The intrinsically disordered tails of PTEN and PTEN-L have distinct roles in regulating substrate specificity and membrane activity

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INTRODUCTION

PTEN (phosphatase and tensin homologue deleted on chromosome 10) is a tumour suppressor, with inactivating mutations that are among the most common in solid tumours [1–3]. Although initially identified as a protein tyrosine phosphatase (PTP) [4], the preferred substrate of PTEN is the lipid second messenger phosphatidylinositol 3,4,5-trisphosphate (PIP3) [5]. PTEN activity directly opposes the activity of the phosphoinositide 3-kinases (PI3K) [13,15–17]. The TI loop (residues 160–171) forms a uniquely deep pocket to accommodate the bulky PIP3 substrate (PIP2)-binding motif (PBM, residues 1–7), a dual-specificity phosphatase domain, a C2 domain, a 50-amino-acid unstructured C-terminal tail and a C-terminal PDZ-binding motif [13,14]. The phosphatase domain has an active site that is formed by the conjunction of three loops: the P loop, the WPD loop and the TI loop. The WPD loop (residues 88–98) is a flexible loop that carries Asp92, a residue critical for substrate catalysis [13,15–17]. The TI loop (residues 160–171) forms a uniquely deep pocket to accommodate the bulky PIP3 substrate [13,16]. The highly conserved P loop (residues 121–131) contains the HCXXGXXKR phosphatase signature motif, including the catalytically crucial cysteine, Cys124, which forms a cysteiny1 phosphoenoyme intermediate that is subsequently hydrolysed [18].

PTEN activity is controlled epigenetically [19], post-transcriptionally and post-translationally [20]. The 47-kDa PTEN protein is subject to phosphorylation [21,22], acetylation [23], oxidation [24], ubiquitination [25] and SUMOylation [26]. Furthermore, recent discoveries have shown that PTEN can be transported from one cell to another, via ubiquitin-mediated exosomal sorting [27] and through secretion of PTEN-long (PTEN-L) [28,29].

The N-terminal extension of PTEN-L (a longer variant of PTEN which results from an alternative translation initiation site) encompasses a poly-alanine secretion signal and a .

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poly-arginine re-entry sequence that enable the protein to be secreted and enter neighbouring cells respectively [29]. PTEN-L also localizes to the mitochondria, where it regulates cytochrome c oxidase function [30]. The N-terminal extension is predicted to be highly disordered and the target of numerous post-translational modifications [31]. PTEN-L was suggested to be in a constitutively active state with regard to PIP2-mediated activation of PTEN, possibly due to an altered structure surrounding the PBM [32].

PTEN activity is closely regulated by phosphorylation at multiple sites by serine/threonine kinases. Six inhibitory phosphorylation sites are located within the PTEN tail on residues Thr\textsuperscript{366}, Ser\textsuperscript{370}, Ser\textsuperscript{380}, Thr\textsuperscript{382}, Thr\textsuperscript{383} and Ser\textsuperscript{385} (Figure 1A). These sites are targeted by the CK2 and GSK3\textbeta [22,23–35], preventing proteosomal degradation of PTEN [36]. Two models of auto-inhibition have been proposed. In one model, the phosphorylated tail interacts with the calcium-binding region (CBR)3 loop of the C2 domain, either electrostatically [35] or non-electrostatically [37], preventing membrane binding and PIP3 dephosphorylation. The second model argues that the phosphorylated tail interacts with the phosphatase domain and the PIP2-binding domain (PBD) [22]. Auto-dephosphorylation of pThr\textsuperscript{366} and pSer\textsuperscript{370} can selectively remove these phosphates [38].

In the present study, we determine how phosphorylation of PTEN and the addition of the N-terminal extension of PTEN-L affect enzymatic properties such as substrate specificity and interfacial catalysis. Furthermore, we use HDX–MS to provide structural insights into how the phosphorylated C-terminal tail...
of PTEN and a putative α-helix in the N-terminal extension of PTEN-L regulate these catalytic properties.

