Allosteric Activation of PTEN Phosphatidylinositol 4,5-Bisphosphate*

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Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is a tumor suppressor that is lost in many human tumors and encodes a phosphatidylinositol 4-phosphate phosphatase specific for the 3-position of the inositol ring. Here we report a novel mechanism of PTEN regulation. Binding of di-C8-phosphatidylinositol 4,5-P2 (PI(4,5)P2) to PTEN enhances phosphatase activity for monodispersed substrates, PI(3,4,5)P3 and PI(3,4)P2. PI(5)P also is an activator, but PI(4)P, PI(3,4)P2, and PI(3,5)P2 do not activate PTEN. Activation by exogenous PI(4,5)P2 is more apparent with PI(3,4)P2 as a substrate than with PI(3,4,5)P3, probably because hydrolysis of PI(3,4,5)P3 yields PI(4)P, which is not an activator. In contrast, hydrolysis of PI(3,4,5)P3 yields a potent activator, PI(4,5)P2, creating a positive feedback loop. In addition, neither di-C4-PI(4,5)P2 nor inositol trisphosphate-activated PTEN. Hence, the interaction between PI(4,5)P2 and PTEN requires specific, ionic interactions with the phosphate groups on the inositol ring as well as hydrophobic interactions with the fatty acid chains, likely mimicking the physiological interactions that PTEN has with the polar surface head groups and the hydrophobic core of phospholipid membranes. Mutations of the apparent PI(4,5)P2-binding motif in the PTEN N terminus severely reduced PTEN activity. In contrast, mutation of the C2 phospholipid-binding domain had little effect on PTEN activation. These results suggest a model in which a PI(4,5)P2 monomer binds to PTEN, initiates an allosteric conformational change and, thereby, activates PTEN independent of membrane binding.

Phosphatase and tensin homologue deleted on chromosome 10 (PTEN)† (also known as MMAC and TEP) was originally identified as a tumor suppressor for gliomas (1–3). We now know that PTEN is deleted or inactivated in many tumor types, including endometrial, melanoma, and prostate (4). The PTEN protein is a phosphatidylinositol phosphate (PIP) phosphatase specific for the 3-position of the inositol ring (5). By lowering levels of phosphatidylinositol 3,4,5-P3 (PI(3,4,5)P3), PTEN inhibits cell proliferation, induces apoptosis and decreases cell motility (6, 7). Knock-out mice lacking PTEN are embryonic lethal, whereas mice with one copy of PTEN have a diminished Fas-mediated apoptosis and a high incidence of cancer (8).

Neural precursor cells from PTEN +/+ mice show enhanced cell motility and invasiveness and are resistant to hydrogen peroxide-induced apoptosis (9). In response to some mitogens, PTEN +/+ lymphocytes proliferate faster than +/+ lymphocytes (8, 10). These results further demonstrate that cells are highly quantitatively dependent on PTEN levels, and hence, understanding regulation of PTEN activity is critical. Here we report a novel mechanism of PTEN regulation in which binding of phosphatidylinositol 4,5-P2 (PI(4,5)P2) to PTEN activates the phosphatase for monodispersed substrates, PI(3,4)P2 and PI(3,4,5)P3, likely by an allosteric conformational change.

EXPERIMENTAL PROCEDURES

Phosphatidylinositol and Inositol Phosphates—All phosphatidylinositol-4-phosphates were purchased from Echelon Research Laboratories (Salt Lake City, UT). Unless noted otherwise, the synthetic lipids used in these experiments have di-C8, saturated fatty acid chains. Measurements of the critical micelle concentration (CMC) are not available in the literature for C8-PIPs and would be cost prohibitive to determine. However, the CMCs for these multiply charged PIPs can be estimated by comparison with the CMC for C8-PI, which is 60 μM (11). Addition of phosphates to the inositol ring would increase head group size and charge and, thereby, increase the CMC (12). For example, the CMC of di-C8-phosphatidylserine is 2.28 mM (13). Because the CMC concentrations in these experiments are much less than the predicted CMCs, these PIPs may be monodispersed and do not form micelles or membranes. This prediction is confirmed by the lack of turbidity observed for all of the PIP solutions used in these experiments. Finally, d-myoinositol-1,4,5-trisphosphate (IP3) was purchased from Sigma.

