A phosphorylation-dependent intramolecular interaction regulates the membrane association and activity of the tumor suppressor PTEN

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The PI 3-phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome 10), one of the most important tumor suppressors, must associate with the plasma membrane to maintain appropriate steady-state levels of phosphatidylinositol 3,4,5-triphosphate. Yet the mechanism of membrane binding has received little attention and the key determinants that regulate localization, a phosphatidylinositol 4,5-bisphosphate (PIP_2) binding motif and a cluster of phosphorylated C-terminal residues, were not included in the crystal structure. We report that membrane binding requires PIP_2 and show that phosphorylation regulates an intramolecular interaction. A truncated version of the enzyme, $PTEN_{1-351}$, bound strongly to the membrane, an effect that was reversed by co-expression of the remainder of the molecule, $PTEN_{352-403}$. The separate fragments associated in vitro, an interaction dependent on phosphorylation of the C-terminal cluster, a portion of the PIP_2 binding motif, integrity of the phosphatase domain, and the CBR3 loop. Our investigation provides direct evidence for a model in which PTEN switches between open and closed states and phosphorylation favors the closed conformation, thereby regulating localization and function. Small molecules targeting these interactions could potentially serve as therapeutic agents in antagonizing Ras or PI3K-driven tumors. The study also stresses the importance of determining the structure of the native enzyme.

iRAP | phosphatase | PI3K | PIP2 | PIP3

PTEN (phosphatase and tensin homologue deleted on chromosome 10) is a lipid phosphatase that dephosphorylates phosphatidylinositol-3,4,5-triphosphate (PIP_3) and opposes PI3K signaling (1). This important tumor suppressor is frequently mutated in endometrium, prostate, and brain cancer, and alterations of PTEN activity leads to Cowden and Bannayan-Zonana syndromes in which patients develop benign hamartomas (2–4). Mice homozygous for a deletion of PTEN die in early embryogenesis whereas heterozygotes have a propensity to develop widespread neoplasms (5). Prostate-directed conditional deletions of PTEN result in metastatic prostate cancer (6–8). Neural and astrocyte-specific deletions lead to greatly enlarged brain size and abnormalities in astrocytes and neurons (9). Cultured mammalian cells lacking PTEN proliferate faster, resist apoptosis, and migrate aberrantly (10–12). *Dictyostelium discoideum* cells lacking PTEN have elevated PIP_3 and are severely defective in chemotaxis (13).

Regulation of PTEN activity, localization, and function are controlled by a variety of mechanisms. NOTCH1, by acting through transcription factors CBF-1 and MYC or HES-1, respectively, has been reported to either increase or decrease PTEN expression (14–16). Another level of regulation is post-translational modification. Acetylation by p300/CBP-associated factor blocks PTEN activity (17). PTEN catalytic activity is negatively regulated by reactive oxygen species under oxidative stress (18–20) by disulfide linkage of catalytic cysteine 124 with cysteine 71 (18). E3 ubiquitin ligase NEDD4–1 can mono- and poly-ubiquitinate PTEN (21). The

mono-ubiquitinated protein displays increased translocation to the nucleus and the poly-ubiquitinated form undergoes rapid degradation. Finally, phosphorylation of the C-terminal residues regulate protein stability and function in cells (22). In addition, PTEN is phosphorylated by a series of kinases, including RhoA-associated kinase (23), casein kinase 2, and glycogen synthase 3b (24–26).

Although the main substrate of PTEN is at the plasma membrane, the enzyme is mainly found in cytosol and the nucleus, but a small fraction is dynamically associated with the inner face of the plasma membrane (27). This interaction is critical, as mutations that do not affect catalytic activity against soluble substrates but impair membrane binding, such as deletion of an N-terminal "PIP_2 binding" motif, lead to a null phenotype in cells (27–29). Consistently, addition of PIP_2 to PIP_3-containing vesicles makes them more effective substrates (30). Furthermore, there are tumor-derived mutations that do not reduce catalytic activity in vitro but effectively inactivate PTEN by preventing membrane association (31). In addition to the PIP_2 binding motif, a globular phosphatase domain, and a C2 domain that binds lipid vesicles, human PTEN has a 51-aa C terminus that contains a cluster of phosphorylation sites. This cluster, thought to be the target of casein kinase 2, is an important regulatory region, as a version of PTEN with alanine substitutions of these phosphorylation sites, designated $PTEN_{A4}$, displays greatly increased membrane association (32). The PIP_2 binding motif and the entire C terminus were in the crystallized protein (33). Thus, the structures and conformations of the enzyme including the regions most critical for membrane association and function in cells are unknown.

Single-molecule imaging studies have shown that the increased steady-state levels of $PTEN_{A4}$ on the membrane results from an increased association rate whereas the lifetimes of PTEN and $PTEN_{A4}$ on the membrane were identical. This behavior is consistent with a model wherein PTEN exists in "closed" and "open" conformations, regulated by phosphorylation, and that the open conformation has a more favorable interaction with the membrane. Here we demonstrate that the C terminus of PTEN directly interacts with the remainder of the molecule in a phosphorylation-dependent manner, as well as plasma membrane binding and activity. Furthermore, we demonstrate that depletion of PIP_2 from the plasma membrane causes a concomitant loss of PTEN binding sites.