**MATERIALS AND METHODS**

**Protein expression and purification**

For expression of recombinant PTEN proteins, 1–8 litres of *Spodoptera frugiperda* Sf9 cells at a density of 1 × 10⁶ cells/ml were infected with an optimized ratio of virus. Expression of PTEN in *Escherichia coli* was found to produce unsatisfactory material that showed pronounced aggregation. All PTEN constructs contain an N-terminal His₆-tag followed by a tobacco etch virus (TEV) cleavage site. After 48 h of infection at 27 °C, the cells were harvested and washed with ice-cold PBS. The pellets were lysed by sonication for 5 min in Buffer A (20 mM Tris/HCl, pH 8.0, 300 mM NaCl, 10 mM imidazole, 5% glycerol, 2 mM 2-mercaptoethanol and 0.5% Triton X-100, with one Complete EDTA-free protease inhibitor tablet added (Roche) per 50 ml of buffer). Lysate was centrifuged for 45 min at 140 000 g. The supernatant was then passed through a 0.45 μm filter (Sartorius Biotech) and then loaded on to a HiTrap FF column (GE Healthcare). The column was washed with up to 30 mM imidazole before being eluted with a 0–100% gradient of Buffer B (20 mM Tris/HCl, pH 8.0, 100 mM NaCl, 5% glycerol, 200 mM imidazole and 2 mM 2-mercaptoethanol). The pooled fractions containing the PTEN were then placed along with an aliquot of His-tagged TEV protease into a 10 000 Da molecular-mass cut-off Snakeskin Dialysis membrane and dialysed in 4 litres of Buffer C (20 mM Tris/HCl, pH 8.0, 200 mM NaCl, 5% glycerol, 2 mM imidazole, 2-carboxyethylphosphine (TCEP)) at 4 °C for 12 h. The ratio of PTEN/TEV in the dialysis was 20:1 (w/w). To remove the His₆–TEV protease, the dialysed solution was subsequently passed back over a 5 ml of HisTrap FF column. Flow-through from the HisTrap column was then dialysed 1:1 with Buffer D (20 mM Tris/HCl, pH 8.0, 10% glycerol and 1 mM DTT), before being loaded on to a 5 ml HiTrap Q column (GE Healthcare) pre-equilibrated in Buffer E (20 mM Tris/HCl, pH 8.0, 50 mM NaCl, 10% glycerol and 1 mM DTT). Protein was eluted with a gradient of 0–100% Buffer F (20 mM Tris/HCl, pH 8.0, 1 M NaCl, 10% glycerol and 1 mM DTT). Pooled fractions were concentrated and then dialysed over a C₄ Van-Guard pre-column (Waters) in 0.1 M sodium acetate, 10 000 units of each kinase per 1000 μl of 40 μM PTEN. The reaction mixture was then injected on to a Superdex 75 10/300 gel-filtration column (or a Superdex 75 16/600 for larger volumes) pre-equilibrated in Buffer G. Fractions containing protein were concentrated and frozen in liquid nitrogen for subsequent analysis. Phosphorylation status was checked with both intact and peptide fragmentation MS.

**Protein dephosphorylation**

Protein was incubated at 30 °C for 90 min with λ-phosphatase as per the manufacturer’s instructions (approximately 50 000 units per reaction). The reaction mixture was then injected on to a Superdex 75 10/300 gel filtration column pre-equilibrated in Buffer G. Fractions containing protein were concentrated and frozen in liquid nitrogen for subsequent analysis. Phosphorylation status was checked with both intact and peptide fragmentation MS.

**Intact MS**

PTEN samples (60 μl at 0.4 μM) were injected on to an ultra-performance liquid chromatography (UPLC) system and passed over a C₁₈ Van-Guard pre-column (Waters) in 0.1% formic acid for 3 min to remove excess salt before elution using a 3λp–100% B gradient of Buffer A (0.1% formic acid) and Buffer B (100% acetonitrile) over 13 min. This eluent was injected on to a Xevo QTOF (Waters) acquiring a mass range from 350 to 1500 m/z, with an ESI source operated at a temperature of 225 °C and a spray voltage of 2.5 kV. Data were deconvoluted using MaxEnt Software (MicroMass).

**Soluble lipid phosphatase activity assay**

Recombinant PTEN was incubated with 50 μM dioctanoyl-PIP₂ (diC₂-PIP₂) (Echelon) in 50 mM Tris/HCl, pH 8.0, and 2 mM DTT for 10 min at 37 °C in a final volume of 25 μl. The reaction was stopped by the addition of 100 μl of Malachite Green Reagent (Millipore). The reaction was then incubated at room temperature, with agitation at 400 rev./min for 15 min to allow colour to develop. The absorbance at 620 nm was measured and the amount of phosphate released was determined by using a K₅H₆PO₄ (Millipore) standard curve. The specific activity of the PTEN was obtained by dividing the amount of phosphate released by the concentration of the enzyme.

