THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 285, NO. 17, pp. 12620–12628, April 23, 2010

Ubiquitination of PTEN (Phosphatase and Tensin Homolog) Inhibits Phosphatase Activity and Is Enhanced by Membrane Targeting and Hyperosmotic Stress*[S]

Received for publication, October 6, 2009, and in revised form, February 19, 2010 Published, JBC Papers in Press, February 22, 2010, DOI 10.1074/jbc.M109.072280

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The PTEN (phosphatase and tensin homolog) tumor suppressor is a phosphatase that inhibits phosphoinositide 3-kinase-dependent signaling by metabolizing the phosphoinositide lipid phosphatidylinositol 3,4,5-trisphosphate ($PtdInsP_3$) at the plasma membrane. PTEN can be mono- or polyubiquitinated, and this appears to control its nuclear localization and stability, respectively. Although PTEN phosphorylation at a cluster of C-terminal serine and threonine residues has been shown to stabilize the protein and inhibit polyubiquitination and plasma membrane localization, details of the regulation of ubiquitination are unclear. Here, we show that plasma membrane targeting of PTEN greatly enhances PTEN ubiquitination and that phosphorylation of PTEN *in vitro* does not affect subsequent ubiquitination. These data suggest that C-terminal phosphorylation indirectly regulates ubiquitination by controlling membrane localization. We also show that either mono- or polyubiquitination *in vitro* greatly reduces PTEN phosphatase activity. Finally, we show that hyperosmotic stress increases both PTEN ubiquitination and cellular $PtdInsP_3$ levels well before a reduction in PTEN protein levels is observed. Both PTEN ubiquitination and elevated $PtdInsP_3$ levels were reduced within 10 min after removal of the hyperosmotic stress. Our data indicate that ubiquitination may represent a regulated mechanism of direct reversible control over the PTEN enzyme.

PTEN[3] is a lipid phosphatase that, through dephosphorylation of the second messenger phosphatidylinositol 3,4,5-trisphosphate ($PtdInsP_3$), inhibits phosphoinositide 3-kinase-dependent signaling (1, 2). This appears to be the dominant mechanism by which PTEN can control cell proliferation, survival, and growth and the reason why PTEN is one of the most frequently lost tumor suppressors in human cancer (3). In contrast to the classical model of two-hit loss of tumor suppressor function, *in vivo* models of tumor formation, best analyzed in the prostate, suggest that PTEN acts in a haploinsufficient and dose-dependent manner (4–6). Several binding partners and regulators of the PTEN protein also appear to act as oncogenes and tumor suppressors principally through their ability to control PTEN (7–12). These findings highlight the importance of mechanisms by which PTEN expression and activity are regulated.

It has been known for some years that PTEN can become polyubiquitinated and that this appears to target PTEN for degradation by the proteasome (13–15). Also, C-terminal phosphorylation of PTEN causes the enzyme to be less ubiquitinated and more stable (13, 14, 16). More recently, NEDD4-1 has been identified as an efficient E3 ubiquitin ligase for PTEN *in vitro* that is capable of controlling PTEN ubiquitination and expression in cells (11). Stemming from this work have been further studies implying much greater complexity in the regulation of PTEN function by ubiquitination. These include the indication that the nuclear/cytoplasmic shuttling of PTEN can be controlled by monoubiquitination (17) and regulated deubiquitination (18) and that NEDD4-1 may not be a universal regulator of PTEN ubiquitination (19), implicating further unidentified E3 ligases such as XIAP (X-linked inhibitor of apoptosis protein) (20). Here, we investigate the link between PTEN phosphorylation and ubiquitination and analyze the effects of ubiquitination on PTEN enzyme activity.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—U87MG glioblastoma cells and HEK293T cells were obtained from the European Collection of Animal Cell Cultures and maintained in the recommended medium (10% fetal bovine serum). Standard cell culture medium, additives, and sera were from Invitrogen. Transfection of U87MG cells was performed using TransIT-LT1 reagent (Mirus) when U87MG cells were ~50% confluent. Plasmids encoding PTEN, the PTEN A3 mutant (S380A/T382A/T383A), or the myristoylated PTEN construct and/or FLAG-tagged ubiquitin were used in transfection as indicated. After transfection, cells were treated with or without inhibitor(s) for the indicated times. Cells were then washed with phosphate-buffered saline and lysed. The following agents were purchased from Calbiochem: proteasome inhibitor I (PSI), calpeptin, and the CK2 inhibitors DMAT and 4,5,6,7-tetrabromo-

* The work performed in the Inositol Lipid Signalling Laboratory was supported by the Medical Research Council, the Association for International Cancer Research, and the Division of Signal Transduction Therapy Consortium (AstraZeneca, Boehringer Ingelheim, GlaxoSmithKline, Merck KGaA, and Pfizer).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

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[3] The abbreviations used are: PTEN, phosphatase and tensin homolog; $PtdInsP_3$, phosphatidylinositol 3,4,5-trisphosphate; PSI, proteasome inhibitor I; DMAT, 2-dimethylamino-4,5,6,7-tetrabromo-1*H*-benzimidazole; GST, glutathione *S*-transferase; MAPK, mitogen-activated protein kinase.