Results

As previously shown, the localization of $PTEN_{WT}$-YFP and $PTEN_{A4}$-YFP are dramatically different. In HEK293T cells, mem-
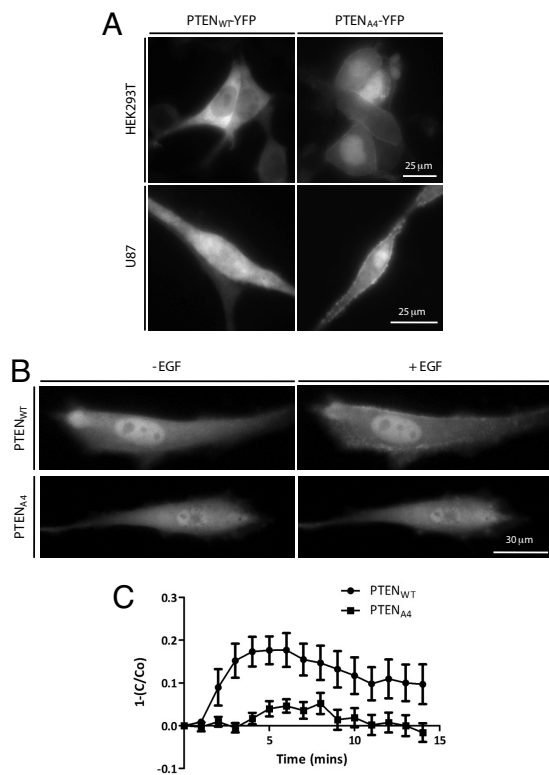
Fig. 1. PTEN cellular activity is proportional to the extent of membrane association. (*A*) HEK293T (*Upper*) and PTEN-null U87 cells (*Lower*) were transiently transfected with either PTEN$_{WT}$-YFP or PTEN$_{A4}$-YFP. Localization of the respective proteins was assessed. (*B*) U87 cells expressing PH$_{AKT}$-GFP were co-transfected with either PTEN$_{WT}$ or PTEN$_{A4}$. PH$_{AKT}$-GFP localization was monitored after addition of 20 ng/mL EGF to serum starved cells. Examples are representative of 20 respective cells. Whereas two cells co-expressing PTEN$_{WT}$ and PH$_{AKT}$-GFP failed to respond to EGF stimulation, all PTEN$_{A4}$ co-transfected cells failed to respond. (*C*) Depletion of the cytosolic GFP signal was measured. Cytoplasmic GFP intensity was compared with the initial value (C/C$_0$) and subtracted from 1. Error bars represent SEM.

brane association of PTEN$_{WT}$-YFP was not discernible by epifluorescence, whereas PTEN$_{A4}$-YFP with alanine substitutions S380A, T382A, T383A, and S385A showed significant localization to the membrane (Fig. 1*A*). This effect did not require catalytic activity, as PTEN$_{C124S,A4}$-YFP, which lacks lipid and protein phosphatase activity, showed even greater association with the membrane (data not shown). Quantitative analysis of multiple cells expressing PTEN$_{WT}$-YFP or PTEN$_{A4}$-YFP indicated a membrane to cytosol ratio of 0.02 ± 0.03 ($n = 7$) versus 0.24 ± 0.07 ($n = 8$), respectively. Levels of PTEN$_{A4}$-YFP in the cytosol were lower and a significant pool was localized to the nucleus. A similar difference was also found when PTEN$_{WT}$-YFP and PTEN$_{A4}$-YFP were expressed in U87 cells. The membrane to cytosol ratios were 0.01 ± 0.02 ($n = 7$) and 0.22 ± 0.07 ($n = 5$) for PTEN$_{WT}$-YFP and PTEN$_{A4}$-YFP, respectively [supporting information (SI) Fig. S1].

Because the PTEN substrate PIP$_3$ is localized at the membrane, we reasoned that, in a cellular context, PTEN$_{A4}$ would display greater activity than PTEN$_{WT}$. We examined the relative ability of PTEN$_{WT}$ and PTEN$_{A4}$ to reduce PIP$_3$ levels by monitoring EGF-induced translocation of the PIP$_3$ probe PH$_{AKT}$-GFP to the membrane in individual cells. U87 cells transfected with PH$_{AKT}$-GFP alone showed high levels of membrane association (Fig. S2). Following co-transfection with PTEN$_{WT}$ or PTEN$_{A4}$, these levels were no longer detectable (Fig. 1*B Left*). When cells co-expressing PTEN$_{WT}$ were stimulated with 20 ng/mL EGF, PH$_{AKT}$-GFP translocated to the plasma membrane coincident with cytosolic deple-
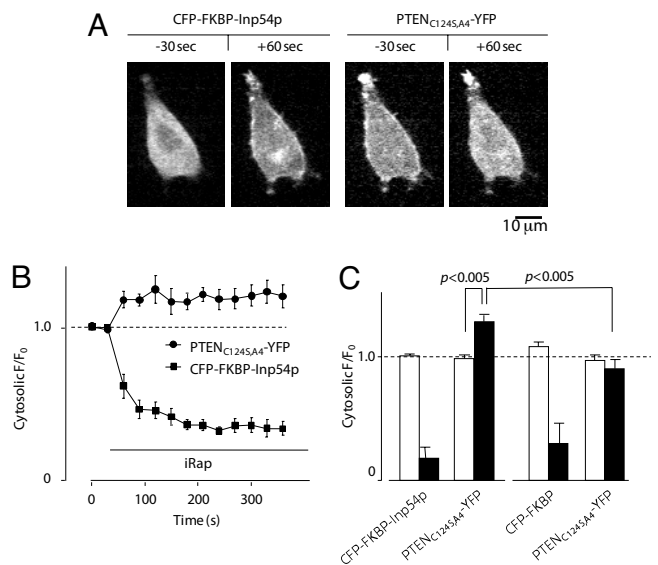