**Lipid vesicle preparation**

Lipid components were mixed together while in organic solvent (chloroform or chloroform/methanol). The solvent was then...
evaporated under a stream of nitrogen. Vesicles were composed of 5% brain PIP_2, 20% brain phosphatidylserine (PS), 45% brain phosphatidylethanolamine (PE), 15% brain phosphatidylcholine (PC), 10% cholesterol and 5% sphingomyelin (w/v; Avanti Polar Lipids). The lipid film was allowed to dry for 1 h under vacuum and then resuspended in 20 mM HEPES, pH 7.4, 100 mM KCl and 1 mM EGTA. The lipids were bath sonicated for 10 min, then subjected to ten freeze–thaw cycles between liquid nitrogen and a 43°C water bath. The liposomes were finally extruded 11 times through a 100-nm filter. Vesicles were frozen in liquid nitrogen and stored at −80°C, before being defrosted at room temperature before use.

**Protein/lipid FRET assays**

Vesicles were prepared exactly as for the lipid kinase assays, except that 10% dansyl-PS was substituted for 10% of the brain phosphatidylserine. Vesicles were diluted with 20 mM HEPES, pH 7.4, 100 mM KCl and 1 mM EGTA to give a final concentration of 50 μg/ml. PTEN was thawed and centrifuged at 13000 rev./min at 4°C to remove any precipitate. PTEN was diluted in 20 mM HEPES, 200 mM NaCl and 2 mM TCEP to a final concentration of 4 μM. Protein was serially diluted in 2.3 ratio in the above buffer. The lipid solution (5 μl) was then mixed with 5 μl of the protein solution at various concentrations. The protein/lipid mixture was allowed to equilibrate for 10 min, while being agitated at 450 rev./min at room temperature. Reactions were measured with a PHERAStar HTS microplate reader (BMG Labtech) using a 280 nm excitation filter with 350- and 520-nm emission filters to measure tryptophan and dansyl-PS FRET emissions respectively. The FRET signal is I/I_o, where I is the intensity at 520 nm and where I_o is the intensity of the solution containing only lipid (i.e. without protein). Binding curves were fitted with a one site-specific-binding model (GraphPad Prism version 5.00).

**PIP_2-containing lipid vesicle phosphatase assay**

PIP_2-containing vesicles with the composition described above were resuspended at a concentration of 1 mg/ml and phosphorylated by incubation for 2 h with P13K, purified as detailed previously [42]. The reaction was carried out in 20 mM Tris/HCl, pH 7.4, 50 mM NaCl, 50 mM KCl, 1 mM EGTA, 3 mM MgCl_2 and 0.1 mM ATP, before being quenched with 10 mM EDTA. The resulting PIP_2-containing vesicles were then incubated with PTEN for 3 min at 25°C and quenched with 22 mM H_2O_2 (Sigma). The quenched reaction was agitated for 10 min at 500 rev./min at 25°C, before being spotted on to a nitrocellulose membrane. After the droplet had dried, the membrane was washed four times with TBS/Tween 20 solution (TBST) (50 mM Tris/HCl, pH 7.4, 150 mM NaCl and 0.1% Tween 20) over the course of 1 h, before being blocked with TBST + 2 mg/ml BSA for 1 h at 4°C. The membrane was then incubated with 0.5 μg/ml Alexa Fluor 488-labelled GRP1-PH domain in TBST + 2 mg/ml BSA overnight at 4°C as described previously [58]. The membrane was washed six times in TBST and dried at 25°C before being imaged using a Typhoon imager (GE Healthcare). The intensities were quantified using ImageQuant (GE Healthcare).

**Surface dilution assay**

The assay was conducted as described above, but with two lipid stock solutions, a 1 mg/ml 5% PIP_2 plasma-membrane-mimicking composition and a 2 mg/ml 2.5% PIP_2 plasma-membrane-mimicking composition. PIP_2 was generated as above. Reactions were conducted with 10 nM PTEN over a 40-min period. PIP_3 levels were determined using a GRP1-PH domain as described above.