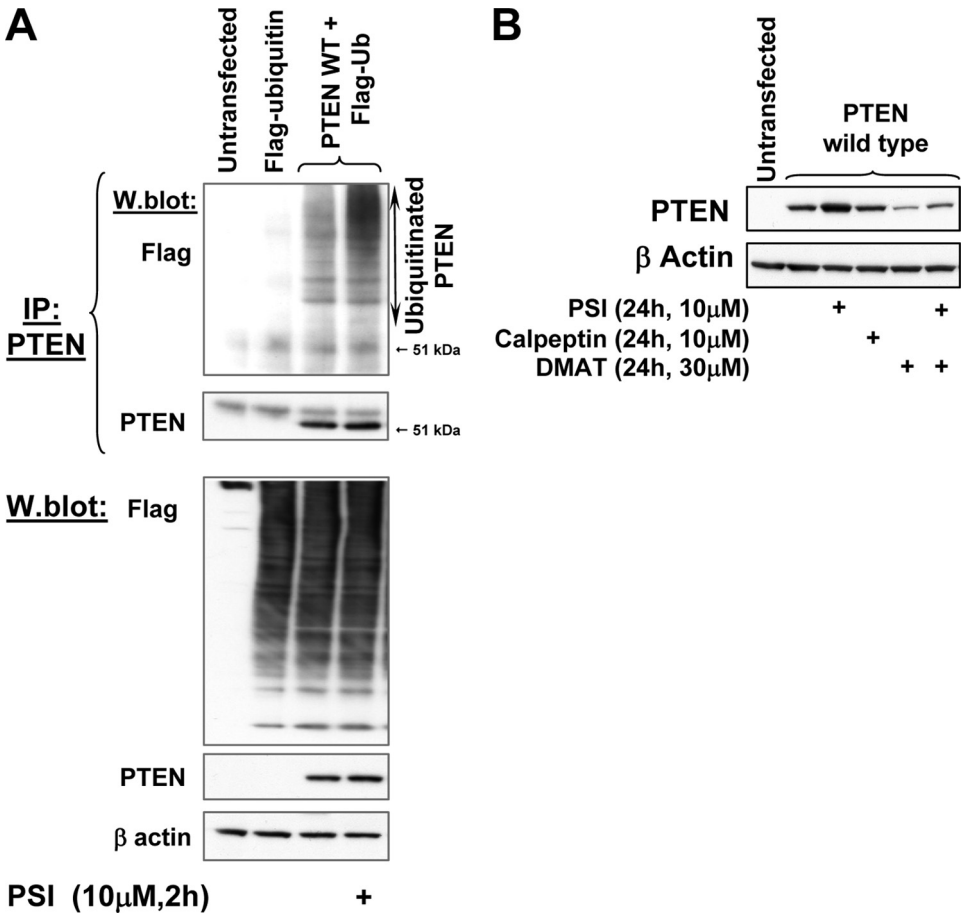

FIGURE 1. **Cellular assay for PTEN ubiquitination.** *A*, PTEN-null U87MG glioblastoma cells were transfected with expression vectors encoding FLAG-ubiquitin (*Flag-Ub*) either alone or in combination with a vector encoding untagged wild-type PTEN (*PTEN WT*). After 24 h, a sample of cells coexpressing FLAG-ubiquitin and PTEN was then treated with 10 μM PSI for 2 h. Cells were lysed, and PTEN was immunoprecipitated (*IP*) before Western blotting (*W.blot*) these immunoprecipitates with antibodies raised against the FLAG epitope and PTEN. An anti-FLAG Western blot of the lysates shows cellular protein ubiquitination levels in the FLAG-ubiquitin-expressing cells. *B*, U87MG cells transfected as indicated with vectors encoding wild-type PTEN were treated for 24 h with 10 μM PSI, 10 μM calpeptin, or 30 μM DMAT before PTEN expression was analyzed by Western blotting for PTEN and β-actin. DMAT inhibits CK2 (22), whereas PSI inhibits the proteasome (23).

benzotriazole. The specificity of these inhibitors has been studied previously and described (21–23). 293T cells were transfected using a standard calcium phosphate protocol.

DNA Expression Vectors—Mammalian expression vectors for untagged PTEN were originally generated by cloning the PTEN cDNA from pCR2.1-TOPO-PTEN (24) into pcDNA3.1$^+$ (Invitrogen) as a BamHI-XbaI fragment. The generation of myristoylated hemagglutinin-PTEN vectors has been described previously (25), and the production of a second myristoylated vector was performed by non-directional EcoRI restriction enzyme cloning from pEGFP-PTEN (24) into this site of the myristoylated vector pCMF. The generation of mutants PTEN C124S and PTEN A3 has been described previously (24, 26). A mammalian expression vector for FLAG-ubiquitin was kindly provided by Philip Cohen (University of Dundee). A bacterial expression vector for the E3 ligase NEDD4-1 was produced as follows. The 5′-end of NEDD4-1 was amplified from I.M.A.G.E. expressed sequence tag 5575951 (Geneservice) using Phusion DNA polymerase (Finnzymes) and primers NEDD4 F1 (5′-AAAAGAATTCATGGCAACTTGCGCGGTGGAGGTGTTCGGG) and NEDD4 R1 (5′-TGTTCCCTCCAGTCATTCACATCAACATCTCCCAGTCCACACATAAG). The remaining 3′-end was cloned by PCR from I.M.A.G.E. expressed sequence tag 6138012 using primers NEDD4 F2 (5′-GTGTGGACTGGGAGATGTTGATGTGAATGACTGGAGGGAAC) and NEDD4 R2 (5′-TTTTCTCGAGCTAATCAACTCCATCAAAGCCCTGGGTGTTTTCAATTGCCATCTGA). The full-length PCR fragment was assembled and cloned into pCR2.1 (Invitrogen) and sequenced to completion. Full-length NEDD4-1 was subsequently excised using EcoRI and XhoI and inserted into pGEX6P-1.

Preparation of Whole Cell Extracts, SDS-PAGE, and Western Blotting—Cells were washed twice with ice-cold phosphate-buffered saline and lysed in ice-cold lysis buffer (25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1 mM EGTA, 1 mM EDTA, 5 mM sodium pyrophosphate, 10 mM β-glycerophosphate, and 50 mM sodium fluoride) containing 0.1% 2-mercaptoethanol and protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Equal amounts of proteins were separated by SDS-PAGE using precast 4–12% gradient gels (Invitrogen) and blotted onto polyvinylidene difluoride membranes (Polyscreen, PerkinElmer Life Sciences). Most reagents for electrophoresis and blotting were purchased from Invitrogen, and standard manufacturers' protocols were followed. Mouse anti-PTEN monoclonal antibody (clone A2B1) was purchased from Santa Cruz Biotechnology. Polyclonal antibodies to phospho-Ser^{380}/Thr^{382}/Thr^{383} PTEN and phospho-Ser^{380} PTEN were purchased from Cell Signaling Technologies. Anti-FLAG antibody M2 and β-actin antibody were purchased from Sigma.

