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)P₃) 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)P₃ production in human 293 cells without affecting insulin-induced phosphoinositide 3-kinase activation. Further, transfection of the catalytically inactive mutant of PTEN (C124S) caused PtdIns(3,4,5)P₃ accumulation in the absence of insulin stimulation. Purified recombinant PTEN catalyzed dephosphorylation of PtdIns(3,4,5)P₃, specifically at position 3 on the inositol ring. PTEN also exhibited 3-phosphatase activity toward inositol 1,3,4,5-tetrasphosphate. Our results raise the possibility that PTEN acts in vivo as a phosphoinositide 3-phosphatase by regulating PtdIns(3,4,5)P₃ levels. As expected, the C124S mutant of PTEN was incapable of catalyzing dephosphorylation of PtdIns(3,4,5)P₃ 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). 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 HCXGXXR(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-Tyr), (5). Based on these observations we thought it possible that PTEN could catalyze the dephosphorylation of acidic nonproteinaeous 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)P₃ is an important second messenger involved in cell growth signaling (7). PtdIns(3,4,5)P₃ is specifically produced from PtdIns(4,5)P₂ by PI 3-kinase upon stimulation by a variety of ligands (7). Recent studies have identified that PtdIns(3,4,5)P₃ 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)P₃ in vivo is still unclear (10, 11). In the present study we demonstrate that recombinant PTEN has PtdIns(3,4,5)P₃ 3-phosphatase activity. In addition, we provide evidence that PTEN may act in vivo as a regulator of PtdIns(3,4,5)P₃, which produces a substrate that can be recycled by PI 3-kinase.

EXPERIMENTAL PROCEDURES

Determination of PtdIns(3,4,5)P₃ 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′-CCGGTACCCGCCACCATGGACTACAAGGACGACGATGACAAGACAGCCATC-ATCGGATCCGAAGCTT) and 3’ primer (5′-CCGGTACCTACGACTCATTTTTGA-TTGTGA-3′). The product was cleaved with KpnI and SalI and ligated into the KpnISalI 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 was 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 [32P]Pi (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 CH₃OH/CHCl₃/1% HClO₄ (50/25/18, v/v/v). The lipid extract was dried and separated on a TLC plate as described (13) to determine the amount of [32P]PtdIns(3,4,5)P₃. 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 immunoprecipitates, the sample was incubated for 10 min at 37 °C in 33 mM Tris-HCl (pH 7.4), 2.5 mM EGTA, 5 mM MgCl₂, 300 mM NaCl, 0.1 mM EDTA, 2 mM Na pyrophosphate, and 0.25 mM Na orthovanadate. The reaction was terminated by the addition of 0.47 ml of 0.4 M HClO₄ and the lipid products 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 NdeISalI fragment from PTEN/pT7-7 (12) into the SalI 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 lyso buffer (20 mM Tris-HCl [pH 8.2], 2 mM EDTA, 2 mM DTT, 300 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride) and lysed by sonication. The crude lysate was diluted to contain 220 ml of the lyso 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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(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 dialyzed 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)P3 phosphatase assay was performed at 37 °C in a buffer (20 μl) consisting of 100 mM Tris-HCl (pH 8), 10 mM DTT, [γ-32P]PtdIns(3,4,5)P3, and 1 μg of purified PTEN. The reaction was terminated by the addition of 0.47 ml of CH3OH/ClCH2COOH/6% HCOOH (30/15/2, v/v/v). Then the phospholipids were extracted and separated on a TLC plate as described (13). To prepare [γ-32P]PtdIns-(3,4,5)P3, the phosphorylation of PtdIns(4,5)P2 by PI 3-kinase using [γ-32P]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 using anti-p85 antibody (Uptara Biotechnology, Inc.) as described (14). For identification of the dephosphorylation site (see Fig. 2B), dephosphorylation of PtdIns(3,4,5)P3 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)P3 (BIOMOL), 0.15 mg/ml of phosphatidylyserine, and 1 μg of purified PTEN. The reaction was terminated by the addition of 0.47 ml of CH3OH/ClCH2COOH/6% HCOOH (30/15/2, v/v/v). Then 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 [3H]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 [3H]inositol phosphate (0.02 μCi) and 1 μg of enzyme. After an incubation of 30 min, the reaction was terminated by the addition of 1 ml of the stop solution. Then the dephosphorylated product from the substrate, the sample was applied to AG1-X8 column (0.5 ml) equilibrated with the stop solution. Dephosphorylated [3H]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 mM HCOONH4/0.1 mM HCOOH, 0.4 mM HCOONH4/0.1 mM HCOOH, and 0.2 M HCOONH4/0.1 mM HCOOH for [3H]inositol 1, 4-bisphosphate, [3H]Ins(1,4,5)P3, and [3H]Ins(1,3,4,5,6)P5, respectively. Recombinant human VHR, Cdc25B, and PTIP1 were kindly gifted from Harris Vikis, Elizabeth Gottlin, and Jin Zhou, respectively. For kinetic analysis (see Fig. 3B), inositol phosphatase activity was assayed in the same buffer as described above using 10–150 μM Ins(1,3,4,5)P4 instead of [3H]inositol phosphate. After an incubation of 3 min at 37 °C, the reaction was terminated by the addition of 1 ml of ice-cold water, and the amount of dephosphorylation of PtdIns(3,4,5)P3 produced was estimated using BIO-TRAK Ins(1,4,5)P3 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-nitrophosphophosphate and other artificial protein substrates poorly, having the highest catalytic activity with the highly negatively charged, multiply phosphorylated polymer of (Glu-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 phosphatidic lipid species.