Fig. 2. PTEN membrane binding requires PIP$_2$. (*A*) HeLa cells were transiently co-transfected with CFP-FKBP-Inp54p and PTEN$_{C124S,A4}$-YFP. Localization of respective proteins was observed 60 seconds after iRap treatment. (*B*) Change in cytosolic fluorescence intensity of CFP-FKBP-Inp54p and PTEN$_{C124S,A4}$-YFP was analyzed upon iRap addition. (*C*) Effect of Inp54p on PTEN$_{C124S,A4}$-YFP localization was assessed by transient co-transfection of probe lacking PIP$_2$ phosphatase domain (i.e., CFP-FKBP). Open and closed bars represent cytosolic levels (F/F$_0$) before and after addition of iRap, respectively. Error bars represent SEM.

tion of the probe. Membrane levels remained elevated for 15 min (Fig. 1 *B* and *C* and Movie S1). In contrast, stimulation of cells co-expressing PTEN$_{A4}$ elicited only a slight transient decrease in cytoplasmic levels of PH$_{AKT}$-GFP, whereas no observable membrane translocation was observed (Movie S2). The extent of inhibition up to 15 min was 87%. Based on these inferred PIP$_3$ levels, we estimated PTEN$_{A4}$ was at least sevenfold more active than PTEN$_{WT}$. Furthermore, as PTEN$_{A4}$ is typically expressed at slightly lower levels then PTEN$_{WT}$, the relative activity of PTEN$_{A4}$ is likely to be higher. PTEN$_{A4}$ was also effective at lowering steady-state levels of phosphorylated AKT1 (data not shown and ref. 35). These observations suggest that activity is dependent on extent of membrane binding and that phosphorylation of the C-terminal cluster has an inhibitory effect on membrane association and activity.

The relatively higher levels of PTEN$_{A4}$ binding to the membrane allowed us to ask whether PIP$_2$ was required for the interaction. Although its PIP$_2$ binding motif is required for membrane localization of PTEN and addition of PIP$_2$ to PIP$_3$-containing vesicles make them more effective substrates, a requirement of PIP$_2$ for PTEN binding to the membrane in cells has not been demonstrated to our knowledge. To assess the need for PIP$_2$, we expressed PTEN$_{C124S,A4}$-YFP in HeLa cells equipped with an engineered system previously shown to conditionally deplete PIP$_2$ from the inner leaflet of the plasma membrane (34). The cellular distribution of PTEN$_{C124S,A4}$-YFP was continuously monitored whereas PIP$_2$ was rapidly depleted by forced translocation of a PIP$_2$-specific phosphatase, inositol polyphosphate-5-phosphatase (Inp54p), to the plasma membrane. As the probe was recruited to the membrane, PTEN$_{C124S,A4}$-YFP rapidly re-localized to the cytosol, indicating that membrane-associated binding sites for PTEN had disappeared (Fig. 2 *A* and *B*). PTEN$_{C124S,A4}$-YFP re-localization was not observed when a dimerization probe lacking a phosphatase domain was used (Fig. 2*C*). These studies strongly suggest that PIP$_2$ is required for high-affinity binding of PTEN$_{C124S,A4}$-YFP to the membrane.

We hypothesized that the mechanism of inhibition depends on a direct association of the C-terminal region with the rest of the

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Fig. 3. PTEN$_{1-351}$ binds strongly to the plasma membrane and co-expression of the phosphorylated C-terminal domain, PTEN$_{352-403}$, blocks this interaction. (*A*) Schematic of PTEN$_{WT}$-YFP, PTEN$_{1-351}$-CFP, PTEN$_{352-403}$-YFP, and PTEN$_{352-403(A4)}$-YFP. (*B*) Depiction of the strategy used to assess the effects of PTEN$_{352-403}$ on membrane binding of PTEN$_{1-351}$. (*C*) U87MG cells were transiently transfected with PTEN$_{1-351}$-CFP alone or co-transfected with PTEN$_{352-403}$-YFP or PTEN$_{352-403(A4)}$-YFP for 48 h. Images of cells expressing both constructs were taken using CFP and YFP filter sets, and localization of the respective constructs was assessed.