Preparation of PTEN Proteins—Recombinant human PTEN was prepared using the expression vector, pGEX-3X (Amersham Biosciences). The GST-PTEN fusion protein was expressed in DH5α bacteria, and the cells were lysed with a French press. The GST-PTEN protein was purified using glutathione-Sepharose beads (Amersham Biosciences) and dialyzed overnight at 4 °C against 100 mM Tris, 250 mM NaCl, 10 mM dithiothreitol, pH 7.5. Using Coumassie Blue-stained SDS-PAGE gels, concentrations of GST-PTEN proteins were determined by comparison with bovine serum albumin standards. Aliquots of the purified proteins were stored at −20 °C for up to 2 weeks.

PTEN mutants were prepared with the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Lipid Phosphatase Assay—The PIP phosphatase assay of Maehama et al. (14) was adapted to a 96-well format. PIPs were dissolved in the dialysis buffer described above. GST-PTEN (0.3 μg per assay) was added to initiate the reaction (total volume of 22.5 μL per well of 96-well enzyme-linked immunosorbent assay microplates (model 3389) from Corning Inc.). The reaction was carried out at 37 °C and terminated with 195 μL per well of BIOMOL GREEN (BIOMOL, Plymouth Meeting, PA), which is a malachite green solution that shows increased absorbance in the presence of free phosphate. The green color was allowed to develop for 30 min, and the absorbances for the phosphatase products were measured at 595 nm with a microplate reader. Each data point was assayed in duplicate, and all experiments were repeated at least three times.

Phosphatidylinositol 3,4,5-trisphosphate; CMC, critical micelle concentration; IP3, d-myoinositol 1,4,5-trisphosphate.
RESULTS

To better understand the mechanism by which PTEN hydrolyzes monodispersed PIPs, we measured reaction rates for varying concentrations of PI(3,4,5)P3. As seen in Fig. 1, release of free phosphate was more rapid for higher concentrations of PI(3,4,5)P3. However, the kinetic curves did not follow the typical Michaelis-Menten form, especially at higher PI(3,4,5)P3 levels. These kinetic curves were sigmoidal, indicating that the enzymatic activity increases as the reaction progresses, and suggest a novel mechanism for the regulation of PTEN.

One possible explanation is that the PTEN product, PI(4,5)P2, is a positive regulator of PTEN activity. In fact, binding of PI(4,5)P2 is known to regulate a number of other membrane-associated proteins (15). To test this hypothesis, we added varying concentrations of PI(4,5)P2 in addition to a constant amount of PI(3,4,5)P3. Addition of 22.5 or 45 μM PI(4,5)P2 increased the reaction rate (Fig. 2A). In parallel experiments using PI(3,4)P2 as the substrate, addition of the PI(4,5)P2 had a more dramatic effect on the reaction rate (Fig. 2B). In the absence of PI(4,5)P2, there was little hydrolysis of the PI(3,4)P2, consistent with an earlier report that PI(3,4)P2 is a poor substrate compared with PI(3,4,5)P3 (16). Hence, the apparent lack of PI(3,4)P2 hydrolysis by PTEN probably reflects the absence of the PI(4,5)P2 activator rather than the poor capacity of PI(3,4)P2 to serve as a substrate.

We next measured PTEN activation as a function of PI(4,5)P2 concentration (Fig. 2C). PI(4,5)P2 increased the initial rates of PI(3,4)P2 hydrolysis in a dose-dependent and saturable manner. PTEN activation was half-maximal at 20.2 ± 3.8 μM PI(4,5)P2. In these assays, the total concentration of PI(3,4)P2 and PI(4,5)P2 was substantially below the predicted CMC (see “Experimental Procedures”), indicating that activation of PTEN was not induced by or dependent upon formation of a membrane or mixed micelle with PI(3,4)P2. These data suggest that activation was initiated by binding of PI(4,5)P2 to a specific site on PTEN and not by anchoring PTEN to an artificial membrane or micelle.