**Hydrogen/deuterium exchange (HDX) MS**

HDX reactions were conducted with 10 μl of protein in dilution buffer (20 mM HEPES, pH 7.5, 150 mM NaCl and 2 mM TCEP) and initiated by the addition of 40 μl of D_2O buffer solution (10 mM HEPES, pH 7.5, 50 mM NaCl, 2 mM TCEP and 92% D_2O), to give a final concentration of 74% D_2O. Final protein concentrations were 1 μM. For HDX–MS membrane interaction experiments, lipid vesicles were mixed with the D_2O buffer. Four time points of exchange (3, 30, 300 and 3000 s) were terminated by the addition of a quench buffer (final concentration 0.6 M guanidinium chloride and 0.8% formic acid). Samples were rapidly frozen in liquid nitrogen and stored at −80°C until MS analysis.

**Measurement of deuterium incorporation**

Protein samples were rapidly thawed and injected on to a UPLC system immersed in ice as previously described [42]. The protein was run over an immobilized pepsin column (Applied Biosystems; Poroszyme, catalogue number 2-3131-00) at 130 μl/min for 3 min and the peptides were collected on to a Van Guard pre-column trap (Waters). The trap was subsequently eluted in line with an Acquity 1.7 μm particle, 100 mm × 1 mm C_18 UPLC column (Waters), using a gradient of 5–36% B (buffer A 0.1% formic acid, buffer B 100% acetonitrile) over 20 min. Eluent from the column was injected on to a Xevo QTOF (Waters) acquiring over a mass range from 350 to 1500 m/z for 30 min, using an ESI source operated at a temperature of 225°C and a spray voltage of 2.5 kV.

**Protein digestion and peptide identification**

Peptide identification was done by running MS/MS experiments using a 5–36% B gradient over 120 min with a Xevo QTOF (Waters). This was supplemented with a 20-min gradient separation to identify and correct the retention time for all samples. The MS tolerance was set to 3 ppm with an MS/MS tolerance at 0.1 Da. The resulting MS/MS datasets were analysed with the Mascot search within Proteome Discoverer (Thermo Scientific). All peptides with a Mascot score > 20 were analysed using HD-Examiner Software (Sierra Analytics). The full list of peptides was then manually validated by searching a non-deuterated protein sample’s MS scan to test for the correct m/z state and check for the presence of overlapping peptides. Ambiguously identified peptides were excluded from all subsequent analysis. The first round of analysis and identification were performed automatically by the HD-Examiner software, but all peptides (deuterated and non-deuterated) were manually verified at every state and time point for the correct charge state, m/z range, presence of overlapping peptides and any deviation from the expected retention time. All HDX experiments were carried out in triplicate.

**Mass analysis of peptide centroids**

All results are presented as relative levels of deuterium incorporation and no correction for back exchange is applied.
because no fully deuterated protein sample could be obtained. All percentage differences quoted in the Results section are the maximal changes in HDX seen at any time point of the analysis, unless explicitly stated otherwise.

**Size-exclusion chromatography–multi-angle light scattering (SEC–MALS)**

PTEN solution (100 μl of 20–50 μM protein) was injected on to a Superdex 200 GL 10/300 gel-filtration column (GE Healthcare) equilibrated in 20 mM HEPES, pH 7.4, 200 mM NaCl and 2 mM TCEP. Samples were run at 0.5 ml/min and eluted into a Dawn Heloex (Wyatt) (for light-scattering measurements) and Optilab rEX (Wyatt; for refractive index measurements). The system was calibrated using a BSA standard prior to samples being run.

**RESULTS**

**Phosphorylation of the C-terminal tail differentially inhibits PTEN towards distinct substrates**

PTEN was expressed in Spodoptera frugiperda Sf9 cells and purified to determine how the protein phosphatase and lipid phosphatase activities of PTEN were regulated by post-translational modifications of the C-terminal tail. To produce homogeneously and selectively phosphorylated PTEN, several constructs were produced. First, a construct containing six mutations, T366A, S370A, S380A, T382A, T383A and S385A (PTEN-6A), was prepared to prevent background heterogeneous phosphorylation of the tail, as previously described [22,37,39] (Figure 1A). We also generated two constructs that preserved only a portion of the tail phosphorylation sites: PTEN-2A with mutations T366A and S370A (mimicking auto-dephosphorylated PTEN [38]) and PTEN-4A with mutations S380A, T382A, T383A and S385A. We also expressed both full-length PTEN as well as a truncated PTEN lacking the tail (PTEN–ΔTail). We used λ- phosphatase to dephosphorylate PTEN or CK2 and GSK3/β kinases to phosphorylate it. PTEN constructs were further purified after λ- phosphatase or kinase treatment before biochemical and structural analysis. Proteins produced in this manner were homogeneously dephosphorylated or phosphorylated, as verified by LC–MS of intact proteins (Supplementary Figure S1). Using SEC–MALS, both the phosphorylated and the non-phosphorylated PTEN were shown to be monodisperse, with masses in agreement with what would be expected for monomers (Supplementary Figure S2).

