

## Phosphorylation of the PTEN Tail Acts as an Inhibitory Switch by Preventing Its Recruitment into a Protein Complex\*

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**PTEN is a tumor suppressor protein that functions, in large part, by dephosphorylating the lipid second messenger phosphatidylinositol 3,4,5-trisphosphate and by doing so antagonizing the action of phosphoinositide 3-kinase. PTEN structural domains include an N-terminal phosphatase domain, a lipid-binding C2 domain, and a 50-amino acid C-terminal tail that contains a PDZ binding sequence. We showed previously that phosphorylation of the PTEN tail negatively regulates PTEN activity. We now show that phosphorylated PTEN exists in a monomeric "closed" conformation and has low affinity for PDZ domain-containing proteins. Conversely, when unphosphorylated, PTEN is in an "open" conformation, is recruited into a high molecular weight complex (PTEN-associated complex), and strongly interacts with PDZ-containing proteins such as MAGI-2. As a consequence, when compared with wild-type PTEN, the phosphorylation-deficient mutant form of PTEN strongly cooperates with MAGI-2 to block Akt activation. These results indicate that phosphorylation of the PTEN tail causes a conformational change that results in the masking of the PDZ binding domain. Consequently, the ability of PTEN to bind to PDZ domain-containing proteins is reduced dramatically. These data suggest that phosphorylation of the PTEN tail suppresses the activity of PTEN by controlling the recruitment of PTEN into the PTEN-associated complex.**

*PTEN* (also known as *MMAC1/TEP1*) is a tumor suppressor gene localized to the chromosome 10q23 region (see Refs. 1–3, and reviewed in Ref. 4). The *PTEN* protein product (PTEN) is a lipid phosphatase that dephosphorylates the D3 position of phosphatidylinositol 3,4,5-trisphosphate (5). Although it has protein phosphatase activity against focal adhesion kinase (6)

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PTEN acts as a tumor suppressor, as a lipid phosphatase, and as an antagonist of PI3K<sup>1</sup>/Akt signaling (reviewed in Refs. 4 and 7). Loss of PTEN leads to elevated levels of phosphatidylinositol 3,4,5-trisphosphate and consequent Akt activation. PTEN is comprised of an N-terminal phosphatase domain (PHD) within which lies the consensus phosphatase signature motif, a C2 domain that binds lipid vesicles and a C-terminal "tail" that contains a PDZ binding domain (PDZbd) (see Ref. 8, and reviewed in Ref. 7). Several PDZ domain-containing proteins interact with PTEN (MAGI-1, -2, and -3, hDLG, MAST205) (9–12). MAGI-2 and -3, in particular, can enhance the activity of PTEN as measured by inhibition of Akt (9, 10).

We showed previously that phosphorylation of the PTEN tail negatively regulates its function as an antagonist of PI3K signaling (13). Here, we show that phosphorylated PTEN is in a "closed" conformation and migrates as a monomer in gel filtration columns. In contrast, unphosphorylated PTEN is in an "open" conformation, is found in a high molecular weight complex (>600 kDa) (PTEN-associated complex; PAC) and unlike phosphorylated PTEN strongly interacts with PDZ domain-containing proteins such as MAGI-1 and -2. As a result unphosphorylated PTEN acts synergistically with MAGI-2 to down-regulate Akt activity. Based on these results, we propose that phosphorylation of the PTEN tail regulates its activity by preventing it from participating in the PAC.

### EXPERIMENTAL PROCEDURES

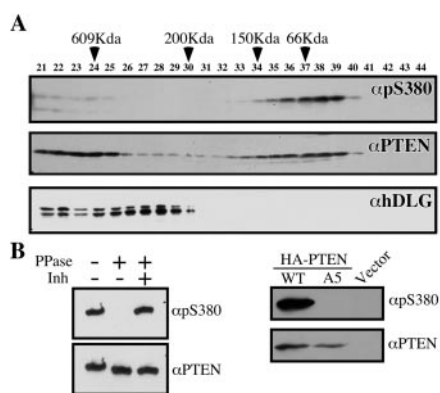
**Plasmids**—pSG5L-HA-PTEN;WT, pSG5L-HA-PTEN;A4, pSG5L-HA-PTEN;A5, and pCDNA3-T7-Akt were described previously (13). pCDNA3-GFP-PTEN;WT and GFP-PTEN;A4 were subcloned from pSG5L-HA-PTEN;WT and HA-PTEN;A4. pCDNA3-HA-MAGI-2 was kindly provided by Dr. Charles Sawyers.

