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Analysis of Site-specific Phosphorylation of PTEN using Enzyme-Catalyzed Expressed Protein Ligation

Samuel H. Henager^a, Stephanie Henriquez^a, Daniel R. Dempsey^b, Philip A. Cole^b

^[a]Department of Pharmacology and Molecular Sciences, Johns Hopkins School of Medicine, 725 N. Wolfe St., Baltimore, MD, 21205 (USA)

^[b]Division of Genetics, Departments of Medicine and Biological Chemistry and, Molecular Pharmacology, Harvard Medical School and Brigham and Women's Hospital, 25 Shattuck St., Boston, MA 02115 (USA)

Abstract

The activity and localization of PTEN, a tumor suppressor lipid phosphatase that converts the phospholipid PIP3 to PIP2, is governed in part by phosphorylation on a cluster of four Ser and Thr residues near the C-terminus. Prior enzymatic characterization of the four mono-phosphorylated (1p) PTENs using classical expressed protein ligation (EPL) was complicated by the inclusion of a non-native Cys at the ligation junction (aa379), which may alter the properties of the semisynthetic protein. Here we apply subtiligase-mediated EPL to create wt 1p-PTENs. These PTENs are more autoinhibited than previously appreciated, consistent with Tyr379's role in driving autoinhibition. Alkaline phosphatase sensitivity analysis revealed that these autoinhibited 1p conformations are kinetically labile. In contrast to the Cys mutant 1p-PTENs, which are poorly recognized by an anti-phospho-PTEN Ab, three of the four wt 1p-PTENs are recognized by a commonly used anti-phospho-PTEN Ab.

Graphical Abstract

PTEN (Phosphatase and TENsin homolog deleted on chromosome TEN) is a lipid phosphatase that negatively regulates the PI3K/AKT pathway by removing the 3'-phosphate from phosphatidyl 3',4',5'-triphosphate (PIP3) to form PIP2, thus preventing AKT activation and its pro-growth effects.^[1, 2] Inactivating mutations in PTEN have been found in many brain, breast, and prostate cancers,^[3] and germline mutations in PTEN are associated with cancer-predisposition syndromes such as Cowden's disease.^[4] PTEN is a 403-residue protein comprised of an N-terminal phosphatase domain, a C2 domain, and a C-terminal 52-residue tail.^[5] This tail is highly acidic, and is known to be phosphorylated at several

positions by multiple kinases including casein kinase 2 (CK2) and glycogen synthase kinase 3-beta (GSK3 β).^[6, 7] The published structure of PTEN omitted the C-terminal tail to facilitate crystallization, so its structure is unknown.^[5] The details of how C-terminal phosphorylation of PTEN affects the structure and activity of PTEN are incompletely understood. The most well-studied phosphorylation occurs at a cluster of serine and threonine residues near the very end of the tail (Ser-380, Thr-382, Thr-383, and Ser-385).

Previously, semisynthetic PTEN has been generated via classical expressed protein ligation (EPL)^[8] with varying levels of phosphorylation at the Ser-380/Thr-382/Thr-383/Ser-385 cluster. Classical EPL involves a chemoselective ligation of an N-Cys synthetic peptide with a recombinant protein thioester generated via intein rearrangement. Biochemical and biophysical analyses of the semisynthetic phosphorylated and non-phosphorylated PTENs revealed that C-tail phosphorylation drives a “closed” conformation, in which the phospho-tail interacts with the C2 and phosphatase domains of PTEN^[9, 10] and weakens phospholipid interaction.^[9, 11] These semisynthetic PTENs all contained a Y379C mutation to facilitate the native chemical ligation reaction required for EPL,^[8, 12] but it was later found that this mutation is linked to anomalous PTEN membrane association *in vivo*.^[13]

Subsequently, wild-type tetra-phosphorylated PTEN was created using enzyme-catalyzed EPL involving the engineered protein ligase, subtiligase.^[14] ^[15–17] Subtiligase catalyzes ligation between the C-terminus of a protein or peptide containing a C-terminal ester or thioester, and the free N-terminus of another peptide.^[14–18] Subtiligase can accommodate a wide variety of residues at the ligation junction,^[14, 15, 17] and this sequence tolerance was harnessed to create tetra-phosphorylated (4p) PTEN without the Y379C mutation. Analysis of this PTEN showed that it adopted a closed conformation and was less active toward PIP₃ to a much greater degree than was seen with C379 tetra-phosphorylated PTEN.^[14] This difference between C379 and Y379 4p-PTEN's suggests that previous studies ^[10] with mono-phosphorylated (1p) PTENs, which incorporated the Y379C mutation, may also not fully capture the effect of tail phosphorylation on PTEN. To more accurately understand the effect of tail mono-phosphorylation events on PTEN activity and structure, we again exploited enzyme-catalyzed EPL to create each of the wild-type semisynthetic mono-phosphorylated PTENs (Schema 1), and below we describe their production and the analysis of their enzymatic properties.