To compare the activities and specificities of differentially phosphorylated PTEN constructs, phosphatase assays were conducted against four substrates: a phosphotyrosine-containing acidic polypeptide, the soluble lipid diC8-PIP3, inositol tetrakisphosphate (IP4) and lipid phosphatase substrates was restored (Figures 1B–1D), but PTEN remained inhibited against liposome-incorporated PIP3 substrate (Figure 1E; Supplementary Figure S3). This suggests that pThr366 and pSer370 may have a role in occluding the active site of PTEN, whereas pSer380, pThr382, pThr383 and pSer385 have a role in occluding the membrane-binding surface. Phos-PTEN-4A, phosphorylated on only two sites, pThr366 and pSer370, showed similar activities with all substrates to that of dephosphorylated PTEN-4A, with only a slight reduction in activity against liposome-incorporated PIP3, (Figures 1B–1E).

**Phosphorylation of the C-terminal tail inhibits membrane binding**

To determine whether the substrate specificities of differentially phosphorylated PTENs are due to altered affinities for liposomes, we conducted protein/lipid FRET binding experiments [40] using liposomes with a composition mimicking plasma membranes [41] (Figure 1F). Dephosphorylated PTEN (subsequently referred to as wtPTEN) binds to these liposomes, but Phos-wtPTEN had no measurable membrane binding. PTEN–ΔTail maintained membrane affinity, and all dephosphorylated constructs showed an affinity similar to that of wtPTEN, suggesting that neither the tail nor the six threonine/serine residues have crucial roles in contacting the membrane. Phos-PTEN-4A (phosphorylated on Thr366 and Ser370) showed a slight decrease in membrane binding (Figure 1F) which may be due to electrostatic repulsion. Consequently, a slight reduction in liposome-incorporated PIP3 phosphatase activity was observed (Figure 1E). Phos-PTEN-2A (phosphorylated on Ser380, Thr382, Thr383 and Ser385) showed dramatically decreased membrane binding, exhibiting an affinity indiscernible from phosphorylated wtPTEN, accompanied with a great reduction in activity against liposome-incorporated PIP3.

**HDX–MS shows that the phosphorylated C-terminal tail forms intramolecular interactions with both the C2 and the phosphatase domains**

To understand the molecular details behind phosphorylation-mediated auto-inhibition, we carried out HDX–MS and compared the HDX levels of the dephosphorylated enzyme with those of the fully phosphorylated enzyme, as described previously [42]. Further details of the experiments can be found in the Supplementary Online Data (see Supplementary Figure S4 and the deuteration incorporation for all experiments are shown in Supplementary Figures S5–S8). The changes in exchange described in the text are the largest seen at any time point. Using HDX–MS it was possible to determine the location of conformational changes that occur upon tail phosphorylation (Figure 2A). Phosphorylation produced decreases in HDX (defined as changes greater than both 0.7 Da and 6% at any time point) in regions of phosphatase domain, as well as part of the C2 domain. In the phosphatase domain, five peptides (residues 4–21, 35–45, 82–99, 111–134 and 155–177) showed decreases in HDX in regions of phosphatase domain, as well as part of the C2 domain. In the phosphatase domain, five peptides (residues 4–21, 35–45, 82–99, 111–134 and 155–177) showed decreases in exchange. These peptides span the N-terminal PBM, a positively charged loop termed here the ‘arginine loop’ (the loop spanning pβ2–pε1, residues 35–49 [13]), the WPD loop, the P loop and the T1 loop respectively. In the C2 domain, the Cε2 loop (residues 321–342) also shows a reduced HDX (peptide 319–341) upon phosphorylation. Overall, these decreases in HDX suggest that the fully phosphorylated C-terminal tail makes contact at the C2/phosphatase interface and more extensive contacts within the phosphatase domain, interacting extensively with all three loops of the active site and the PBM.
Dephosphorylation of Thr366 and Ser370 exposes parts of the active site