protein that prevents membrane binding. To test this hypothesis, we removed residues 352 through 403, the flexible region beyond the C2 domain, and fused the remainder of the protein to CFP (Fig. 3 *A* and *B*). Strikingly, when expressed in U87 cells, the association of PTEN$_{1-351}$-CFP with the plasma membrane was greatly increased, with a distribution similar to that observed for PTEN$_{A4}$-YFP (compare Figs. 1*A* and 3*C Upper*). Similar results were obtained in HEK293T cells (data not shown). To further demonstrate that the C-terminal region inhibited the interaction of PTEN$_{WT}$ with the membrane, we co-expressed PTEN$_{352-403}$-YFP and found that it reversed the enhanced membrane binding of PTEN$_{1-351}$-CFP (Fig. 3*C* and Fig. S7). Importantly, the C-terminal portion of PTEN$_{A4}$, PTEN$_{352-403(A4)}$-YFP, did not reduce the increased PTEN$_{1-351}$-CFP membrane binding, indicating that phosphorylation of S380, T382, T383, and/or S385 are critical for the presumed intramolecular interaction. Whether co-expressed with PTEN$_{1-351}$-CFP or independently expressed, neither PTEN$_{352-403(WT)}$-YFP nor PTEN$_{352-403(A4)}$-YFP showed significant membrane association (Fig. 3*C Lower*). These observations suggest that a phosphorylation-dependent intramolecular interaction modulates PTEN membrane association and activity.

To directly test the phosphorylation-dependent interaction of the C- and N-terminal portions of the protein, we carried out a series of co-expression and co-immunoprecipitation experiments. We observed that PTEN$_{1-351}$-CFP-FLAG was able to effectively co-immunoprecipitate PTEN$_{352-403}$-YFP (Fig. 4*A*). However, it was not able to appreciably co-immunoprecipitate PTEN$_{352-403(A4)}$-YFP. The C-terminal fragment was invariably expressed at much higher levels, but approximately stoichiometric amounts of the N- and C-terminal fragments were bound in the immunoprecipitates

Fig. 4. The phosphorylated C-terminal domain, PTEN$_{352-403}$, interacts specifically with PTEN$_{1-351}$. (*A*) HEK293T cells were transiently co-transfected with PTEN$_{1-351}$-CFP-FLAG and either PTEN$_{352-403}$-YFP or PTEN$_{352-403(A4)}$-YFP. Cell lysates were immunoprecipitated and probed with anti-GFP antibody. (*B*) Lysates from HEK293T cells expressing PTEN$_{352-403}$-YFP or PTEN$_{352-403(A4)}$-YFP were analyzed by Western blot using anti-phosphorylated PTEN (i.e., S380) antibody. (*C*) Interaction of PTEN$_{1-351}$-CFP-FLAG with single alanine substituted PTEN$_{352-403}$-YFP (S380A, T382A, T383A, or S385A) was assessed. (*D*) Lysates from HEK293T cells expressing either PTEN$_{1-351}$-CFP-FLAG or PTEN$_{352-403}$-YFP were mixed in a 1:1 ratio. The mixture was subjected to previously described immunoprecipitation and Western blot analysis using anti-GFP antibody. The data were compared with co-expression experiments. (*E*) Cell lysate from HEK293T cells transfected with PTEN$_{352-403}$-YFP-FLAG were divided and immunoprecipitated. The immunoprecipitates were treated with and without protein phosphatase, washed, and mixed with cell lysates from HEK293T cells expressing PTEN$_{1-351}$-CFP. The mixture was immunoprecipitated and probed using anti-GFP antibody. (*F*) The cell lysate before first immunoprecipitation was analyzed by Western blot using anti-phospho-PTEN (i.e., S380) antibody and compared with immunoprecipitated fractions with and without phosphatase treatment.

(ratio of C-terminal to N-terminal, 0.79 ± 0.14; $n = 5$). To verify that PTEN$_{352-403}$-YFP was phosphorylated, we expressed it in HEK293T cells and performed immunoblotting with antibody specific for S380. As shown in Fig. 4*B*, PTEN$_{352-403}$-YFP was phosphorylated, whereas, as predicted, PTEN$_{352-403(A4)}$-YFP was not. These observations confirm a phosphorylation-dependent interaction between the N- and C-terminal regions of the protein. We then examined whether alanine substitutions of single phosphorylation sites would disrupt the interaction. PTEN$_{1-351}$-CFP-FLAG was co-expressed with PTEN$_{352-403}$-YFP containing S380A, T382A, T383A, or S385A substitution, and immunoprecipitated using anti-FLAG affinity beads. It was observed that substitution of any single phosphorylated residue in the cluster resulted in a loss of affinity for PTEN$_{1-351}$-CFP (Fig. 4*C*), suggesting all of the sites are required for optimal interaction.

We next tested whether the interaction of PTEN$_{1-351}$ with PTEN$_{352-403}$ required co-translation or whether the mature

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proteins could associate. We performed a series of lysate mixing experiments to test the interaction and to determine whether it is phosphorylation-dependent. PTEN$_{1-351}$-CFP-FLAG, PTEN$_{352-403}$-YFP, or PTEN$_{352-403(A4)}$-YFP were separately expressed in HEK293T cells. The appropriate lysates were mixed and the samples were then immunoprecipitated using anti-FLAG affinity beads. Fig. 4D shows that PTEN$_{1-351}$-CFP interacted selectively with PTEN$_{352-403}$ but not with PTEN$_{352-403(A4)}$. Furthermore, the ratios of PTEN$_{352-403}$YFP co-immunoprecipitated with PTEN$_{1-351}$-CFP-FLAG were similar whether they were co-expressed or mixed after lysis. Thus, the interaction does not require co-translation and can occur at dilutions of more than 500 fold compared with the cellular volume, indicating a specific, high-affinity binding reaction.

