacia Biotech) following the manufacturer's recommended protocol. Kinetic constants were determined using KaleidaGraph software (Abelbech).

RESULTS AND DISCUSSION

Although PTEN has the consensus sequence of a PTPase, it dephosphorylates *p*-nitrophenylphosphate and other artificial protein substrates poorly, having the highest catalytic activity with the highly negatively charged, multiply phosphorylated polymer of $(Glu\text{-}Tyr)_n$ (5, 6). This observation raised the distinct possibility that PTEN might utilize acidic substrates other than Tyr or Ser/Thr phosphoproteins. In order to explore this possibility, we transfected PTEN into 293 cells and analyzed the changes in cellular phospholipids.

Suppression of Insulin-induced PtdIns(3,4,5)P_3 Production by Overexpression of PTEN—PtdIns(3,4,5)P_3 is an important second messenger in the regulation of cell growth (7). In human 293 cells, insulin stimulates PI 3-kinase activity (Fig. 1*C*, *lanes 1* and *2*), resulting in an increase in PtdIns(3,4,5)P_3 (Fig. 1*A*, *lanes 1* and *2*). When PTEN was overexpressed in the 293 cells, the insulin-induced PtdIns(3,4,5)P_3 levels were significantly reduced in a dose-dependent manner (Fig. 1, *A* and *B*), whereas

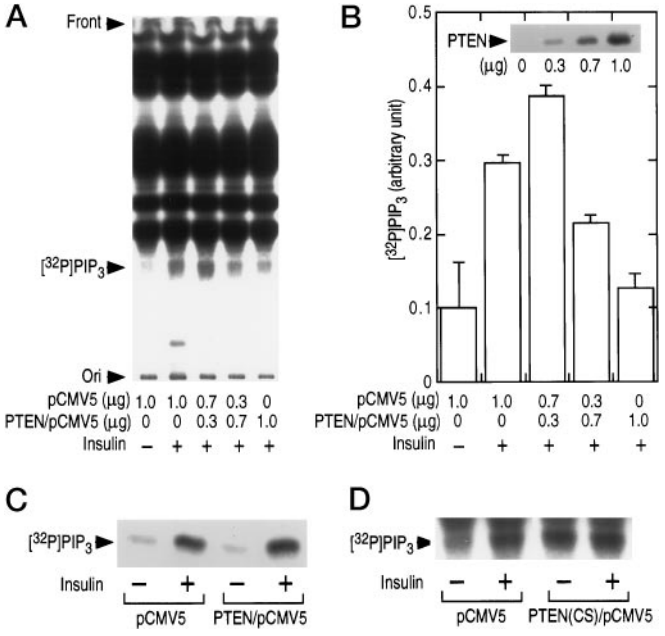

FIG. 1. **Effects of PTEN expression on insulin-induced PtdIns(3,4,5)P_3 production and PI 3-kinase activation in 293 cells.** *A*, *B*, and *D*, human 293 cells were transfected with indicated amount of pCMV5 and PTEN/pCMV5 (*A* and *B*) or 1 μg of PTEN(CS)/pCMV5 (*D*), then serum-starved, and labeled with [^{32}P]P_i as described under "Experimental Procedures." The cells were incubated for 2.5 min in the presence or absence of insulin, and then the lipids were extracted and separated on a TLC plate as described under "Experimental Procedures." The plate was subjected to autoradiography (*A* and *D*), and the radioactivity of [^{32}P]PtdIns(3,4,5)P_3 (*PIP_3*) was estimated using a PhosphorImager (*B*). *Error bars* represent the differences between duplicate determinations in a typical study. Immunoblot of expressed FLAG-tagged PTEN using anti-FLAG antibody is shown in the *inset* of *panel B*. *C*, human 293 cells were transfected with indicated vector, starved, and stimulated by insulin as described under "Experimental Procedures." After the stimulation, the cells were lysed, and the PI 3-kinase activity of the immunoprecipitants with anti-phosphotyrosine antibody was assayed as described under "Experimental Procedures." Similar results were obtained in a repeated experiment.

no effect on the activation of PI 3-kinase was observed (Fig. 1*C*, *lanes 2* and *4*). Because PtdIns(3,4,5)P_3 is specifically produced by PI 3-kinase, this result suggests that PTEN directly effects the turnover of PtdIns(3,4,5)P_3. Additionally, overexpression of the catalytically inactive mutant (C124S) of PTEN (see Fig. 3*A*) caused an accumulation of PtdIns(3,4,5)P_3 in the absence of insulin stimulation (Fig. 1*D*, *lanes 1* and *3*), whereas overexpression of the mutant did not affect PI 3-kinase activity (data not shown). These results suggest that PTEN potentially regulates PtdIns(3,4,5)P_3 levels in cells without alteration of the insulin-stimulated PI 3-kinase activity.