Ubiquitination of PTEN in U87MG Cell Culture—Whole cell extracts were prepared using lysis buffer containing fresh 0.2 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 mM *N*-ethylmaleimide (Sigma). PTEN was immunoprecipitated using monoclonal antibody A2B1 precoupled with agarose beads. The proteins bound to beads were released by boiling in SDS-PAGE sample buffer (Invitrogen) for 10 min. The samples were then resolved by 4–12% SDS-PAGE, followed by immunoblot analysis using anti-FLAG antibody M2 or anti-PTEN antibody,

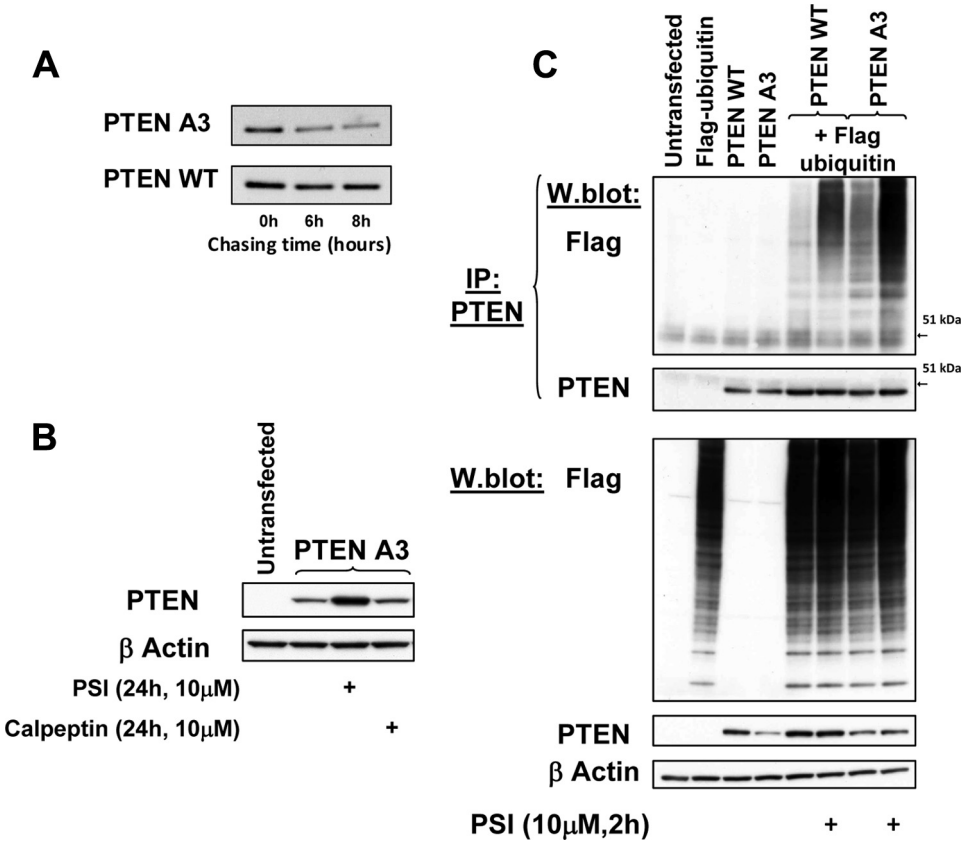

FIGURE 2. **Mutation of the PTEN C-terminal phosphorylation sites reduces PTEN stability and enhances ubiquitination.** *A*, the cellular stability of the wild-type PTEN (*PTEN WT*) and PTEN A3 (S380A/T382A/T383A) proteins was analyzed by metabolic ^{35}S-amino acid labeling of PTEN-null U87MG cells transduced with viruses encoding either wild-type PTEN or PTEN A3 and chasing with unlabeled amino acid-containing growth medium for the indicated times. PTEN proteins were visualized by autoradiography after immunoprecipitation. *B*, U87MG cells transiently expressing PTEN A3 were treated as indicated for 24 h with either 10 μM PSI or 10 μM calpeptin before Western blotting for PTEN expression along with a β-actin control. *C*, the ubiquitination of wild-type PTEN and PTEN A3 was compared in U87MG cells. U87MG cells were transfected with vectors encoding FLAG-tagged ubiquitin and either wild-type PTEN or PTEN A3 24 h before cells were treated with PSI at 10 μM for 2 h. Cells were lysed, and PTEN was immunoprecipitated (*IP*) before Western blotting (*W.blot*) these immunoprecipitates and the lysates with antibodies raised against the FLAG epitope, PTEN, and β-actin.

either mouse monoclonal A2B1 or goat polyclonal N19 (Santa Cruz biotechnology).

In Vitro Ubiquitination, Phosphorylation, and PTEN Assays—In vitro ubiquitin assays were carried out at 30 °C for 1 h in reaction buffer (50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 5 mM $MgCl_2$, 2 mM ATP, 100 μM ubiquitin (Boston Biochem), 0.05 μM His_6-UBE1, 1 μM GST-UbcH5b, 1 μM NEDD4-1, and 500 ng of PTEN recombinant protein). The reaction was terminated by the addition of lithium dodecyl sulfate sample loading buffer (Invitrogen) and 5 mM 2-mercaptoethanol, boiling for 10 min, and running the samples on SDS-polyacrylamide gel. The measurement of PTEN activity after *in vitro* ubiquitination was performed as follows. *In vitro* ubiquitin assays were carried out at 30 °C for 1 h in ubiquitination buffer, and then PTEN was immunoprecipitated using anti-PTEN monoclonal antibody A2B1 in 50 mM Tris-HCl (pH 7.5) buffer containing 1% Nonidet P-40, 150 mM NaCl, and 5 mM 2-mercaptoethanol and washed twice with reaction/wash buffer (25 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EGTA, and 2 mM dithiothreitol) before PTEN assays were performed on the immune complexes. Control immunoprecipitations and phosphatase assays were performed using sham-ubiquitinated PTEN that had been treated in parallel with one component of the ubiquitination assay omitted. Purified His_6-UBE1 (human) expressed in insect cells and bacterially expressed GST-UbcH5b (human) were provided by James Hastie and Hilary McLauchlan (Division of Signal Transduction Therapy, University of Dundee).