**Cell Lines, Cell Culture, and Transfections**—ACHN, 786-O, and U2-OS cells were maintained in Dulbecco's modified Eagle's medium containing 4,500 mg of glucose/ml, 2 mM L-glutamine, 10% fetal clone (HyClone), and penicillin and streptomycin. 786-O were transfected using Fugene reagent (Roche Molecular Biochemicals), and U2-OS were transfected by the calcium phosphate method as described previously (14).

**Antibodies**—Monoclonal HA-11 anti-HA (Babco), Y-11 polyclonal anti-HA (Santa Cruz Biotechnology), anti-hDLG (Transduction Laboratories), anti-T7 (Invitrogen), C54 anti-PTEN (15), and 6H2.1 anti-PTEN monoclonal (a gift of Dr. J. Lees) were used at 1:1000 for Western blotting and 1:500 for immunoprecipitations. To generate specific antibodies against phosphoserine 380 (pS380) the peptides CEPDHYRYpS-DTTSDDP and CEPDHYRYSDTTSDDP (PTEN residues 373–388) were synthesized (Tufts Synthesis Facility). Rabbits were immunized with phosphorylated peptide by Upstate Biotechnology, and immune sera was affinity-purified using the Sulfolink kit (Pierce). Protein extracts, immunoblots, and immunoprecipitations were performed as described (13).

**Phosphatase Treatment**—Immunoprecipitations were performed as described (15). Bound complexes were washed with TNN (50 mM Tris, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, 5 mM EDTA, pH 8.0) and with phosphatase buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1 mM MgCl<sub>2</sub>, 1 mM dithiothreitol) and then incubated with or without alkaline phosphatase (Roche Molecular Biochemicals). Where indicated phosphatase inhibitors (50 mM NaF, 5 mM sodium orthovanadate, 50 mM β-glycerol phosphate) were added.

<sup>1</sup> The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; PDZbd, PDZ binding domain; PAC, PTEN-associated complex; HA, hemagglutinin; WT, wild-type; GFP, green fluorescent protein; PHD, phosphatase domain; MAGI, membrane-associated guanylate kinase inverted.



**FIG. 1. Phosphorylated PTEN is not recruited into a high molecular weight complex.** *A*, fractions from the S-300 gel filtration column were immunoblotted with anti-PTEN ( $\alpha$ PTEN; C54) and then stripped and re-probed with anti-pS380 ( $\alpha$ pS380) and anti-hDLG ( $\alpha$ hDLG). *B*, left, PTEN was immunoprecipitated from protein extracts from ACHN cells with anti-PTEN (6H21) and left untreated or treated with phosphatase with or without inhibitors (*PPase*, *Inh*) and immunoblotted with the indicated antibodies. *B*, right, U2-OS cells were transfected with pSG5L-HA-PTEN;WT or HA-PTEN;A5. Cell extracts were immunoblotted with the indicated antibodies.

**Gel Filtration**—Frozen rat livers were homogenized in TNN, centrifuged, and passed through 0.8, 0.45, and 0.22- $\mu$ m filters. Extracts were applied to Sephacryl S-300 or S-200 (16  $\times$  60; Amersham Biosciences) columns and were developed with phosphate-buffered saline at 0.5 ml/min at 4  $^{\circ}$ C. 2-ml fractions were collected. After the void volume (fractions 1–20) fractions 21 through 44 were collected and immunoblotted.

**Protease Sensitivity Assay**—Immunoprecipitations were prepared with Y-11 or HA-11. Where indicated samples were dephosphorylated prior to digestion. The beads were washed with TNN and 50 mM  $\text{NH}_4\text{HCO}_3$ , split, and incubated with 0, 5, 50, or 500 ng of sequencing grade trypsin. Samples were separated by gel electrophoresis and immunoblotted.