This approach allowed us to directly show that the intramolecular interaction of PTEN$_{1-351}$ and PTEN$_{352-403}$ is phosphorylation-dependent. In a similar lysate mixing experiment, we separately expressed PTEN$_{352-403}$-YFP-FLAG and PTEN$_{1-351}$-CFP in HEK293T cells. The PTEN$_{352-403}$-FLAG was immunoprecipitated from the lysate and treated with λ-protein phosphatase, while a parallel sample was similarly treated in the absence of protein phosphatase. The samples were washed, lysate containing PTEN$_{1-351}$-CFP was added, the reaction was allowed to proceed, and the beads were collected again. As shown in Fig. 4E, the phosphatase treatment resulted in a nearly complete loss of the interaction. Consistently, Western blot analysis of PTEN$_{352-403}$-YFP-FLAG treated with λ-phosphatase verified the elimination of S380 phosphorylation (Fig. 4F). These observations confirm the findings from the alanine substitutions and definitively show that the interaction can be regulated by reversible phosphorylation.

To show that there is a phosphorylation-dependent intramolecular interaction within the native PTEN molecule, we designed a series of experiments illustrated in Fig. S3A. We envision that phosphorylation of the C-terminal region promotes a "closed" conformation that would be expected to prevent interaction of an exogenous phosphorylated C-terminal fragment. Conversely, PTEN$_{A4}$ is expected to be in an "open" conformation in which the endogenous C-terminal portion would not interfere with its exogenous counterpart (Fig. S3A). These possibilities were assessed by comparing the interaction of PTEN$_{352-403}$-YFP-FLAG with either PTEN$_{WT}$-YFP or PTEN$_{A4}$-YFP. As shown in Fig. S3B, PTEN$_{A4}$-YFP showed significantly greater association with PTEN$_{352-403}$-YFP-FLAG than did PTEN$_{WT}$-YFP. Also, PTEN$_{A4}$-YFP association with PTEN$_{352-403}$-YFP was comparable to that of PTEN$_{1-351}$-CFP. All of these observations are consistent with a phosphorylation-dependent intramolecular interaction with the native PTEN molecule.

We next investigated the determinants involved in the binding of PTEN$_{352-403}$ to PTEN$_{1-351}$. As shown earlier, PTEN$_{C124S,A4}$ binding to the membrane requires PIP$_2$, and previous studies have indicated that binding is mediated by the N-terminal PIP$_2$ binding motif. We set out to determine whether the C-terminal region interacted with this critical amino-terminal region. We removed the first 10 N-terminal amino acids from PTEN$_{1-351}$, which are required for membrane binding, and assessed the ability of PTEN$_{11-351}$-CFP-FLAG to effectively immunoprecipitate PTEN$_{352-403}$-YFP, as previously described. As shown in Fig. 5A, the N-terminal truncation had no effect on the ability of PTEN$_{11-351}$-CFP-FLAG to interact with PTEN$_{352-403}$-YFP. However, in separate experiments, deletion of the first 15 aa, the triple mutation K13R, R14A, and R15A, or the single substitution K13A, prevented the interaction of FLAG-PTEN$_{1-353}$ with PTEN$_{354-403}$-GFP or FLAG-PTEN$_{A4}$ with PTEN$_{354-403}$-GFP (Fig. S4 A and B). Thus, the entire N-terminal PIP$_2$-binding motif is necessary for membrane association, whereas only the most C-terminal-charged residues are needed for the intramolecular interaction.

We then explored whether the phosphorylated cluster might interact with the rest of the protein by docking near the catalytic
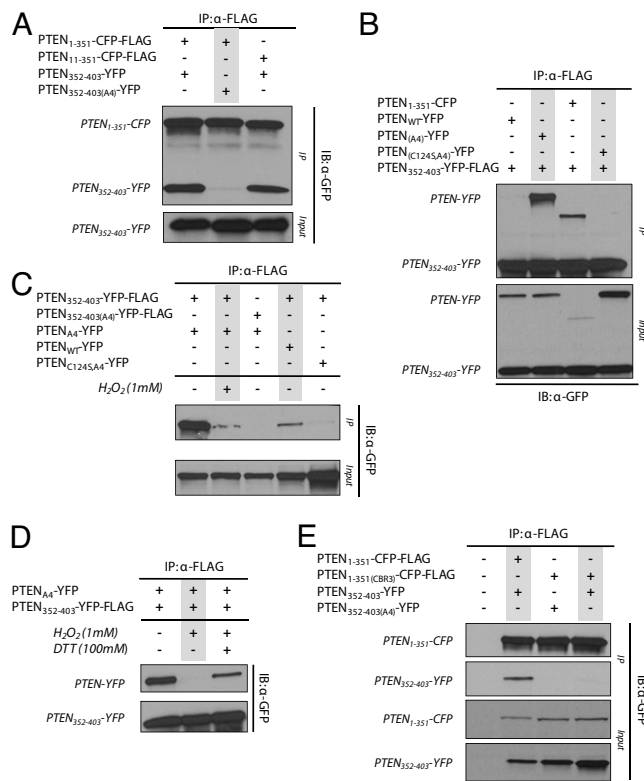