To produce the native ligation junction semisynthetic PTENs (Y379-PTENs) with phosphorylation at Ser380, Thr382, Thr383, or Ser385, the corresponding mono-phosphorylated PTEN C-tail peptides aa378–402 (RYS**DTT**SDPENEPFDEDQHTQITK-biotin, phosphorylated residues in bold) were prepared using solid phase peptide synthesis. These were ligated to recombinant PTEN thioester aa1–377 (r-PTEN) using subtiligase. The enzyme-catalyzed ligation with the pSer380 C-tail peptide proceeded to about 30% conversion whereas those with pThr382, pThr383, and pSer385 reached 40–45% conversion based on Coomassie-stained SDS-PAGE (see Figures S1–S4). We surmise that the subtiligase-mediated reaction is somewhat impeded by the pSer380 which is closest to the Arg378 ligation site, leading to the lower yield. Purification of each of the desired semisynthetic mono-phosphorylated Y-PTENs (Tyr at aa379) to greater than 90% homogeneity could be achieved using avidin affinity chromatography based on the inclusion

of a biotin attached to a Lys at aa402 (see Figures S1–S4). The Y-PTENs were analyzed by western blot using a commercially available polyclonal anti-phospho-PTEN antibody that was previously generated with a triphosphorylated-pSer380,pThr382,pThr383-peptide (Figure 1a). It was previously reported with phosphorylated Cys379-PTEN (C-PTEN) forms that only pSer380 was detected over background with this antibody.^[10] In contrast, the pSer380, pThr383, and pSer385 Y-PTEN forms were each readily illuminated with the anti-phospho-PTEN antibody, whereas pThr382-Y-PTEN was the only mono-phosphorylated Y-PTEN that showed background signal with this antibody (Figure 1a). These results indicate that most of the mono-phosphorylated PTENs will contribute to the overall analysis of phospho-PTEN state in cellular studies. They also highlight the importance of having the natural sequence around the phospho-sites for characterizing phospho-antibody reagents.

We evaluated the phosphatase activity of the four mono-phosphorylated PTEN forms using the soluble PIP3 substrate, di-C6-PIP₃. Mammalian PIP3 commonly includes long chain fatty acids, C18-C20, but these phospholipids show low solubility and are commonly studied in vesicles, complicating enzymologic analysis. Six carbon fatty acid di-C6-PIP3 is often used as a surrogate for kinetic studies as these phospholipids are more soluble and previous work on PTEN has validated their utility.^[14] In the PTEN enzymatic assays, all four mono-phosphorylated Y-PTEN forms showed a decrease in catalytic activity relative to truncated recombinant PTEN (t-PTEN, aa 1–378) or non-phosphorylated full-length semisynthetic PTEN (n-Y-PTEN) (Figure 1b and Table 1). pSer380-Y-PTEN showed a 3-fold reduction in k_{cat}/K_M relative to Y-n-PTEN, which is similar to what had been seen previously with pSer380-C-PTEN.^[10] However, pThr382-, Thr383-, and pSer385-Y-PTENs each showed a 6- to 9-fold catalytic reduction compared with n-Y-PTEN, which are markedly greater declines than the 2- to 3-fold inhibition seen with the corresponding mono-phosphorylated C-PTENs.^[10] The catalytic behaviors of pSer380-, pThr382-, and pThr383-Y-PTENs showed near-saturation with 160 μ M of di-C6-PIP3 substrate, indicating that phospho-mediated effects for these forms is driven by a reduction in k_{cat} . In contrast, pSer385-Y-PTEN exhibited a linear increase in activity with increasing di-C6-PIP₃, which suggests that the pSer385 impact on catalysis derives primarily from an increase in K_M . Notably, a similar pattern was observed previously with pSer385-C-PTEN.^[10]