The preparation of 3-^{33}P-labeled phosphoinositide lipid substrates, the expression and purification of GST-PTEN, the removal of the GST tag, and the further purification of untagged PTEN have been described previously (42). The production of the phosphorylated peptide polymer [^{33}P]poly(Glu-Tyr) with insulin receptor kinase has also been described previously (26). Except where noted, $PtdInsP_3$ assays were performed using substrate vesicles prepared by sonication of 100 μM phosphatidylcholine, 10 μM phosphatidylinositol 4,5-trisphosphate, 10 nM unlabeled $PtdInsP_3$, and 100,000 dpm [3-^{33}P]$PtdInsP_3$. These were incubated in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, and 10 mM dithiothreitol with washed immunoprecipitates for 1 h at 30 °C. Poly(Glu-Tyr(P)) phosphatase assays were conducted in 50 mM Tris-HCl (pH 7.4), 1 mM EGTA, and 10 mM dithiothreitol with 500 ng of enzyme and 100,000 dpm (~1 μg) phosphorylated substrate per assay also at 30 °C for 1 h. Reactions were terminated directly by the addition of 500 μl of ice-cold 1 M perchloric acid and 100 μg/ml bovine serum albumin, left on ice for 30 min, and spun at 15,000 × *g* at 4 °C for 10 min. The supernatant was removed, and ammonium molybdate was added to a final concentration of 10 mg/ml. After extraction with 2 volumes of 1:1 (v/v) toluene/isobutyl alcohol, the upper phase was removed, and radioactivity was determined by scintillation counting. PTEN was phosphorylated using purified CK2 following previous protocols (27, 28).

Analysis of Cellular PTEN Stability—U87MG cells were infected with baculoviruses encoding PTEN and then, 24 h after transduction, washed and incubated for 1 h in methionine/cysteine-free medium containing 10% (v/v) dialyzed fetal bovine serum. Cells were labeled with [^{35}S]methionine/cysteine (70 μCi/ml; Amersham Biosciences) for 2 h. After three washes with complete culture medium without label, cells were incubated in more culture medium and chased for different times before lysis. PTEN proteins were isolated by immunoprecipitation and resolved by SDS-PAGE. The labeled PTEN present at each time point was quantified using a Fuji FLA-2000 phosphorimager and AIDA software.

RESULTS

Phosphorylation of PTEN Increases Stability and Inhibits Ubiquitination in Cells—To better understand the role of ubiquitination in regulating PTEN function, we established assays for PTEN ubiquitination in cells and *in vitro*. These cell-based experiments were performed using the PTEN-null glioblastoma cell line U87MG, coexpressing recombinant PTEN with FLAG epitope-tagged ubiquitin. In these cells, only when both PTEN and FLAG-ubiquitin were coexpressed was a smear of high molecular weight polyubiquitinated PTEN observed when PTEN immunoprecipitates were blotted with anti-FLAG antibody (Figs. 1*A* and 2*B*). Similar results were obtained when anti-FLAG immunoprecipitation was followed by anti-PTEN Western blotting (data not shown). This apparent polyubiquitinated protein accumulated when cells were treated with a proteasome inhibitor for 2 or 24 h, correlating with the increased abundance of PTEN protein caused by similar treatment for 24 h (Fig. 1, *A* and *B*, and supplemental Fig. S1) and in accordance with previous data (13–15). PTEN levels were not affected by cellular treatment with calpeptin, an inhibitor of calpain proteases. Initially, we chose to use this system to investigate the details of the connection between C-terminal phosphorylation and ubiquitination. PTEN can be stabilized by phosphorylation of a cluster of serine and threonine residues by the kinase CK2, correlating with reduced polyubiquitination (13, 14, 29, 30). In agreement with this model, treatment of U87MG cells expressing wild-type PTEN with well validated selective inhibitors of CK2, 4,5,6,7-tetrabromobenzotriazole and DMAT (22), led to a significant reduction in PTEN expression level, which could be reversed by a proteasome inhibitor (Fig. 1*B* and supplemental Fig. S1). Similarly, blocking C-terminal phosphorylation by mutation of three residues in this cluster of serine and threonine residues to alanine (PTEN A3) led to a marked destabilization of PTEN in amino acid pulse-chase labeling experiments (Fig. 2*A*). This mutant protein was also stabilized by treatment of cells with a proteasome inhibitor (Fig. 2*B*), and when the ubiquitination of PTEN A3 was analyzed, enhanced ubiquitination of this mutant relative to the wild-type protein was observed (Fig. 2*C*).

Membrane Localization Enhances PTEN Ubiquitination—It has been shown that mutation of the C-terminal phosphorylation sites of PTEN, including work with the PTEN A3 mutant, leads to enhanced plasma membrane localization (31, 32). This, along with other lines of evidence, indicates that the C-terminal phosphorylation of PTEN leads to a conformational switch, whereby the phosphorylated tail binds to the C2 and phosphatase domains and inhibits membrane binding (29, 31–35). This leaves a question as to whether phosphorylation inhibits ubiquitination directly through this conformational change or perhaps indirectly through, for example, inhibiting the co-localization of PTEN with a plasma membrane-localized ubiquitin ligase. To test this latter hypothesis, we analyzed the ubiquitination of PTEN proteins artificially targeted to the plasma membrane by the addition of the N-terminal myristoylation/palmitoylation signal from Lck (25, 36). Both wild-type and phosphatase-dead PTEN proteins were ubiquitinated to a far greater level when myristoylated compared with their corresponding non-myristoylated proteins (Fig. 3*A*) despite being C-terminally phosphorylated to a similar degree (Fig. 3*B*). The N-terminal myristoylation signal contains no lysine residues, and similar results were obtained using two different myristoylated PTEN expression constructs with different linker regions, one of which has no additional lysine residues other than those in PTEN.