Fig. 5. Evaluation of binding determinants of PTEN$_{1-351}$ and PTEN$_{352-403}$. (A) Interaction of the C-terminal domain of PTEN with its N-terminal tail was assessed by co-expression and co-immunoprecipitation of PTEN$_{11-351}$-CFP-FLAG with PTEN$_{352-403}$-YFP. (B) The interaction of catalytically inactive PTEN with its C-terminal domain was assessed by co-expression of PTEN$_{C124S,A4}$-YFP with PTEN$_{352-403}$-YFP-FLAG. Lysates were immunoprecipitated and analyzed by Western blot. PTEN$_{C124S,A4}$-YFP association with PTEN$_{352-403}$-YFP-FLAG was compared with PTEN$_{352-403}$-YFP-FLAG affinity for PTEN$_{WT}$-YFP, PTEN$_{A4}$-YFP, and PTEN$_{1-351}$-CFP. (C) HEK293T cells were co-transfected with the indicated constructs. Cells expressing PTEN$_{352-403}$-YFP-FLAG and PTEN$_{A4}$-YFP were pre-treated with 1 mM H$_2$O$_2$ for 5 min and immunoprecipitated. Relative expression of full-length PTEN variants were also analyzed by Western blot using anti-GFP antibody. (D) HEK293T cells co-expressing PTEN$_{A4}$-YFP and PTEN$_{352-403}$-YFP-FLAG were treated with 1 mM H$_2$O$_2$ for 5 min before lysis. Equal volumes of the lysate were subjected to immunoprecipitation with anti-FLAG affinity beads with and without the presence of 100 mM DTT. (E) To evaluate the possible interaction of PTEN$_{352-403}$-YFP with the CBR3 loop of the C2 domain, lysines K260, K263, K266, K267, and K269 were alanine substituted to generate PTEN$_{1-351(CBR3)}$-CFP-FLAG, then co-expressed with PTEN$_{352-403}$-YFP in HEK293T cells and analyzed by Western blot using anti-GFP antibody. Results were compared with PTEN$_{1-351}$-CFP-FLAG co-expression with PTEN$_{352-403}$-YFP and PTEN$_{1-351(CBR3)}$-CFP-FLAG co-expression with PTEN$_{352-403(A4)}$-YFP.

pocket of the enzyme. Initial evidence of this possibility arose serendipitously when we used the catalytically dead version of PTEN, PTEN$_{C124S,A4}$. We observed that PTEN$_{C124S,A4}$-YFP did not interact with PTEN$_{352-403}$-YFP-FLAG (Fig. 5B). To further explore this phenomenon, we exploited the observations of Lee et al. (18, 20). In studies investigating the effects of radical oxygen species on PTEN activity, they discovered that H$_2$O$_2$ can reversibly inhibit PTEN activity by covalently linking Cys-71 to Cys-124. Cells co-expressing PTEN$_{352-403}$-CFP-FLAG and PTEN$_{A4}$-YFP were treated with 1 mM H$_2$O$_2$. We found that, following H$_2$O$_2$ pre-treatment, PTEN$_{A4}$ displayed notably lower binding to the exogenous C-terminal region (Fig. 5C). This inhibition could be partially reversed by inclusion of 100 mM DTT in the immunoprecipitation buffer (Fig. 5D). Furthermore, in separate experiments, mutation of charged residues at the start of the TI loop, R161A, K163A, and K164A prevented the interaction of
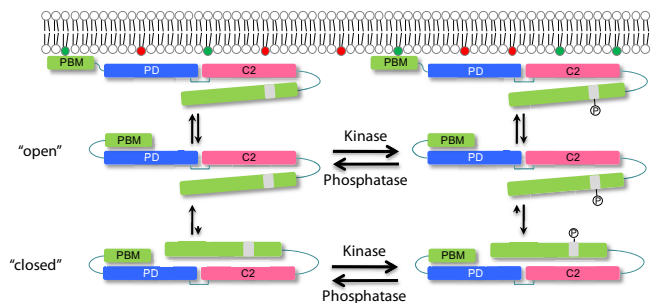
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Fig. 6. Phosphorylation-dependent switch between open and closed conformations regulates rate of association of PTEN with the membrane. PTEN molecules can exist in open or closed conformations, and open molecules bind to the membrane. Membrane shows scattered molecules of PIP_2 (green), which are required for binding, and PIP_3 (red), which are PTEN substrates. Phosphorylation of the C-terminal cluster tilts the equilibrium toward the closed conformation, whereas most dephosphorylated molecules are in the open conformation. Alanine substitution of the C-terminal cluster or removal of the C-terminal region locks all of the molecules in an open conformation, promoting membrane association.

$FLAG-PTEN_{1–353}$ with $PTEN_{354–403}$-GFP (Fig. S4A). These observations indicate that the phosphatase domain, particularly access to the catalytic pocket, is required for the interaction with the C-terminal region.