## RESULTS

**PTEN Is Recruited into a Protein Complex**—PTEN is a lipid phosphatase whose main substrate is localized to the plasma membrane. A prediction is that PTEN needs to be localized to the membrane to perform its function. There are two possible mechanisms by which PTEN could be targeted to the membrane. First, PTEN can bind to lipid vesicles through the C2 domain (8). However, the fact that the addition of a myristoylation signal increases the ability of PTEN to induce a proliferation arrest (see Ref. 16, and data not shown) suggests that a second mechanism for recruitment may exist. Second, PTEN contains a PDZ binding domain at the tail that can interact with PDZ domain-containing proteins (9–12). Such PDZ proteins serve as scaffolds to build membrane-localized multiprotein complexes (17). Thus, recruitment into a protein complex could be a regulatory mechanism by which PTEN is translocated to the plasma membrane. To test the hypothesis that PTEN is recruited into a protein complex, cell extracts prepared from rat liver were separated by gel filtration over a Sephacryl S-300 column. PTEN was found in two peaks when detected by immunoblotting. The first peak eluted with a molecular mass greater than 600 kDa whereas the second eluted with a molecular mass of 65 kDa (Fig. 1A). The latter peak is consistent with the predicted size of monomeric PTEN, whereas the former appears to represent a higher order complex. These results suggest that a fraction of PTEN is found in a protein complex (PAC). Several PDZ domain-containing proteins bind to PTEN including hdlg and MAGI-1, -2, and -3 (9–12). To determine whether any of these proteins were present in the PAC we investigated whether PTEN and hDLG co-fractionated in gel filtration experiments. Although hDLG

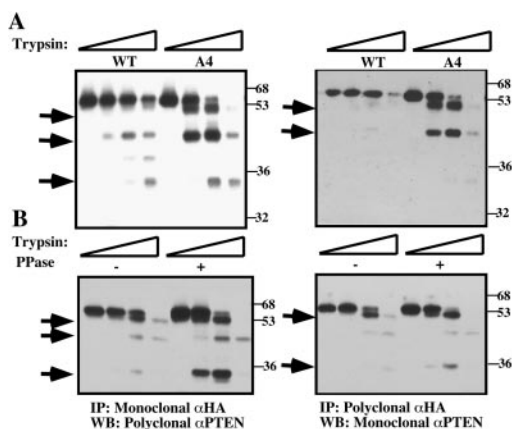
could be detected in high molecular weight fractions it did not co-migrate with the PAC (Fig. 1A). Attempts to recapitulate these experiments with MAGI proteins were hampered by the lack of reagents sensitive enough to detect the endogenous proteins in these extracts. Thus, it is not clear whether any one or all of these proteins are physiologically bound to PTEN *in vivo*. Interestingly, the p85 regulatory subunit of PI3K also co-fractionated with PTEN (data not shown). Although one column is not enough to determine whether PI3K p85 is present in the PAC these data raise the interesting possibility that both PI3K and the inhibitor PTEN are present in the PAC.

**PTEN Phosphorylation Blocks Its Recruitment into a Protein Complex**—We and others showed previously that mutation of phosphorylation sites within the PTEN tail leads to a decrease in protein stability and an increase in PTEN activity (13, 18). An interesting possibility is that phosphorylation of PTEN regulates its association or participation in the PAC. To this end a phospho-specific antisera against phosphoserine 380 (anti-pS380) was raised. Experiments done previously with individual phosphorylation site mutants indicated that of the phosphorylation sites found *in vivo* serine 380 is the more critical for regulating PTEN function (see Ref. 13, and data not shown). Anti-pS380 detected PTEN in the untreated immunoprecipitates and in those containing phosphatase inhibitors. However, no signal was detected when the extracts were treated with phosphatase (Fig. 1B). Additionally, anti-pS380 could detect PTEN;WT but not PTEN;A5 (alanine substitutions at Ser-370, Ser-380, Thr-382, Thr-383, and Ser-385) or PTEN;S380A (see Fig. 1B, and data not shown). Thus the anti-pS380 antibody is phosphospecific.