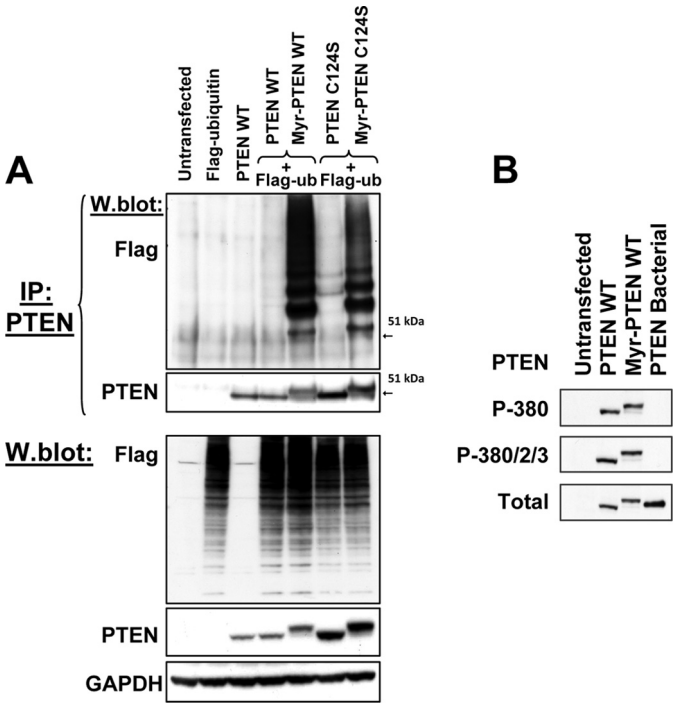

FIGURE 3. **Membrane targeting through N-terminal myristoylation enhances PTEN ubiquitination.** *A*, U87MG cells were transfected with vectors encoding FLAG-tagged ubiquitin (*Flag-ub*) and either untagged wild-type PTEN (*PTEN WT*) or N-terminally myristoylated wild-type (*Myr-PTEN WT*) or phosphatase-dead (*Myr-PTEN C124S*) PTEN. An N-terminal myristoylation signal and linker (33 amino acids in total) was used to target these fusion proteins to the plasma membrane, resulting in a larger slower migrating protein. Cells were lysed, and PTEN was immunoprecipitated (*IP*) before Western blotting (*W.blot*) these immunoprecipitates with antibodies raised against the FLAG epitope and PTEN. An anti-FLAG Western blot of the lysates shows cellular ubiquitination levels in the FLAG-ubiquitin-expressing cells. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase. *B*, equal amounts of immunoprecipitated wild-type PTEN and myristoylated wild-type PTEN were analyzed along with unphosphorylated bacterially expressed PTEN using phosphospecific antibodies for the C-terminal CK2 phosphorylation sites of PTEN and total protein antibodies.

PTEN Ubiquitination Inhibits Catalytic Activity in Vitro—We then chose to directly address the effect of phosphorylation on ubiquitination *in vitro* (Fig. 4). Recombinant purified PTEN was first phosphorylated with purified CK2 and immunoprecipitated before being ubiquitinated *in vitro* (Fig. 4*A*). *In vitro* phosphorylation of PTEN with CK2 consistently achieved a stoichiometry of >3 and often approaching 4. Ubiquitination used recombinant purified Ubc5Hb and NEDD4-1 as the ubiquitin E2 and E3 components, respectively. In these experiments, we used both purified untagged ubiquitin and a mutant in which each lysine residue within ubiquitin is mutated to arginine (referred to as "ubiquitin K all R") to block the formation of polyubiquitin chains. Phosphorylation had little or no inhibitory effect on subsequent ubiquitination, with some experiments with wild-type ubiquitin showing an increase in high

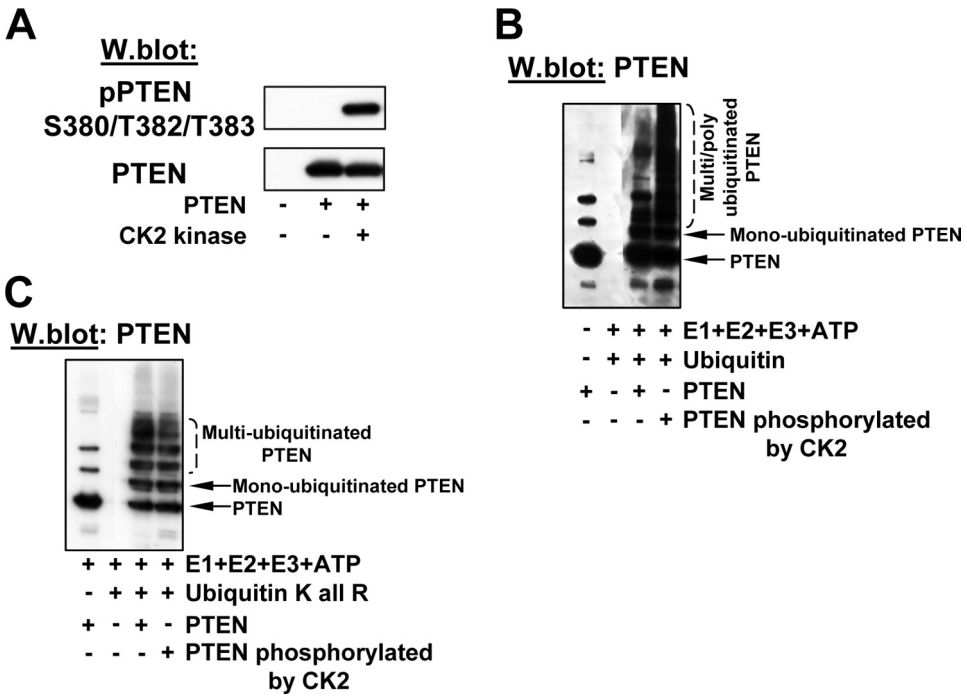

FIGURE 4. **PTEN phosphorylation does not inhibit subsequent ubiquitination *in vitro*.** *A*, PTEN was phosphorylated *in vitro* to a stoichiometry of >3 or sham-phosphorylated in the presence or absence of CK2 enzyme as described previously (28). These proteins were then analyzed by Western blotting (*W.blot*) with anti-PTEN and anti-phospho-Ser^{380}/Thr^{382}/Thr^{383} PTEN antibodies. *B* and *C*, phosphorylated and sham-phosphorylated PTEN proteins were immunoprecipitated and subjected to a ubiquitination assay *in vitro* using either purified ubiquitin (*B*) or the ubiquitin K all R mutant (*C*). Ubiquitination of PTEN was investigated by Western blotting for PTEN.

molecular weight polyubiquitination relative to unphosphorylated PTEN (Fig. 4, *B* and *C*).