We next examined the possible role of the CBR3 loop and the $C\alpha2$ region and its adjacent loop of the C2 domain in binding of $PTEN_{1–351}$ to $PTEN_{352–403}$. We reasoned that, as the CBR3 is required for PTEN membrane association, the C-terminal region might regulate CBR3 membrane accessibility. We disrupted the CBR3 loop by alanine substitution of lysine residues K260, K263, K266, K267, and K269. $PTEN_{1–351(CBR3)}$-CFP-FLAG was co-expressed with $PTEN_{352–403}$-YFP and immunoprecipitated with anti-FLAG affinity beads. Following Western blot analysis, we observed that these mutations of the CBR3 loop completely disrupted the association of the C-terminal fragment with the rest of the protein (Fig. 5E). In a similar experiment, substitutions of CBR3 loop residues K263A, M264A, L265G, K266A, K267A, and K269A also disrupted the association. Conversely, alanine substitution of $C\alpha2$ and its adjacent loop had no effect (Fig. S4A). The observations suggest that the interaction of the C-terminal region with the remainder of the protein is in part dependent on intramolecular interactions with its C2 domain.

Discussion

The interaction of PTEN with the membrane requires a short N-terminal PIP_2 binding motif and is negatively regulated by phosphorylation of a cluster of residues in the C-terminal region of the protein. In this report we showed that indeed PIP_2 is part of the binding site and that PTEN activity in cells is correlated with the level of membrane association. Furthermore, we have found that the negative regulation is caused by an intramolecular interaction between the phosphorylated C-terminal region and the phosphatase and the C2 domains. Our observations are consistent with a model in which PTEN switches between open and closed states and phosphorylation of the C-terminal cluster favors the closed conformation (Fig. 6). First, we demonstrated that removal of the C-terminal region enhances PTEN membrane localization in a variety of cell types, and that co-expression of a phosphorylatable C-terminal fragment blocks membrane binding. Second, we confirmed the suggested intramolecular interaction between the N- and C-terminal regions by showing that two separate fragments, $PTEN_{1–351}$ and $PTEN_{352–403}$, respectively, could be co-immunoprecipitated whether they are co-expressed or expressed separately and mixed. Eliminating phosphorylation by alanine substitution or phosphatase treatment of the C-terminal fragment prevented the

association. Furthermore, perturbations of the catalytic pocket or mutation of the CBR3 loop in the C2 domain prevented the interaction of the two fragments. Removal of the N-terminal PIP_2 binding motif did not. Third, substitution of the phosphorylated cluster, in context of the full-length protein, greatly increased access for exogenous phosphorylated C-terminal fragment.

We propose that there is a steady-state distribution between open and closed conformations of PTEN (Fig. 6). Phosphorylated molecules are found predominantly in the closed conformation, whereas un-phosphorylated molecules are found predominantly in the open conformation. Open molecules encountering the membrane bind transiently, whereas the closed molecules fail to associate. Therefore, only a small fraction of phosphorylated WT PTEN is associated with membrane at steady state. Substitution of the phosphorylated cluster would be expected to shift the distribution toward the open conformation and thereby increase the rate of association with the membrane. Consistently, previous studies have shown that non-phosphorylatable PTEN has a more rapid rate of association with the membrane but its lifetime on the membrane is identical to WT (27). Similarly, removal of the C-terminal region would be expected to render PTEN in the open conformation. As we have shown here, this manipulation greatly increases association with the membrane and co-expression of the phosphorylated C-terminal region closes the molecule and reverses this effect. We think it is unlikely that the phosphorylation/dephosphorylation events are directly linked to each cycle of binding. Both $PTEN_{WT}$ and $PTEN_{A4}$ dissociate at identical rates, yet clearly $PTEN_{A4}$ cannot receive a phosphate as it dissociates. We assume that a similar situation holds for $PTEN_{1–351}$, although we have not directly measured its lifetime. Nevertheless, future work is needed to determine the rates of phosphate turnover and compare them with binding lifetime.

Our studies extend previous work showing that the conserved PIP_2 binding motif on the N terminal of PTEN is required for membrane association and that, in mixed micelles, inclusion of PIP_2 enhances phosphatase activity against PIP_3 (30). The latter effect was abolished by the tumor-derived mutation K13E within the PIP_2 binding motif. We show here that rapid depletion of PIP_2 from the plasma membrane triggered by forced recruitment of Inp54p causes $PTEN_{C124S,A4}$ to immediately re-localize to the cytosol. This shows that PIP_2 comprises one element of the binding site for PTEN. Taken together, these observations suggest an association of the PIP_2 binding motif with PIP_2. Interestingly, only a portion of the PIP_2 binding motif was required for the phosphorylation-dependent intramolecular interaction between the C-terminal region and the remainder of the protein.