PtdIns(3,4,5)P_3 3-Phosphatase Activity of PTEN—To investigate the possibility that PTEN has PtdIns(3,4,5)P_3 phosphatase activity, the recombinant enzyme was expressed in *E. coli* and purified to homogeneity (data not shown). Radiolabel was incorporated in position 3 of the substrate, [^{32}P]PtdIns(3,4,5)P_3, using PtdIns(4,5)P_2, PI 3-kinase and [γ-^{32}P]ATP. When [^{32}P]PtdIns(3,4,5)P_3 was incubated with the purified PTEN, the radiolabel of [^{32}P]PtdIns(3,4,5)P_3 rapidly disappeared from the lipid phase (Fig. 2*A*, *inset*) while coincidentally appearing in the aqueous phase (Fig. 2*A*), suggesting the release of inorganic phosphate. In order to conclusively prove that the only phosphate that had been cleaved was at position 3 of the phosphoinositide, as opposed to other possible cleavages that could also generate a water-soluble radiolabel, we used the product of the reaction of PTEN as a substrate for PI 3-kinase. When the product was treated with PI 3-kinase,

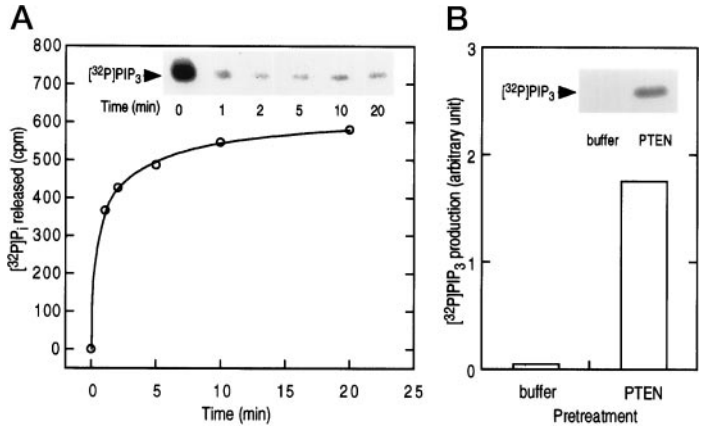

FIG. 2. **PtdIns(3,4,5)P_3 3-phosphatase activity of PTEN.** *A*, time course of PTEN-catalyzed dephosphorylation of [^{32}P]PtdIns(3,4,5)P_3 (*PIP$_3$*). At the indicated time, the reaction was terminated, and [^{32}P]PtdIns(3,4,5)P_3 was extracted and separated on a TLC plate as described under "Experimental Procedures." *Inset*, autoradiography of the plate. Released [^{32}P]P_i in the aqueous phase was pooled during the extraction, and the radioactivity was counted. *B*, PtdIns(3,4,5)P_3 pretreated (30 min) with PTEN or corresponding buffer was subjected to the phosphorylation by PI 3-kinase using immunopurified PI 3-kinase and [γ-^{32}P]ATP as described under "Experimental Procedures." After the incubation for 30 min, the reaction was terminated, and the phospholipids were extracted and separated on a TLC plate. The radioactivity of PtdIns(3,4,5)P_3 produced was determined using a PhosphorImager. *Inset*, autoradiography of the plate. Similar results were obtained in a repeated experiment.