Next, we were able to use the *in vitro* ubiquitination assay to analyze the effects of ubiquitination on PTEN catalytic activity. PTEN has been described to be both polyubiquitinated and monoubiquitinated (17). Therefore, we addressed the effects of monoubiquitination by performing ubiquitination *in vitro* using the ubiquitin K all R mutant, with each lysine residue replaced by arginine, blocking the formation of polyubiquitin chains (represented in Fig. 5*A*). Experimentally, ubiquitin K all R led to an apparent block in the polyubiquitination of PTEN, with a range of molecular weights being evident that presumably correspond to monoubiquitination and multisite monoubiquitination (Fig. 5*B*). Purified PTEN was put through this *in vitro* ubiquitination reaction in parallel with control samples, in which ubiquitin was omitted from the incubation. PTEN was then immunoprecipitated, washed, and assayed against both $PtdInsP_3$ lipid vesicles and the peptide polymer substrate poly-(Glu-Tyr(P)), with, in each case, greatly reduced activity being observed with ubiquitinated PTEN relative to a sham-ubiquitinated control (Fig. 5, *C* and *D*). Similarly, when the PTEN product of the monoubiquitination assay was immunoprecipitated and assayed, it was also found to show a similar loss of activity to polyubiquitination, suggesting that either mono- or polyubiquitination may greatly inhibit PTEN activity. In this case, although the mean activity against both substrates was reduced, this difference achieved statistical significance only using the lipid substrate (Fig. 5*D*). Given the incomplete reaction in the ubiquitination assay and that, in this series of experiments, the degree of ubiquitination appeared to correlate closely with the degree of PTEN activity loss, this suggests that the ubiquitination of PTEN in these experiments must lead to almost complete inactivation. Western blotting of both the completed crude ubiquitination assay and the subsequent PTEN immunoprecipitates demonstrated that the ubiquitination procedure did not affect the efficiency of PTEN immunoprecipitation (supplemental Fig. S2).

PTEN Ubiquitination Can Be Stimulated by Hyperosmotic Stress and Correlates with $PtdInsP_3$ Levels—Finally, we wanted to test the hypothesis that ubiquitination directly inactivates PTEN catalytic activity in cell-based experiments. Previous experiments in our laboratory had shown that treatment of HEK293T cells with hyperosmotic stress led to a reduction in PTEN expression levels over a period of several hours (Fig. 6 and data not shown). Therefore, we analyzed the ubiquitination of PTEN in HEK293T cells treated with 0.5 M sorbitol. We found that hyperosmotic stress rapidly increased PTEN polyubiquitination (Fig. 6*A*). This treatment also led to a modest decrease in PTEN protein levels, ~50% reduction in 4 h (Fig. 6, *A*–*C*), which could be blocked by proteasome inhibition (Fig. 6*B*).

The phosphorylation of the downstream $PtdInsP_3$-regulated kinase Akt could not be used as an indicator of the cellular $PtdInsP_3$ level in this case, as hyperosmotic stress appeared to block the activation of Akt (Fig. 6*C*), as described in a previous study despite a reported increase in the cellular $PtdInsP_3$ level (37). Therefore, we used direct measurements of $PtdInsP_3$ levels in these cells as a readout of PTEN activity. Sorbitol treatment of HEK293T cells caused cellular $PtdInsP_3$ levels to increase rapidly (Fig. 6*C*), correlating with the stimulated ubiquitination, long before effects on PTEN expression level became evident. In these experiments, sorbitol stimulation led to an increase in the activating phosphorylation of the recognized hyperosmotically responsive kinase p38 MAPK at Thr^{180}/Tyr^{182} but did not affect the phosphorylation of PTEN at Ser^{380}/Thr^{382}/Thr^{383} (Fig. 6*C*). Furthermore, both PTEN ubiquitination and cellular $PtdInsP_3$ levels were reduced within 10 min if cells were returned to normal medium after 1 h of sorbitol treatment (Fig. 6, *D* and *E*). These data support a direct connection between PTEN ubiquitination, cellular PTEN activity, and cellular $PtdInsP_3$ levels.

DISCUSSION

Our data support two novel conclusions. First, PTEN ubiquitination directly inhibits its phosphatase activity. Second,