These data confirm that each C-terminal phosphorylation event contributes to the overall inhibition of PTEN, but to a larger degree than seen previously.^[10] This increased inhibition is presumably due to contacts mediated by the phenolic sidechain of Tyr379 that are lost with the thiomethyl sidechain of Cys379. In addition, the substrate concentration dependence pattern and the magnitude of the inhibitory effects hint that there may be three distinct interactions between the phosphorylated residues on the tail and the core of PTEN. In one of these, phosphoSer380, the modest-size of the effect (3-fold) and the substrate saturation behavior is distinct from the impacts of the other three C-tail phosphorylations. The phosphoThr382 and phosphoThr383 inhibitory actions, 6-fold and 9-fold, respectively, are similar to each other, larger than the phosphoSer380 effect, but like phospho380, don't significantly influence the reaction K_M . In contrast, phosphoSer385 stands out as it raises the K_M but shows an apparent k_{cat} in the same range as that of the non-phosphorylated PTEN form. Cross-linking data^[10] suggest that phosphoSer385 may be close to the active site of PTEN. If it in fact occupies the active site, or otherwise blocks PIP3 from accessing

the active site, this could drive the observed increase in K_M . When phosphoThr382/phosphoThr383 or phosphoSer380 bind to their respective sites, it could trigger a conformational shift in the active site such that catalysis is reduced, but substrate binding is less affected. As noted previously, combinations of these phosphorylations appear to show partial additivity in inhibiting PTEN catalytic activity and particular patterns of phosphorylation may allow for a gradation of PIP3 hydrolytic impact.^{[9],[10]}

Prior studies have shown with pSer380-C-PTENs that the hydrolysis of pSer380 by the non-selective enzyme, alkaline phosphatase can be used as a probe of the state of conformational closure of the phospho-PTEN forms.^[10] In this approach, intramolecular interactions with the phospho groups limit exposure to alkaline phosphatase, reducing the rate of dephosphorylation. We therefore investigated the sensitivity of pSer380, pThr383, and pSer385 mono-phosphorylated Y-PTEN forms to alkaline phosphatase action. Each of these PTEN phospho-forms exhibited rapid dephosphorylation by alkaline phosphatase with half-lives of 1–3 minutes (Figure 1c and Table 1), rates close to the denatured state.^{[9],[10]} These rates are ~10-fold faster than that of the folded tetraphosphorylated C-PTEN state and ~40-fold faster than the tetraphosphorylated Y-PTEN state. These results suggest a relatively rapid dissociation of the phospho-tails from the body of PTEN, efficiently accessing an open conformation. A direct comparison of the alkaline phosphatase reactions with mono-phosphorylated and tetra-phosphorylated PTEN forms is complicated by the fact that there are four phospho-equivalents that are removed in the latter, and three of these will still give western blot signal with anti-phospho-PTEN antibody. Nevertheless, we speculate that the alkaline phosphatase assay with the phospho-PTEN forms indicates that the kinetic stability of the interaction between the phospho-tail and the core of PTEN is low and the open and closed phospho-PTEN forms are in rapid equilibrium, rendering the single phosphates amenable to speedy removal by alkaline phosphatase.

In summary, we have prepared and analyzed the enzymatic properties of each of the mono-phosphorylated forms of the key regulatory C-tail phospho-cluster in the signaling lipid phosphatase PTEN using the recently described enzyme-catalyzed expressed protein ligation. By producing these semisynthetic forms with the natural sequence in the phospho-cluster region, we have revealed more sizable inhibitory actions of these phosphates than previously understood. Based on the pattern of inhibition, our findings suggest that there may be three distinct surfaces of interactions mediated by the three phosphates and the PTEN core. Experiments with alkaline phosphatase hint that the open and closed mono-phosphorylated PTEN forms rapidly interconvert so that the phosphates can be quickly removed by a phosphatase. Such rapid dephosphorylation could enable fast activation of PTEN under conditions relevant for phospholipid cell signaling.

Experimental Section

r-PTEN expression and purification

A pFastBac1 baculovirus vector containing the PTEN-intein-CBD fusion (PTEN residues 1 – 377) was used to make bacmid and then baculovirus in SF-21 insect cells using standard methods,^[9, 10, 14] and the baculovirus was then used to infect High Five insect cells with M.O.I. 1.0. After growing infected High Five cells in Express Five SFM Media (Gibco) for