Given the prediction of a specific binding site for PI(4,5)P2, we tested other PIPs for PTEN activation. PI(5)P was not a substrate for PTEN but did promote the hydrolysis of PI(3,4,5)P3 (Fig. 3A). In contrast, PI(4)P did not activate PTEN phosphatase activity. Neither PI(4)P nor PI(5)P changed the end point for the reaction, further indicating that they are not substrates for PTEN and do not alter the specificity of PTEN for phosphates on 3-position of the inositol ring. We also found that PI(3,5)P2 is a poor substrate for PTEN (Fig. 3B). Addition of PI(4,5)P2 increased the enzymatic activity, but even in the presence of PI(4,5)P2, PI(3,5)P2 is a relatively poor substrate compared with PI(3,4,5)P3 or PI(3,4)P2. Since PI(4,5)P2 enhances PTEN hydrolysis of PI(3,4,5)P3, PI(4,5)P2 cannot be a strong activator. However, because the product of PI(3,4,5)P3 hydrolysis is a strong activator, we cannot say whether PI(3,4)P2 is a weak activator. These results show that PI(4,5)P2 and PI(5)P are potent activators, but PI(4)P, PI(3,4)P2 and PI(3,5)P2 have little or no activating capacity at 45 μM. Hence, PTEN is activated by a subset of PIPs, consistent with binding of activators to a specific site on PTEN.

We next tested whether the glycerol backbone and fatty acid chains of PI(4,5)P2 are required for PTEN activation. As noted, PI(4,5)P2 with eight-carbon (C8) fatty acid chains increased PTEN hydrolysis of PI(3,4,5)P3 (Fig. 4A). In contrast, PI(4,5)P2 with four-carbon (C4) fatty acid chains did not appreciably activate PTEN. In addition, IP3, which is the soluble headgroup from PI(4,5)P2, did not increase the phosphatase reaction. Hence, the interaction between PI(4,5)P2 and PTEN requires specific, ionic interactions with the phosphate groups on the inositol ring as well as hydrophobic interactions with the fatty acid chains, likely mimicking the physiological interactions that PTEN has with the polar surface headgroups and the hydrophobic core of phospholipid membranes.
Since PI(4,5)P₂ likely binds to an exclusive site on PTEN, the next goal was to identify residues critical for activation and phosphatase action. The two most obvious candidates are the known lipid-binding sites, which include the proposed N-terminal domain and the C2 lipid-binding domain (17). Studies of other PI(4,5)P₂-binding proteins have led to a consensus sequence for PIP₂ binding (18), and two groups have proposed a PI(4,5)P₂-binding site at the PTEN N terminus that is distinct from the phosphatase active site (16, 19) (Fig. 5A). Finally, there is a C2 lipid-binding domain in the C terminus that binds a variety of lipid types (17). Phospholipid binding to the C2 domain is dependent upon a series of positively charged residues in the CBR3 loop, and M-CBR3, a mutant C2 domain with four alanines substituted for four lysines (Fig. 5A), completely lacked phospholipid membrane binding (17).

We first tested mutations in the N terminus. Mutations of lysine 13 or arginine 14, which are part of the consensus sequence for the PI(4,5)P₂-binding motif, reduced the rates of hydrolysis of PI(3,4,5)P₃ and PI(3,4)P₂ in both the absence and presence of PI(4,5)P₂ (Fig. 5, B and C). In addition, mutation of arginine 15 to alanine occurs in human cancers and is reported to reduce PTEN activity (3, 6). In agreement, we found that the R15A mutant PTEN completely lacked activity in both the presence and absence of the PI(4,5)P₂ activator (data not shown). Finally, the M-CBR3 mutant PTEN showed activation by PI(4,5)P₂, but activity was somewhat diminished (Fig. 5D). These data confirm the critical role of the N terminus in PTEN from the phosphatase active site (16, 19) (Fig. 5A). Finally, there is a C2 lipid-binding domain in the C terminus that binds a variety of lipid types (17). Phospholipid binding to the C2 domain is dependent upon a series of positively charged residues in the CBR3 loop, and M-CBR3, a mutant C2 domain with four alanines substituted for four lysines (Fig. 5A), completely lacked phospholipid membrane binding (17).

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activity and show that the C2 phospholipid-binding site is not required for activation by PI(4,5)P2.