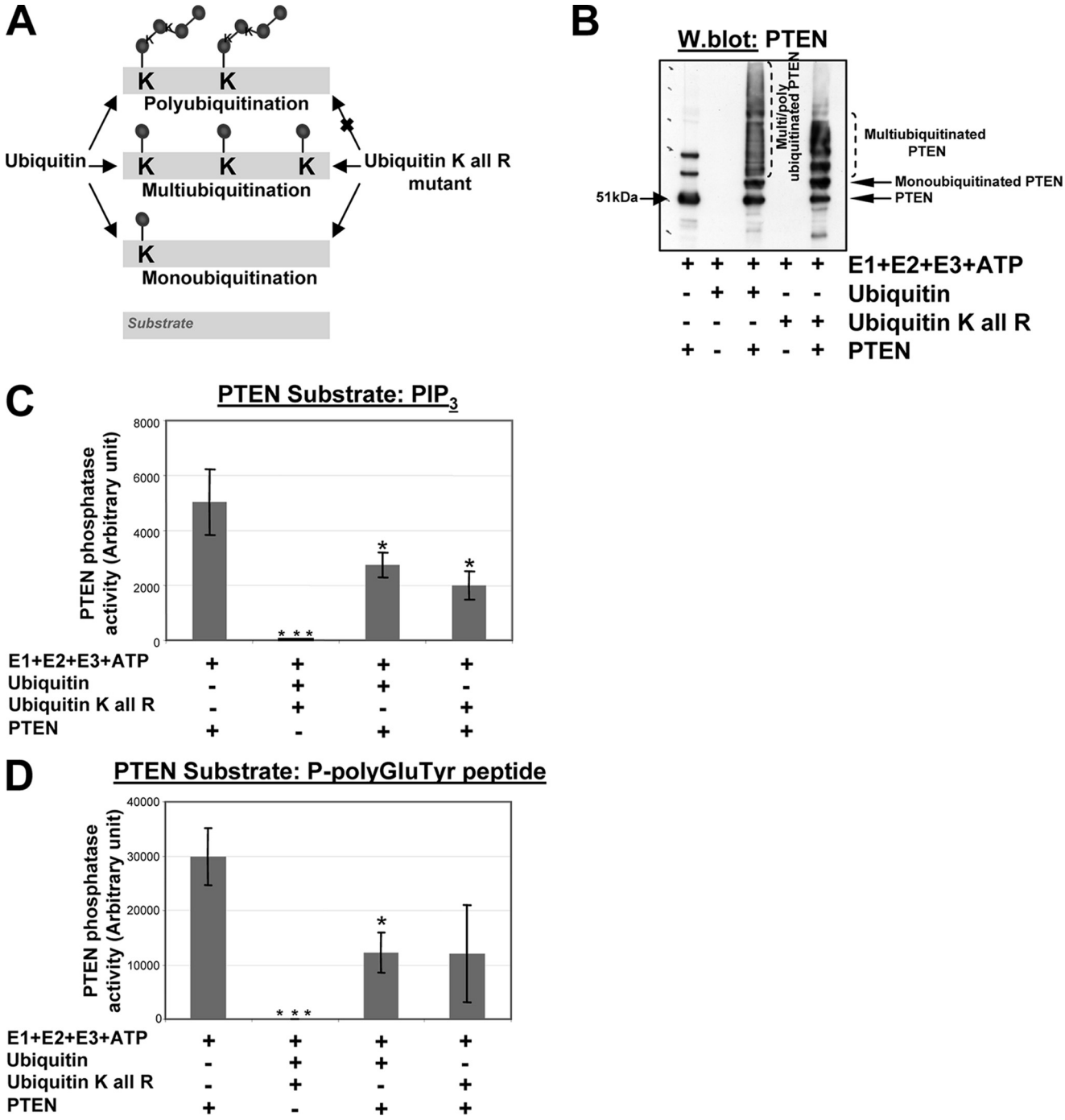

FIGURE 5. **PTEN ubiquitination *in vitro* inhibits phosphatase activity.** *A*, a scheme is shown that distinguishes among polyubiquitination, multiubiquitination (multisite monoubiquitination), and monoubiquitination of different lysine (*K*) residues on a ubiquitinated substrate protein. Using a ubiquitin mutant with each lysine residue mutated to arginine (ubiquitin K all R mutant), the formation of polyubiquitin chains is blocked. *B*, an anti-PTEN Western blot (*W.blot*) is shown comparing an *in vitro* ubiquitinated purified PTEN protein sample with a similar reaction using the ubiquitin K all R mutant and with a sham-ubiquitinated sample. Other immunoreactive bands within this negative control sample represent residual GST-tagged PTEN after an incomplete cleavage of this affinity purification tag. *C* and *D*, PTEN was immunoprecipitated from these ubiquitination and sham ubiquitination reactions along with a no-PTEN control, and each immunoprecipitate was assayed *in vitro* against $PtdInsP_3$ (*PIP_3*) vesicles (*C*) or the peptide substrate poly(Glu-Tyr(P)) (*D*). The mean phosphatase activity ± S.D. from triplicate samples is shown. *Asterisks* represent statistically significant reductions in activity compared with the relevant sham-ubiquitinated control ($p < 0.05$). In control blots, ubiquitination did not interfere with the efficiency of the immunoprecipitation (supplemental Fig. S2).

PTEN ubiquitination is greatly enhanced by hyperosmotic stress and membrane localization, and this latter indirect mechanism is how C-terminal phosphorylation controls PTEN ubiquitination.

Several previous studies have established that C-terminal phosphorylation of PTEN increases the stability of the enzyme and reduces polyubiquitination (13–16). Our data here indicate that a dominant mechanism by which this effect is mediated is indirectly via the recognized effect of phosphorylation in suppressing PTEN localization at the plasma membrane (31, 32, 35). It has recently been reported that the phosphorylated PTEN C terminus inhibits ubiquitination in cells and *in vitro* by