48 h at 27 °C, they were pelleted ($700 \times g$, 10 min, 4 °C) and then resuspended in 1/20 of the medium used for culture, pelleted again (discarding the supernatant), and then flash frozen and stored at -80 °C. Resuspended cells from 200 ml culture were lysed in a 40 ml homogenizer in 30 ml lysis buffer (150 mM NaCl, 50 mM HEPES, 1 mM EDTA, 10% Glycerol, 0.1% Triton X-100) containing three dissolved protease tablets (Roche). The lysate was then centrifuged ($17,500 \times g$, 40 min, 4 °C), and the supernatant was added to a 10-ml bed of powdered cellulose (Sigma). After 1 h of incubation at 4 °C, the lysate was drained from the cellulose and then bound to 5 ml chitin resin (NEB). The resin was then washed with 150 ml washing buffer (250 mM NaCl, 25 mM HEPES, 0.1% Triton X-100, pH 7.5), and incubated overnight in cleavage buffer (250 mM NaCl, 50 mM HEPES, 300 mM MESNA, pH 7.5). The cleavage buffer was eluted from the resin, and the buffer was exchanged with 150 mM NaCl, 50 mM MES, pH 6.5 using an Amicon 10 kDa MWCO filter unit. The PTEN thioester protein produced was shown to be $>80\%$ pure by Coomassie-stained SDS–PAGE and concentrated to greater than 1 mg/ml. If the r-PTEN protein thioester was not used within a day, it was flash frozen and stored at -80 °C. For stable t-PTEN generation, 50 mM Cys was added to the cleavage buffer, and it was otherwise handled as described above.

Peptide synthesis

Peptides corresponding to residues 378–403 of PTEN were synthesized by double coupling residues 386–402 for 1.5 h each, triple coupling residues Asp-384 and Asp-381 for 1.5 h each, and double coupling phosphorylated residues for 3 h each. Phosphate groups were monoprotected by O-benzyl groups during the synthesis. All peptides were deprotected and cleaved from resin using reagent K (82.5:2.5:5:5:5–trifluoroacetic acid:ethane dithiol:water:thioanisole:phenol) then purified using reverse-phase C18 HPLC and lyophilized. Supplementary Table 1 lists the peptides used for PTEN ligations. Peptide structures were confirmed using negative ion mode MALDI mass spectrometry which showed the following masses: for these peptides, calculated m/z [M] 3273.6, measured m/z [M-H] 3271.4 (1p-380-tail), 3271.0 (1p-382-tail), 3271.0 (1p-383-tail), and 3271.4 (1p-385-tail).

PTEN ligation and purification

Ligations were carried out as described previously.^[14] Briefly, a reaction mixture containing r-PTEN thioester (~ 1 mg/ml) C-terminal peptide (~ 10 mg/ml) in buffer (100 mM BICINE, 100 mM CaCl_2 , pH 8.0) was treated with subtiligase (~ 25 μM final). A 3 μl aliquot was saved as a negative control before adding subtiligase. The reaction was split into 150 μl aliquots and incubated at 25 °C. After 4 h, 3 μl of the mixture was taken for SDS–PAGE analysis. The remainder of the mixture was injected onto a Superdex 75 size-exclusion column (GE Healthcare Life Sciences). Size-exclusion chromatography was performed with a flowrate of 0.6 ml/min in a buffer containing 500 mM NaCl, 5 mM DTT, pH 7.0; and 0.3 ml fractions were collected, and 5 μl of each fraction was analyzed by Coomassie-stained SDS–PAGE. Fractions containing ligated PTEN were combined and stored at 4 °C overnight and then loaded onto 500 μl pre-blocked (10 mM biotin followed by 0.1 M Glycine, pH 2.8, followed by 500 mM NaCl, 5 mM DTT, pH 7.0) mono-avidin resin (Thermo) on ice. Resin was washed sequentially with 5 ml of 500 mM NaCl, 50 mM Tris, 10 mM DTT, pH 8.0 for

1 h followed by 5 ml of 1 M NaCl, 50 mM Tris, 10 mM DTT, pH 8.0 for 1 h. Next, the resin was equilibrated with 5 ml 150 mM NaCl, 50 mM Tris, 10 mM DTT, pH 8.0 and then eluted with 10 mM biotin in the same buffer. The resin was incubated with 100 μ l of elution buffer for 10 – 15 min at room temperature, drained, then incubated with an additional 100 μ l of elution buffer. This process was repeated up to a total of 500 μ l. The purified semisynthetic protein was shown to be >90% pure by Coomassie-stained SDS–PAGE (Supplementary Figures 2 – 5)