Next, gel filtration column fractions were re-probed using anti-pS380. As shown in Fig. 1A although two peaks are detected by anti-PTEN, only the low molecular mass (65 kDa) peak was detected by anti-pS380. Similar results were seen in independent experiments where proteins were separated over an S-200 gel filtration column (data not shown). These results suggest that PTEN recruitment to PAC is regulated through phosphorylation. Previous data have shown that PTEN is more active when unphosphorylated; thus these new data suggest that the PTEN found in the PAC is the biologically active form of PTEN.

**Phosphorylation of the PTEN Tail Results in a Conformational Change That Masks the PDZ Binding Domain**—How does phosphorylation regulate PTEN recruitment into the PAC? One possibility is that phosphorylation of the PTEN tail causes a conformational change that diminishes PTEN affinity for its binding partners. To test this hypothesis HA-PTEN and HA-PTEN;A4 (alanine substitutions at Ser-380, Thr-382, Thr-383, and Ser-385) were produced in U2-OS cells, purified by anti-HA immunoprecipitation, and subjected to partial tryptic digestion. Tryptic fragments were detected by immunoblotting with either a monoclonal (6H2.1) or a polyclonal (C54) anti-PTEN antibody that recognize the PTEN C terminus (Fig. 2). Mutation of the phosphorylation sites (PTEN;A4) resulted in increased accessibility of tryptic sensitive sites indicated both by the presence of new cleavage sites and by more efficient digestion at multiple enzyme concentrations (Fig. 2, *top panels*). Similarly when wild-type HA-PTEN was treated with phosphatase it was more sensitive to tryptic digestion (Fig. 2). These results suggest that PTEN undergoes a conformational change when phosphorylated on tail residues.

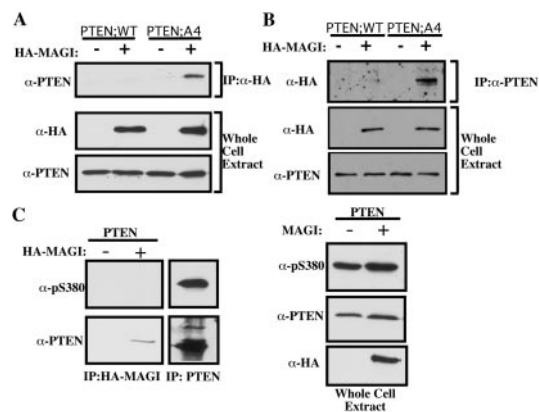
Because tail phosphorylation closed the conformation of PTEN, we investigated whether phosphorylation masked the PDZbd. Several PDZ domain-containing proteins have been shown to bind to the PTEN PDZbd (9–12). To determine whether phosphorylation could regulate the interaction of



**FIG. 2. Phosphoisoforms of PTEN exist in different conformational states.** A, U2OS cells were transfected with pSG5L-HA-PTEN;WT (WT) or HA-PTEN;A4 (A4), and lysates were immunoprecipitated with monoclonal or polyclonal anti-HA antibodies. Immunoprecipitates were digested with trypsin and immunoblotted with 6H2.1 or C54. B, proteins extracted from U2O-S cells transfected with pSG5L-HA-PTEN;WT were immunoprecipitated (IP) and treated or left untreated with  $\lambda$ PPase, digested with trypsin, and immunoblotted as in A. WB, Western blot.