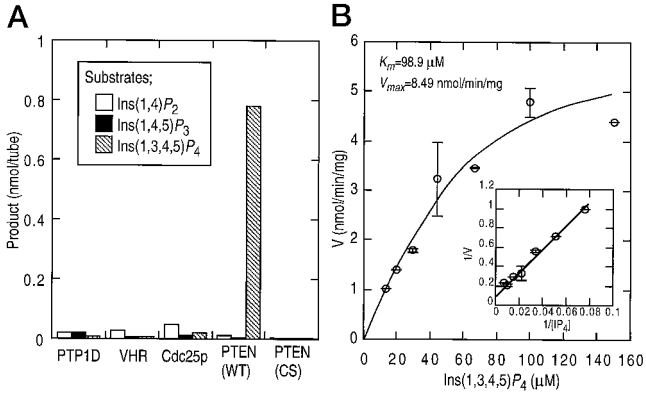

FIG. 3. **Inositol phosphatase activity of PTEN.** *A*, inositol phosphatase activity of various enzymes was assayed using [^{3}H]inositol phosphate as indicated. After the incubation for 30 min, dephosphorylated [^{3}H]inositol phosphate was separated as described under "Experimental Procedures," and then the radioactivity of the dephosphorylated products was counted. Similar results were obtained in a repeated experiment. *WT*, wild type; *CS*, C124S mutant. *B*, initial rate of PTEN-catalyzed dephosphorylation of Ins(1,3,4,5)P_4 was determined as described under "Experimental Procedures" using various concentration of Ins(1,3,4,5)P_4. The Lineweaver-Burk plot is shown in *inset*. The data are presented as the means ± S.E. of triplicate determinations.

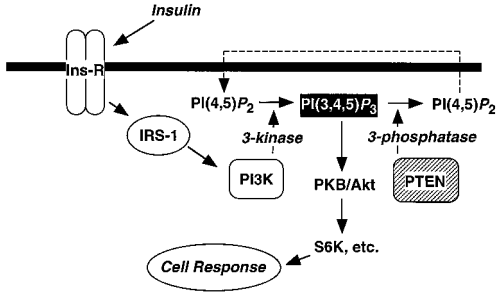

FIG. 4. **Model for the regulation of PtdIns(3,4,5)P_3 level.** See "Results and Discussion" for details. *Ins-R*, insulin receptor; *IRS-1*, insulin receptor substrate-1; *PI3K*, phosphoinositide 3-kinase; *PKB*, protein kinase B; *S6K*, p70 S6 kinase.

[^{32}P]PtdIns(3,4,5)P_3 was reformed, thereby providing further evidence that the two products generated by PTEN were inorganic phosphate and PtdIns(4,5)P_2 (Fig. 2*B*). Under similar conditions, PTEN also exhibited 3-phosphatase activity on phosphatidylinositol 3-monophosphate and phosphatidylinositol 3,4-bisphosphate; however, dephosphorylation of these phosphoinositides occurred at ~20% the rate observed with PtdIns(3,4,5)P_3 (data not shown). PTPases including PTP1D and dual-specific phosphatases (VHR, Cdc25B) exhibited no phosphoinositide phosphatase activity (data not shown).

Inositol Phosphate 3-Phosphatase Activity of PTEN—To more carefully dissect the specific nature of the catalytic activity of PTEN toward PtdIns(3,4,5)P_3, we asked if PTEN displayed activity toward inositol phosphates. PTEN can dephosphorylate Ins(1,3,4,5)P_4, whereas tyrosine-specific (PTP1D) and the dual-specific phosphatases (Cdc25B, VHR) exhibited no activity toward this inositol phosphate (Fig. 3*A*). Again, the PTEN-catalyzed reaction was specific for the position 3 of Ins(1,3,4,5)P_4. Other inositol phosphates that do not have a phosphate at the position 3 on the inositol ring were not dephosphorylated by PTEN (Fig. 3*A*). The dephosphorylated product was identified as Ins(1,4,5)P_3 using the Ins(1,4,5)P_3-binding protein (Fig. 3*B*). These results demonstrate that PTEN also has 3-phosphatase activity toward inositol phosphate. Both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 have been proposed to be a functional second messenger responsible for the intracellular calcium signaling (17). Interestingly, Ins(1,3,4,5)P_4 can associate and activate a GTPase-activating protein (18). In contrast to PtdIns(3,4,5)P_3, Ins(1,3,4,5)P_4 is water-soluble and therefore was used to assess the significance of our *in vitro* observations by determining the kinetic parameters for the PTEN-catalyzed dephosphorylation occurred at position 3 on the inositol ring. The K_m and V_{max} values for Ins(1,3,4,5)P_4 were 98.9 μM and 8.49 nmol/min/mg (k_{cat}, 0.49 min^{-1}), respectively (Fig. 3*B*). The K_m value of 98.9 μM is 250-fold lower than the K_m of *p*-nitrophenylphosphate, which is 25.6 mM. Similar comparisons with the phosphorylated polymer $(Glu\text{-}Tyr)_n$ were difficult to assess because a detailed kinetic analysis was not performed with this substrate (6), and it is likely to be phosphorylated at more than one site. The low V_{max} for the PTEN-catalyzed dephosphorylation of Ins(1,3,4,5)P_4 implies that this may not be the preferred substrate *in vitro* for this enzyme. These *in vitro* assays may not be reflective of the *in vivo* activity of PTEN because the phosphatase could be regulated by phosphorylation, subcellular localization, and/or interaction with other cellular proteins. It is noteworthy that the C terminus of the phosphatase has a consensus PDZ binding site, and we have recently shown that PTEN interacts with several PDZ-containing proteins.[2]