**DISCUSSION**

In this study, we propose that PI(4,5)P2 binds to a site distinct from the phosphatase active site, induces an allosteric conformational change, and, thereby, activates PTEN, leading to a positive feedback loop for PTEN activation. The activator- and substrate-binding sites must be distinct, because the active site is not large enough to bind PI(3,4,5)P3 and PI(4,5)P2 simultaneously (17), and if PI(4,5)P2 did bind to the active site, it would be a competitive inhibitor, which is obviously not the case. Finally, activation of PTEN was half-maximal at 20.2 ± 3.8 μM PI(4,5)P2. Since this value is close to the effective concentration of PI(4,5)P2 in cells, about 10 μM (15), PTEN activation is a likely mechanism for PIP regulation in cells. Our model predicts that PTEN would be preferentially activated at the PI(4,5)P2-bearing plasma membrane or at PI(4,5)P-rich membrane domains. There are some similarities between our model for PTEN activation and that already proposed for protein kinase C. Both require membrane binding through two distinct sites, thereby insuring that activation occurs only at a membrane surface (20).

The activation of PTEN is specific for particular PIPs. PI(5)P and PI(4,5)P2 activate the PTEN phosphatase. However, PI(4)P, PI(3,4)P2, and PI(3,5)P2 do not show apparent activation. This specificity helps us to understand why activation is more apparent with PI(3,4)P2 as a substrate than with PI(3,4,5)P3 as a substrate. Hydrolysis of PI(3,4)P2 yields PI(4)P, which is not an activator. In contrast, hydrolysis of PI(3,4,5)P3 yields a potent activator, PI(4,5)P2. Hence, the reaction accelerates as PI(3,4,5)P3 is hydrolyzed to PI(4,5)P2 (Fig. 1), forming a positive feedback loop.

The analysis of PTEN mutants leads us to three conclusions concerning the mechanism of PTEN activation. First, because the N terminus has an apparent PI(4,5)P2-binding motif (Fig. 5A) and is required for association of Dictyostelium PTEN with the plasma membrane (19), the N terminus has been suggested as a PI(4,5)P2-binding site. We found that activation of PTEN by PI(4,5)P2 is consistent with a single binding site with \( K_{\text{act}} = 20.2 \pm 3.8 \) μM (Fig. 2C), and mutations in the N terminus severely decrease PTEN activity (Fig. 5, B and C). Therefore, the N terminus is the best candidate for the PI(4,5)P2-binding site. Second, the previous assignment (3, 6) of arginine 15 as an essential residue for PTEN phosphatase activity is correct. Even in the presence of 45 μM PI(4,5)P2, which is nearly five times the effective concentration of this activator in cells, the R15A PTEN mutant shows no phosphatase activity. Third, because activation is observed for the M-CBR3 mutant PTEN (Fig. 5D), which lacks phospholipid binding to the C2 domain (17), activation is not through the nonspecific C2 phospholipid-binding site. There are two caveats in this interpretation. First, even though the PTEN N terminus matches the PI(4,5)P2-binding motif exactly, PI(4,5)P2 binding for these motifs is dependent on the three-dimensional protein fold (15). Unfortunately, the first 13 residues of the N terminus are not included in the current PTEN crystal structure; further structural studies are needed to resolve this issue. Second, the N-terminal mutations might affect the folding of the PTEN phosphatase domain rather than altering PI(4,5)P2 binding. We believe that this is not likely, because the PI(4,5)P2-binding motif is distinct from the phosphatase active site. Furthermore, this region of PTEN is poorly ordered (17), implying that the N terminus is flexible and, therefore, unlikely to affect the integrity of the phosphatase active site.

While these data were being prepared for publication, two related publications appeared. First, Schaehtzky et al. (21) reported that myotubulin, which hydrolyzes PI(3,5)P2 to PI(5)P, is activated by its PI(5)P product. It is intriguing that this PIP phosphatase is activated in a similar manner as PTEN, suggesting that positive feedback loops are a common mechanism for regulating PIP phosphatases. Second, McConnellachie et al. (22) found, using membrane-bound substrates, that PI(4,5)P2 activates PTEN. Although the mechanism was not certain, these authors suggested that the activation was due to increased binding of PTEN to the membrane, thereby increasing local concentration of the PI(3,4,5)P3 substrate. In this study, we used synthetic lipids with di-C8 fatty acid chains, and in all cases, these lipids were used at concentrations below the predicted CMC (see “Experimental Procedures”). Hence, at the concentrations used in these experiments, the PIPs do not form micelles or membranes. Therefore, there are no local concentration effects and we propose that activation occurs by a PI(4,5)P2-induced allosteric conformational change. The models presented in this study and McConnellachie et al. (22) are not mutually exclusive, and our view is that both mechanisms are important for the regulation of PTEN.

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