PTEN with PDZ domain-containing proteins the consequence of mutating the PTEN phosphorylation sites on the binding with MAGI-2 was analyzed. U2-OS cells were transfected with plasmids encoding GFP-PTEN;WT or GFP-PTEN;A4 along with either empty vector or HA-MAGI-2. As shown previously PTEN;WT co-immunoprecipitates with MAGI-2 (9); however mutation of the tail phosphorylation sites resulted in a dramatic enhancement in MAGI-2 binding (Fig. 3A). These results were confirmed by immunoprecipitating PTEN and immunoblotting for HA-MAGI-2 (Fig. 3B). Similar results were obtained with MAGI-1 (data not shown) suggesting that the findings are not specific for MAGI-2. Finally, extracts prepared from U2-OS cells transfected with plasmids encoding GFP-PTEN and either vector or HA-MAGI-2 were immunoprecipitated with anti-HA antibody or with anti-PTEN antibody and immunoblotted with either anti-PTEN (pan) or anti-pS380. Here, despite adequate detection of phospho-S380 in both the whole cell extracts and in the direct PTEN immunoprecipitates, no phosphorylated PTEN was detected in complex with MAGI-2 (Fig. 3, left panel) despite the abundant presence of phosphorylated PTEN found in the whole cell extracts (Fig. 3C, right panel). Conversely, we were able to detect PTEN in the immunoprecipitates albeit at lower levels (Fig. 3C). A recent report showed that alanine substitution of threonine 382 and threonine 383 also enhances PTEN binding to MAGI-2 (19). However, alanine substitution of these threonine sites impairs serine 380 phosphorylation (data not shown) thus further experiments will be needed to distinguish direct from indirect effects of mutation of these sites. Taken together these data show that phosphorylation of the tail blocks or inhibits binding of PTEN to MAGI-2 and MAGI-1 suggesting that the PDZbd is masked by the phosphorylation.

**Phosphorylation of the PTEN Tail Blocks Functional Cooperation of PTEN with MAGI-2**—It has been shown previously that MAGI-2 and -3 cooperate with PTEN as measured in assays reflecting PTEN-dependent inhibition of Akt activity. A likely explanation for these results is that MAGI recruits PTEN to the plasma membrane where the substrates of PTEN are localized (9, 10). Our previous results showed that PTEN tail phosphorylation restricts its activity, and data presented here show that PTEN tail phosphorylation prevents MAGI interaction. These data suggest a model in which PTEN-MAGI interaction and therefore PTEN activity is negatively regulated



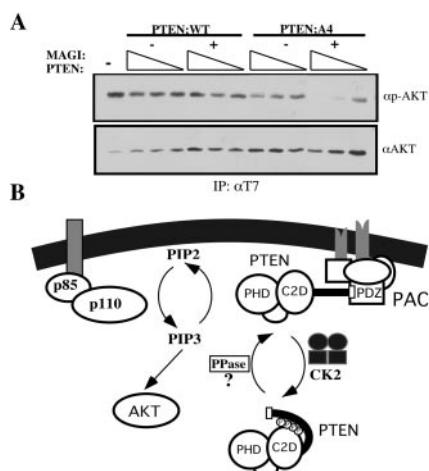
**FIG. 3. Phosphorylation-dependent interaction of MAGI/PTEN.** A, U2-OS cells were transfected with pCDNA3-GFP-PTEN;WT (PTEN;WT) or GFP-PTEN;A4 (PTEN;A4) in the presence or absence of pCDNA3-HA-MAGI-2 (HA-MAGI). Lysates were immunoprecipitated (IP) with HA-11 and immunoblotted with C54. Whole cell extracts were separated and immunoblotted with HA-11 or C54. B, U2-OS cells were transfected as in A. Lysates were immunoprecipitated with C54 and immunoblotted with HA-11. Whole cell extracts were immunoblotted with HA-11 or C54. C, U2-OS cells were transfected with pCDNA3-GFP-PTEN;WT and pCDNA3-HA-MAGI-2, and lysates were immunoprecipitated with HA-11 or 6H21 and immunoblotted with anti-pS380 and C54. Whole cell extracts were immunoblotted with anti-pS380, C54, and HA-11.

by phosphorylation. A prediction of this model is that the enhancement of PTEN function by MAGI-2 would be restricted to the unphosphorylated form of PTEN. To test this hypothesis, the PTEN null cell line 786-O was co-transfected with plasmids encoding PTEN;WT or PTEN;A4 with either the vector alone or with pCDNA3-HA-MAGI-2. Transfected Akt was immunoprecipitated with anti-T7 and immunoblotted with anti-phospho-Akt (Ser-473) to detect active Akt. As shown in Fig. 4A, the ability of PTEN to negatively regulate Akt is maximally achieved when PTEN;A4 and MAGI-2 are co-transfected. These data are consistent with the idea that phosphorylation of the PTEN tail results in an inhibition of PTEN activity, at least in part, by preventing its interaction with PDZ domain-containing proteins such as MAGI-2.