Suppression of Insulin-induced PtdIns(3,4,5)P3 Production by Overexpression of PTEN—PtdIns(3,4,5)P3 is an important second messenger in the regulation of cell growth (7). In human 293 cells, insulin stimulates PI 3-kinase activity (Fig. 1C, lanes 1 and 2), resulting in an increase in PtdIns(3,4,5)P3 (Fig. 1A, lanes 1 and 2). When PTEN was overexpressed in the 293 cells, the insulin-induced PtdIns(3,4,5)P3 levels were significantly reduced in a dose-dependent manner (Fig. 1, A and B), whereas no effect on the activation of PI 3-kinase was observed (Fig. 1C, lanes 2 and 4). Because PtdIns(3,4,5)P3 is specifically produced by PI 3-kinase, this result suggests that PTEN directly affects the turnover of PtdIns(3,4,5)P3. Additionally, overexpression of the catalytically inactive mutant (C1245S) of PTEN (see Fig. 3A) caused an accumulation of PtdIns(3,4,5)P3 in the absence of insulin stimulation (Fig. 1D, 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)P3 levels in cells without alteration of the insulin-stimulated PI 3-kinase activity.

PtdIns(3,4,5)P3 3-Phosphatase Activity of PTEN—To investigate the possibility that PTEN has PtdIns(3,4,5)P3 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, [γ32P]PtdIns(3,4,5)P3, using PtdIns(4,5)P2, PI 3-kinase and [γ-32P]ATP. When [γ32P]PtdIns(3,4,5)P3 was incubated with the purified PTEN, the radiolabel of [γ32P]PtdIns(3,4,5)P3 rapidly disappeared from the lipid phase (Fig. 2A, inset) while coincidentally appearing in the aqueous phase (Fig. 2A), 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,
Phosphoinositide 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 (VHR, Cdc25B) exhibited no activity toward this inositol phosphate (Fig. 3A). 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. 3A). The dephosphorylated product was identified as Ins(1,4,5)P_3 using the Ins(1,4,5)P_3-binding protein (Fig. 3B). 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 with an inositol phosphatase 3-phosphatase activity (data not shown). These observations by 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).

**Insitol 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 (VHR, Cdc25B, VHR) exhibited no activity toward this inositol phosphate (Fig. 3A). 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. 3A). The dephosphorylated product was identified as Ins(1,4,5)P_3 using the Ins(1,4,5)P_3-binding protein (Fig. 3B). 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 with 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 \( V_{\text{max}} \) and \( K_m \) values for Ins(1,4,5)P_3 were 98.9 \( \mu \)M and 8.49 nmol/min/mg (\( k_{\text{cat}} \), 0.49 min \(^{-1} \), respectively (Fig. 3B). The \( K_m \) value of 98.9 \( \mu \)M is 250-fold lower than the \( K_m \) of p-nitrophenolphosphate, which is 25.6 mm. Similar comparisons with the phosphorylated polymer (Glu-Tyr), 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 \( V_{\text{max}} \) 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-
ward all protein substrates is dependent upon an essential cysteine residue that forms a phosphoeymine intermediate during catalysis (1). Because PTEN has the HCXGGXXR(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)P₄ phosphatase activity. Mutation at Cys¹²⁴ of PTEN (C124S) resulted in a complete loss of enzyme activity toward Ins(1,3,4,5)P₄ (Fig. 3A). 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)P₃ 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)P₃ production by overexpression of PTEN is due to its phosphoinositide phosphatase activity. In addition, as shown in Fig. 1D, overexpression of the CS mutant caused PtdIns(3,4,5)P₃ accumulation without insulin stimulation. These results strongly suggest that PTEN can act as a regulator of PtdIns(3,4,5)P₃ 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)P₃ produced by the PI 3-kinase can then activate Akt-mediated signals (Fig. 4). PtdIns(3,4,5)P₃ levels reached a plateau within 3–5 min after the stimulation, and then PtdIns(3,4,5)P₃ is degraded by unknown mechanisms (data not shown). Although our results suggest that PTEN can alter PtdIns(3,4,5)P₃ 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 (SH2 domain-containing inositol 5-phosphatase) is also well known as a regulator of PtdIns(3,4,5)P₃ (10, 19). If PTEN functions in vivo as a PtdIns(3,4,5)P₃ 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)P₃, 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 HCXGGXXR(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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