The activity of PTPases and dual-specific phosphatases to-

[2] Y. Zhao and J. E. Dixon, unpublished data.

ward all protein substrates is dependent upon an essential cysteine residue that forms a phosphoenzyme intermediate during catalysis (1). Because PTEN has the HC*XX*G*XX*R(S/T) motif conserved in all tyrosine or dual-specific phosphatases, we were interested in determining whether the cysteine was essential for the Ins(1,3,4,5)P_4 phosphatase activity. Mutation at Cys^{124} of PTEN (C124S) resulted in a complete loss of enzyme activity toward Ins(1,3,4,5)P_4 (Fig. 3*A*). This mutation also resulted in a loss of phosphoinositide phosphatase activity (data not shown). Additionally, PTEN is extremely labile in the absence of thiols in the assay buffer. Optimum concentration of DTT for the PTEN-catalyzed reactions was 10 mM (data not shown). Therefore, we propose that PTEN-catalyzed dephosphorylation of inositol phosphate and phosphoinositide proceeds via a mechanism that is consistent with that described for other PTPases (1).

Potential Role of PTEN in Intracellular PtdIns(3,4,5)P_3 Regulation—We have established that the recombinant PTEN has phosphoinositide 3-phosphatase and inositol phosphate 3-phosphatase activities. The data shown in Fig. 1 suggest that suppression of insulin-induced PtdIns(3,4,5)P_3 production by overexpression of PTEN is due to its phosphoinositide phosphatase activity. In addition, as shown in Fig. 1*D*, overexpression of the CS mutant caused PtdIns(3,4,5)P_3 accumulation without insulin stimulation. These results strongly suggest that PTEN can act as a regulator of PtdIns(3,4,5)P_3 *in vivo*. Insulin activates PI 3-kinase via tyrosine phosphorylation of insulin receptor substrate-1 catalyzed by the insulin receptor (Fig. 4). PtdIns(3,4,5)P_3 produced by the PI 3-kinase can then activate Akt-mediated signals (Fig. 4). PtdIns(3,4,5)P_3 levels reached a plateau within 3–5 min after the stimulation, and then PtdIns(3,4,5)P_3 is degraded by unknown mechanisms (data not shown). Although our results suggest that PTEN can alter PtdIns(3,4,5)P_3 levels in 293 cells, it is clear that there are other cellular mechanisms that can also alter phosphoinositide concentrations. For example, Guilherme *et al*. (11) reported that a phosphoinositide 5-phosphatase was activated by insulin stimulation, and SHIP (*SH*2 domain-containing *i*nositol 5-*p*hosphatase) is also well known as a regulator of PtdIns(3,4,5)P_3 (10, 19). If PTEN functions *in vivo* as a PtdIns(3,4,5)P_3 phosphatase, it follows that a homozygous deletion/mutation of this tumor suppressor gene could lead to a tumorigenic state through activation of the proto-oncogene, Akt. Specifically, loss of PTEN function would increase cellular levels of PtdIns(3,4,5)P_3, thereby resulting in enhanced activation of Akt. In conclusion, although the physiological function of PTEN needs further clarification, we propose that: (i) members of the PTPase family of enzymes having an active site motif HC*XX*G*XX*R(S/T) such as PTEN are candidates to regulate intracellular levels of nonproteinaceous substrates as has also been reported for the RNA 5′-triphosphatase activity of CEL-1 (20); (ii) the dephosphorylation of phosphoinositide and inositol phosphate by PTEN is specific for position 3 on the inositol ring; and (iii) there are likely to be additional activating and/or localization mechanisms for PTEN within the cell regulating the catalytic activity of this enzyme.

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