We used HDX–MS to determine the molecular mechanism that allows selective activation of Phos-PTEN-2A (T366A, S370A) against soluble substrates but not lipid substrates residing within membranes by comparing the HDX rates of fully phosphorylated PTEN (Phos-wtPTEN) with Phos-PTEN-2A. We found that two peptides, residues 4–21 spanning the PBM, and residues 82–99, spanning the WPD loop, had increased rates of HDX (Figure 2B). The largest increase in exchange (>15%) occurs in the WPD loop peptide, raising the HDX rate for this peptide to that seen in wtPTEN.

Membrane interaction involves exposed loops on both the phosphatase and the C2 domain

To understand the molecular details of how active PTEN interacts with membranes, we carried out HDX–MS experiments on the active wtPTEN in the presence and absence of lipid vesicles. Upon interaction with lipid vesicles, large reductions in the HDX rate were observed around the N-terminus of PTEN (Figure 2C), with numerous peptides showing greater than 25% reduction in HDX. A peptide encompassing residues 2–7 within the PBM had a 42% reduction. Peptide 35–45, which spans the ‘arginine loop’, saw a 39% reduction in HDX, along with peptide 72–81 showing a 19% reduction. Peptide 82–99, in the WPD loop, had a 13% reduction in HDX upon membrane binding. In addition, peptide 155–177, the TI loop, showed a 7% reduction in HDX.

The C2 domain also shows reductions in HDX for two C2 loops (CBRs), peptide 201–215 in the CBR1 (8% reduction) and peptide 258–273 in the CBR3 (11% reduction). Finally, peptide 316–330, incorporating the Co2 loop, shows an 11% reduction in HDX when PTEN is bound to membrane. Our results using HDX–MS on non-labelled intact PTEN indicate that both the phosphatase and the C2 domain play roles in binding and orienting PTEN on membranes.

HDX–MS of PTEN-L indicates an ordered segment in the intrinsically disordered N-terminal extension

PTEN-L-6A was purified from Sf9 cells, as attempts to dephosphorylate PTEN-L were unsuccessful. HDX–MS analysis of PTEN-L-6A (Supplementary Figure S9A) indicated that the first 145 residues of PTEN-L were intrinsically disordered as previously predicted [31], exhibiting very high levels of HDX–MS (>50% exchange at 3 s of exchange at 0°C). However, one region of the N-terminal extension of PTEN-L, residues 145–176, was below this threshold, suggesting that this region may be folded and forming an α-helix, as suggested by secondary structure prediction programs. Interestingly, this region has sequence similarity to the ‘S4’ transmembrane helix of Ci-VSP (Ciona intestinalis Voltage Sensitive Phosphatase), a paralogue of PTEN, further indicating that this region might form an α-helix (Figure 3A). We refer to this region as the ‘membrane-binding helix (MBH)’ for reasons detailed below.

The PBM and the ‘arginine loop’ of PTEN-L-6A showed decreases in HDX compared with wtPTEN (Supplementary Figure S9A). The PBM (residues 177–194 in PTEN-L, 4–21 in PTEN) had a 15% decrease, whereas the arginine loop, (residues 208–217 in PTEN-L, 35–44 in PTEN) had a 7% decrease, suggesting a change in conformation in these regions. Additionally, the C2/PTP interdomain region of PTEN-L exhibited increases in HDX when compared with wtPTEN, suggesting a breakdown of the contacts in this region. A variant of PTEN-L with a deleted MBH (residues Ala141-L to Met174-L...
The tails of PTEN and PTEN-L affect substrate specificity and membrane binding