Alkaline phosphatase sensitivity assay

This was adapted from previous methods.^[9, 10, 14] PTEN (1 μ g) was incubated with 5 μ M calf intestine phosphatase (NEB) in 20 μ l of reaction buffer (50 ng/ μ l PTEN) for up to 3 hours. Reaction buffer consisted of 50 mM Tris, 10 mM BME, pH 8.0. Samples were taken from the reaction at various time points, diluted ten-fold in SDS loading dye, and run on SDS–PAGE for western blot analysis. 10 μ l were loaded for the Cys-PTEN and 2 μ l were loaded for the Tyr-PTEN. Blots were analyzed using anti-phospho-PTEN antibody (Cell Signaling no. 9554) at 1:1,000 dilution. Images were analyzed using ImageJ software, and Prism 6 software (Graphpad) was used to determine phosphorylation half-life fit to a standard exponential decay: $A=A^0e^{-kt}$ where t is the pseudo-first-order rate constant. The half-life ($t_{1/2}$) is calculated according to the equation $t_{1/2}=0.693/k$. Replicates with different preps showed similar half-lives (within 20%).

PTEN phosphatase activity assay

PTEN activity toward a water-soluble substrate (diC6-PIP₃, from Avanti Polar Lipids) was determined by the evolution of inorganic phosphate as measured with a malachite green^[19] detection kit (R and D Biosystems). Assays were conducted as described previously.^[9, 10, 14] Briefly, 0.5 – 1.5 μ g PTEN was incubated with 160 μ M diC6 PIP₃ for 10 minutes in 25 μ l reaction buffer (50 mM Tris, 10 mM BME, pH 8.0) at 30°C. Samples were quenched with malachite green reagent (R and D Biosystems) and absorbance was measured at 620 nm. The data were analyzed using the Michaelis-Menten equation, $v=V_mS/(K_m+S)$ using non-linear curve fitting in Prism 6 software (Graphpad).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments-

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Figure 1.**Characterization of WT 1p-PTEN Constructs**

Monophosphorylated PTEN is less active than non-phosphorylated or truncated PTEN, but adopts a much more open configuration than tetraphosphorylated PTEN. A) Polyclonal phospho-PTEN antibodies are able to recognize mono-phosphorylated PTEN constructs. Top: Western blot analysis of PTEN constructs with polyclonal antibodies raised against tri-phosphorylated (pSer-380, pThr-382, pThr-383) PTEN tail peptide. Values shown are the intensities relative to the 4p-PTEN band. Bottom: Coomassie stained SDS-PAGE analysis of PTEN constructs. Values shown are the intensities relative to the 4p-PTEN band. B) Activity

of n-, t-, 1p380-, 1p382-, 1p383-, and 1p385-PTEN toward soluble di-C6 PIP₃. Values shown are averages and SEM of two replicates. k_{cat}/K_M values are reported in Table 1. C) 1p-PTEN constructs are much more sensitive to alkaline phosphatase than C379-4p-PTEN. Values shown are averages and SEM of two replicates. Representative Western blots show the decrease in phosphorylation with longer phosphatase treatment. C379-4p-PTEN half life = 22.8 ± 2.8 min; 1p380-PTEN half-life = 1.7 ± 0.2 min; 1p383-PTEN half-life = 2.7 ± 0.2 min; 1p385-PTEN half-life = 0.8 ± 0.1 min.

Schema 1.**Subtiligase-mediated Expressed Protein Ligation**

Schematic of subtiligase-catalyzed semisynthesis of mono-phosphorylated PTEN. PTEN is expressed as a fusion with a modified intein and chitin binding domain (CBD). The fusion protein is immobilized on chitin resin and truncated PTEN (r-PTEN) is cleaved with a thiol and isolated. The r-PTEN thioester and a synthetic peptide corresponding to residues 378–402 of PTEN are ligated together by subtiligase, producing a full-length, monophosphorylated semisynthetic PTEN.

Table 1.

Catalytic efficiencies and alkaline phosphatase dephosphorylation half-lives for PTEN constructs.

PTEN	<i>k_{cat}</i> / <i>K_M</i>	Alkaline phosphatase assay half-life
	$\times 10^2 \text{ M}^{-1} \text{ s}^{-1}$	min
t-PTEN	18 ± 2	ND
n-PTEN	18 ± 9	ND
Y379-1p-380	6 ± 5	1.7 ± 0.2
Y379-1p-382	3 ± 2	ND
Y379-1p-383	2 ± 1	2.7 ± 0.2
Y379-1p-385	2 ± 0.1	0.8 ± 0.1
C379-1p-380	6 ± 1	3 ± 2
C379-1p-382	7 ± 1	ND
C379-1p-383	6 ± 4	ND
C379-1p-385	9 ± 3	ND

Catalytic efficiencies and alkaline phosphatase dephosphorylation half-lives for PTEN constructs. ND, not determined. Values for C379 PTEN constructs taken from ref. 10.