The transition between open and closed states favored by phosphorylation depends on direct association of C-terminal region with the phosphatase and C2 domains. Within the phosphatase domain, substitution of the catalytic cysteine by serine (i.e., C124S) completely prevented the interaction. Cross-linking of the C124 to C71 by oxidation with H_2O_2 also blocked the intramolecular interaction, and this effect was reversed by reduction with DTT. Based on our model (Fig. 6), these perturbations would be expected to open the molecule and increase membrane binding. Indeed, both human and *Dictyostelium* versions of $PTEN_{C124S}$ show greater membrane association than their WT counterparts. In the human version, this effect appears to be partially additive, as the combined mutation $PTEN_{C124S,A4}$ displays higher membrane association compared with $PTEN_{C124S}$ or $PTEN_{A4}$. Further studies are needed to determine if the perturbations of the catalytic pocket inhibit C-terminal interaction by conformational changes within the phosphatase domain or loss of protein phosphatase activity. In addition to the phosphatase domain, the integrity of the C2 domain is required as mutations of the CBR3 loop also prevented the intramolecular interaction. Our studies open the way for determination of the exact docking regions by biophysical methods.

During the course of our studies, Odriozola *et al.* presented evidence for an intramolecular interaction within the PTEN mol-

ecule (36). They show that a fragment of PTEN consisting of a portion of the C2 domain and the C-terminal region, $PTEN_{335-403}$, interacts with a partially overlapping fragment consisting of the entire C2 domain and the C-terminal region, $PTEN_{185-403}$. N-terminal truncations into the C2 domain of $PTEN_{185-403}$ did not interfere with this interaction until removal of portions beyond the CBR3 loop. From this, the authors concluded that the CBR3 loop is critical for the interaction, yet, when they mutated all charged residues in the CBR3 loop, leaving only membrane anchoring residues M264 and L265 (33, 37), the interaction remained intact. They also show that the C-terminal domain, $PTEN_{335-403}$, does not interact with the phosphatase domain, $PTEN_{1-185}$. Paradoxically, phospho-mimetic peptides spanning the C-terminal cluster at high concentrations inhibit PTEN phosphatase activity, suggesting a weak interaction between the C-terminal domain and the catalytic pocket of the phosphatase domain. From these data, they derive a model whereby the C-terminal region mediates an auto-inhibitory interaction that inhibits membrane binding and catalytic activity.

Despite the similarity of our models, our data disagree in a number of ways. First, all of their immunoprecipitation studies use a portion of PTEN lacking the phosphatase domain (36). In contrast, we find that subtle perturbations of the phosphatase domain, such as the $PTEN_{C124S}$ mutation, reversible H_2O_2 treatment, or mutation of the TI loop, greatly reduced interactions with the C terminus. Second, in their experiments, mutations of the charged residues in the CBR3 loop did not interfere with $PTEN_{185-403}$ interaction with $PTEN_{335-403}$, whereas in our experiments, similar mutations disrupted the interaction of $PTEN_{1-351}$ with $PTEN_{352-403}$. Third, in our study, phosphorylated closed PTEN bound significantly less C-terminal domain, presumably because of intramolecular competition. Yet in all of their work, overlapping regions of the C2 domain were present in both interacting fragments (36). These details and discrepancies, which may result in part from major differences in assays used, will be important in future strategies to manipulate PTEN localization and function.

Our findings have important implications for the function of PTEN. High levels of PIP_3 promote uncontrolled cell growth and migration, and must be restricted by proper regulation. Because PIP_3 is on the membrane, the localization of PTEN is expected to effectively control catalytic activity in cells. We show that phosphorylation of the C terminus controls an intramolecular interaction that determines the fraction of PTEN associated with the membrane and thereby the set point for cellular PIP_3. In fact, there is some evidence that phosphorylation of PTEN can be regulated (35). It is likely that the set point is regulated in different tissues by controlling the level of phosphorylation of the cluster. Furthermore, the conformational switch in PTEN provides an attractive target. For instance, with further knowledge of the molecular interaction, peptides or small molecules can be designed to inhibit the intramolecular interaction of the C-terminal tail and increase membrane association and activity. Alternatively, opening of the molecule could be achieved by inhibiting C-terminal phosphorylation. Finally, open versions of PTEN could be used for gene therapy.

Materials and Methods

Detailed description of the materials and methods are provided in *SI Text*. Plasmids were prepared by standard PCR and cloning procedures. HEK293 and U87 cells were cultured using ATCC media and procedures. Fluorescence microscopy was carried out using an Olympus IX71 inverted microscope with appropriate CFP/YFP filter sets. For immunoprecipitation and Western blotting, HEK293T cells expressing described constructs were lysed in buffer containing 10 mM Tris-HCl (pH 7.5), 140 mM NaCl, 5 mM EDTA, and 0.1% Nonidet P-40 and supplemented with 1 mM DTT, as well as phosphatase and protease inhibitor mixture.

Inducible PIP_2 depletion using CFP-FKBP-Inp54p, and dual color (CFP/YFP) confocal imaging of cells have been described previously (34). Briefly, HeLa cells were transfected with dimerization probes, CFP-FKBP-Inp54p, and Lyn11-FRB together with YFP-labeled $PTEN_{C124S,A4}$. Cells were treated with iRap, which triggered a rapid translocation of CFP-FKBP-Inp54p to the plasma membrane. Fluorescent signals from CFP and YFP in HeLa cells were alternately collected every 30 seconds on a spinning-disk confocal microscope.

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