#### DISCUSSION

The results presented here provide evidence for a mechanistic model of how phosphorylation of the PTEN tail modulates PTEN biological function (Fig. 4B). Phosphorylation of the PTEN tail can switch PTEN from an open to a closed conformation and prevent its recruitment into a higher molecular mass complex, PAC. This phosphorylation-dependent conformational change of the PTEN tail appears to mask the PDZbd and prevent PTEN interaction with PDZ-containing proteins. The PDZ domain is a small 80–100-residue modular domain usually found in proteins that serve as scaffolds for the assembly of multiprotein complexes. Thus, the PDZ-containing proteins found in the PAC may recruit several other molecules to form a multiprotein complex. A question that remains is which, if any, of the known PDZ proteins are in the PAC complex. In addition, the PAC appears to contain p85, the regulatory subunit of PI3K. This raises the possibility that other members of the PI3K pathway might also be found in the PAC as a mechanism for enhancing signaling efficiency. The biochemical purification and identification of the endogenous PAC will begin to address these questions.

There are several additional lines of evidence favoring a regulatory role for the PTEN tail. First, the structure of PTEN PHD and C2 domain were resolved by x-ray crystallography (8). In these studies the C-terminal tail was highly susceptible



**FIG. 4. MAGI enhancement of PTEN biological function depends on phosphorylation.** A, 786-O cells were transfected with pCDNA3-T7-Akt (0.5  $\mu$ g), pSG5L-HA-PTEN;WT or HA-PTEN;A4 (0.1, 0.05 or 0.01  $\mu$ g), and pCDNA3-HA-MAGI-2 (0.2  $\mu$ g). Akt was immunoprecipitated (IP) with anti-T7 and immunoblotted with phospho-Akt ( $\alpha$ p-AKT). The same blot was then stripped and re-probed with anti-pan Akt ( $\alpha$ AKT). B, model for phosphorylation-dependent regulation of PTEN activity.

to protease digestion perhaps indicating the flexible nature of this domain. These data support the notion that the conformation of the C terminus might change upon phosphorylation. Second, the tail is highly conserved in evolution from *Xenopus* to humans but diverges in *Caenorhabditis elegans* and *Drosophila melanogaster* indicating that a new role for the PTEN tail was acquired during evolution. Third, the recently discovered PTEN homologue, transmembrane phosphatase with tensin homology/PTEN2 (20, 21), is predicted to contain a PHD and a C2 domain (21) but lacks the tail. Instead it contains an N-terminal extension consisting on four transmembrane domains that targets transmembrane phosphatase with tensin homology/PTEN2 to the Golgi (21). These observations suggest that an ancestral gene containing the PHD and the C2 domain diverged and that additional domains, such as the PTEN tail, were acquired to achieve additional regulation. In the case of human PTEN it appears that the phosphorylation-induced conformational change that regulates recruitment into the PAC is one such evolutionary acquisition.

It has been shown previously that the PTEN tail is phosphorylated *in vitro* by CK2 (18). Using an unbiased approach to purify the PTEN tail kinase activity from cells we found that CK2 co-purified with the PTEN tail kinase activity (data not shown) suggesting that CK2 is the major cellular PTEN kinase. CK2 is a constitutive kinase making it likely that the rate-

limiting step, in switching between phosphorylated and unphosphorylated PTEN, is governed by a phosphatase. This phosphatase, upon activation, presumably dephosphorylates the PTEN tail changing its conformation and thus increasing the amount of complexed PTEN. An interesting possibility is that the PTEN tail phosphatase is itself found in PAC.

In conclusion, we propose a model to explain how phosphorylation of the PTEN tail regulates its activity. Once the PTEN tail is phosphorylated there is a change in the conformation of the protein that prevents PTEN interaction with PDZ domain-containing proteins and recruitment of PTEN into a complex. Recruitment is likely important for localization of PTEN close to the plasma membrane and thus is transduced into a change in PTEN activity.

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