Figure 3 Characterization of PTEN-L

(A) Schematic representation of PTEN-L. The 173-residue N-terminal extension contains the six alanine residue signal sequence. The predicted $\alpha$-helix between residues 151-L and 174-L (highlighted) has sequence similarity with the PTEN-family members Ci-VSP and TPTE (transmembrane phosphatase with tensin homology), identified here as the MBH. (B) Phosphatase activity against lipid vesicles containing a long-chain PIP$_3$ substrate. PIP$_3$-containing vesicles (5%) were incubated with PI3K to generate PIP$_3$, which was subsequently dephosphorylated with various concentrations of PTEN. Experiments shown in (B)–(E) were conducted at least three times in triplicate. (C) Protein/lipid FRET of PTEN-L-6A and wtPTEN. (D) Interfacial kinetics experiment, showing different behaviours of wtPTEN, PTEN-L and PTEN-L-$\Delta$MBH. The reactions were conducted with 10 nM protein for 30 min. At this point the reactions were supplemented with more PIP$_3$-containing liposomes to a 4-fold molar excess of liposomes to protein. (E) Surface dilution of PIP$_3$ also highlights the different interfacial kinetics of wtPTEN, PTEN-L and PTEN-L-$\Delta$MBH. The bulk concentration of PIP$_3$ was kept constant by varying the concentration of the carrier lipids. The surface concentration of PIP$_3$ was altered between 5% or 2.5%. Although wtPTEN and PTEN-L-$\Delta$MBH show only minor perturbations in phosphatase rate between the two surface concentrations of PIP$_3$, PTEN-L sees a drastic reduction in phosphatase activity upon dilution of PIP$_3$’s surface concentration.

PTEN-L-6A was as active as dephosphorylated wtPTEN against soluble diC$_8$-PIP$_3$ (Supplementary Figure S9B), but not as active against membrane incorporated PIP$_3$, to a level similar to wtPTEN. Deleting the MBH (PTEN-L-$\Delta$MBH) increased activity against membrane incorporated PIP$_3$, to a level similar to wtPTEN. Despite its apparent lower activity in hydrolysing membrane PIP$_3$, protein/lipid FRET measurements indicated that PTEN-L-6A bound to liposomes more tightly than wtPTEN (Figure 3C). We postulated that the reduced activity might be because PTEN-L is less readily dissociates from vesicles and cannot move to new PIP$_3$-containing vesicles, i.e., PTEN-L works in a scooting rather than hopping mode of catalysis [43]. To determine the interfacial kinetic mode of PTEN-L, we conducted two modified PIP$_3$ liposome assays. In the first assay, a second aliquot of substrate was added to produce a 4-fold excess of liposomes relative to PTEN after the initial reaction had completed (Figure 3D). Surprisingly, PTEN-L and wtPTEN exhibited different interfacial kinetic behaviour. The addition of fresh substrate caused a burst of activity with wtPTEN, whereas PTEN-L failed to dephosphorylate the additional liposomes, indicating that wtPTEN works in a ‘hopping’ mode of interfacial kinetics, in agreement with previous studies [44], whereas PTEN-L-6A works in a ‘scooting’ mode. In a second assay, we measured the phosphatase rate of wtPTEN and PTEN-L with the PIP$_3$ surface concentration of the membrane at 5% and 2.5%, while maintaining a constant bulk PIP$_3$ concentration. The reduction in surface concentration greatly affected the phosphatase rate of PTEN-L (Figure 3E), whereas wtPTEN was affected to a much smaller extent. The results indicate that PTEN-L is carrying out a scooting catalysis, whereas wtPTEN shows predominately hopping behaviour. In both experiments, PTEN-L-$\Delta$MBH exhibited the same behaviour as wtPTEN, suggesting that the MBH is responsible for this change in interfacial kinetics.

PTEN-L has a vastly altered membrane-binding footprint

To determine how the N-terminal extension of PTEN-L caused a change in interfacial kinetics, we compared HDX of PTEN-L-6A in the presence and absence of lipid vesicles (Figure 4; Supplementary Figure S8). The membrane-binding footprint was

deprecated), PTEN-L-$\Delta$MBH, had a HDX profile almost identical with that of wtPTEN (Supplementary Figures S9C and S9D).

The MBH of PTEN-L causes PTEN-L to become a membrane scooter rather than a hopper

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very different from that of wtPTEN, with the MBH having the largest changes in HDX (MBH peptides 150–162, 166–176 and 167–176 decreasing 12%, 20% and 22% respectively, PTEN-L numbering). Additionally, PTEN-L showed decreases in HDx in areas associated with membrane binding for wtPTEN, although most changes were of a smaller magnitude. For example, a PBM-containing peptide (residues 177–194 in PTEN-L, 4–21 in PTEN) decreased 12% in PTEN-L as compared with 28% for the same peptide in PTEN, a peptide covering the arginine loop (208–215 in PTEN-L, 35–42 in PTEN) decreased 11% when PTEN-L binds to liposomes, compared with 32% in PTEN and a peptide spanning the Cα2 loop (6% decrease for 492–514 in PTEN-L, 11% decrease for 319–341 in PTEN). Additionally, the CBR3 loop of PTEN-L no longer showed a decrease in exchange when bound to membranes.