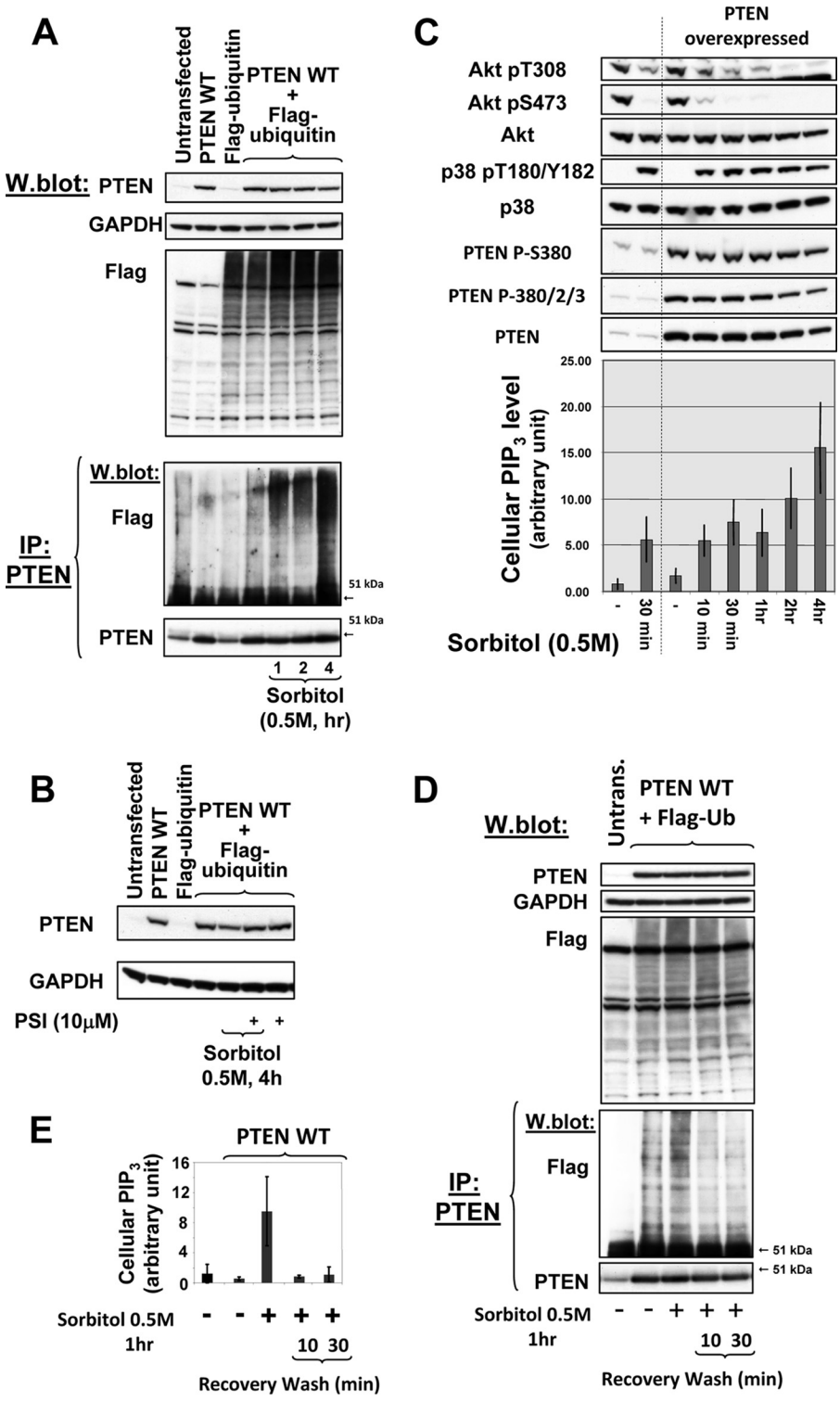

FIGURE 6. **Osmotic stress induces PTEN ubiquitination and increases PtdInsP$_3$ levels.** HEK293T cells were transfected as indicated with vectors encoding wild-type PTEN (*PTEN WT*) and/or FLAG-tagged ubiquitin (*Flag-Ub*). *A*, cells were treated with 0.5 M sorbitol for 1, 2, or 4 h. Cells were then lysed, and PTEN was immunoprecipitated (*IP*) before Western blotting (*W.blot*) these immunoprecipitates and lysates with antibodies raised against the FLAG epitope, PTEN, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). *B*, cells were treated with sorbitol (0.5 M, 4 h) alone or in the presence of PSI (10 μM), and PTEN expression was analyzed by Western blotting along with a glyceraldehyde-3-phosphate dehydrogenase loading control. *C*, cells were treated with sorbitol as indicated (0.5 M, 10 min to 4 h). Cellular PtdInsP$_3$ (*PIP$_3$*) content was measured using a time-resolved fluorescence resonance energy transfer sensor complex by comparison with a standard curve of known PtdInsP$_3$ concentrations as described previously (41). Parallel lysates were analyzed for the phosphorylation of Akt, PTEN, and the hyperosmotically responsive protein kinase p38 MAPK by Western blotting with phosphospecific and total protein antibodies. In these experiments, ~60% of cells were successfully transfected. *D*, after transfection as indicated, some cells were treated with 0.5 M sorbitol for 1 h as indicated, and two sets of samples were then washed with normal medium lacking sorbitol for 10 or 30 min. Cells were lysed, and PTEN was immunoprecipitated before Western blotting of these immunoprecipitates and lysates with antibodies raised against the FLAG epitope, PTEN, and glyceraldehyde-3-phosphate dehydrogenase. *E*, cells transfected as indicated with wild-type PTEN were treated with sorbitol for 1 h before some samples were washed with normal medium for 10 or 30 min. Cellular PtdInsP$_3$ content was then assessed as described previously (41).

NEDD4-1 (38). Because the phosphorylated C terminus inhibits its membrane binding of PTEN (31), these cellular data seem consistent with our conclusions. The reported effects of PTEN C-terminal deletion on ubiquitination *in vitro* appear to us far less strong than those in cells (38). From this we conclude that although both mechanisms may apply, the direct enhancement of ubiquitination *in vitro* caused by deletion of the C-terminal tail is weaker than the effect of membrane localization in cells.

Recent data indicate that the regulation of PTEN via ubiquitination is complex. Both mono- and polyubiquitination appear to have separable effects on PTEN function, with mono- and polyubiquitination targeting PTEN for nuclear localization and proteasomal degradation, respectively (11, 17, 18). Although NEDD4-1 has been identified as an E3 ligase capable of controlling PTEN abundance in several cultured cells (11), subsequent data suggest that this may not be a universal effect and that other E3 ligases may exist, such as XIAP (19, 20). Additionally, other proteins implicated in ubiquitin-dependent signaling have been identified as PTEN-interacting proteins, including the NEDD4-related proteins WWP2 and UbcH7 (39, 40). Our demonstration that ubiquitination inhibits PTEN activity implies that, if PTEN is polyubiquitinated and targeted for degradation, it is far less active once ubiquitinated even before it is degraded by the proteasome. Because our data suggest that this ubiquitination may occur at the plasma membrane, where the lipid substrate for PTEN is found, they also favor the speculation that ubiquitination may act as a mechanism to control PTEN activity. Ubiquitination is well recognized as a reversible modification, and the identification of HAUSP/USP7 as a PTEN-deubiquitinating enzyme supports this model for PTEN (18). The regulated inactivation of PTEN would appear significant whether PTEN is polyubiquitinated and targeted for degradation or monoubiquitinated either for targeting to the nucleus or simply as a mechanism of reversible inactivation at other cellular locations.

In summary, our data imply that PTEN ubiquitination occurs at the plasma membrane or at least is indirectly enhanced by PTEN membrane localization and that either mono- or polyubiquitination inhibits PTEN catalytic activity. These findings raise the possibility that PTEN ubiquitination may be driven by extracellular stimuli and may be a mechanism of reversible control over the enzyme during hyperosmotic stress and other conditions. Given the emerging complexity of mechanisms controlling PTEN ubiquitination, this seems likely to have significant implications for future functional studies.

Acknowledgments—We thank Hilary McLauchlan, James Hastie, and the staff in the Division of Signal Transduction Therapy (University of Dundee) for providing purified antibodies and recombinant protein kinases and Philip Cohen for sharing reagents and critical reading of the manuscript.

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