**DISCUSSION**

PTEN plays important tumour-suppressing roles as both a lipid and a protein phosphatase [8], and the membrane permeability of PTEN-L presents an opportunity to commandeer these activities as a therapeutic agent. We have shown that phosphorylation of the C-terminal tail switches PTEN’s substrate specificity and that a helix within the N-terminal extension of PTEN-L markedly alters the enzyme kinetics on membranes. Development of PTEN-L as a therapeutic agent will require consideration of these two disordered segments and the effect they have on ability of PTEN to function as a tumour suppressor.

We find that fully phosphorylated PTEN is completely inactive and incapable of binding membranes. However, selective dephosphorylation of only two residues, Thr366 and Ser700, causes the active site of PTEN to become partially exposed, allowing cytosolic PTEN to dephosphorylate its growing list of soluble substrates (both phosphoserine/threonine and phosphotyrosine residues) [4,45,46], including CREB [47], IRS1 [8], She [48], components of the MAPK pathway [49] and Dishevelled [50]. Furthermore, this selective activity may have an impact on an emerging role of PTEN in dephosphorylating soluble protein-bound PIP3, such as PIP3 bound to SF-1 within the nucleus [51]. Our data indicate that phosphorylation of Thr366 and Ser700 alone is not sufficient to occlude the active site, as Phos-PTEN-4A was still capable of dephosphorylating soluble substrates. HDX–MS suggests that dephosphorylation of Thr366 and Ser700 results in localized conformational changes; however, these localized changes are apparently not great enough to have been detected by using access to alkaline phosphatase as a measure of conformational change [35].

By using HDX–MS to map both the membrane-binding interface and the interface between the phosphorylated C-terminal tail with the rest of PTEN, we determined the structural basis for the substrate switch of PTEN. The phosphorylated tail interacts predominantly with the N-terminal PBM, the active site of the phosphatase domain and the Cα2 loop of the C2 domain. Previous studies have determined the CBR3 loop of the C2 domain as the binding partner for the phosphorylated tail, through either electrostatic [35,52] or non-electrostatic cryptic binding motifs [14,37]. However, these studies are based on the mutagenesis or deletion of the CBR3 loop. Our HDX–MS experiments, carried out on the native protein, are not consistent with the phosphorylated tail interacting with CBR3. This is in agreement with a previous study that highlighted the need for an intact active site and PBM to allow for the intramolecular binding event [22]. Here, HDX shows that the mechanism for inhibition involves occlusion of the active site by the phosphorylated tail, preventing substrate catalysis, rather than the prevention of PTEN membrane binding through occlusion of the CBR3 loop. The proximity of pThr366 and pSer700 to the active site may explain their propensity for auto-dephosphorylation [38].

Our results also provide a comprehensive picture of how PTEN binds to membranes. For membrane binding and PTEN activity assays, we used a complex mixture of lipids that mimics the composition of the plasma membrane [41], as studies have shown that altering the relative proportions of various lipid moieties can effect both PTEN’s ability to interact with the membrane and its subsequent lipid phosphatase activity [35,44]. Attention has focused on the CBR3 loop as the dictating element of membrane interaction [9,10,15,35,37,52], but we observe a more complex picture, with very large reductions in HDX throughout the phosphatase domain and similar reductions in HDX in two of the three CBR loops, as well as in the Cα2 loop. Mutations in the Cα2 loop decrease activity in vitro and membrane localization in cells [13,53]. Within the phosphatase domain, our data highlight the membrane-binding contribution of the ‘arginine loop’, which forms a positively charged patch, composed of Arg41, Arg47, Arg74 and Lys80. Finally, although both the WPD and the TI loops are protected by membrane binding, the P loop, which contains the catalytically crucial cysteine, is not. These disparate regions of PTEN, i.e. the arginine loop, the TI loop, the CBR3 loop, the Cα2 loop and the PBM, have all been implicated in membrane interaction as modelled by molecular dynamic simulations [54]. Together, our results indicate that a large surface of PTEN interacts with the membrane.

From our results, we propose that PTEN can exist in three distinct activation states (Figure 2D) depending on the phosphorylation status of the tail: a fully phosphorylated state that is inactive against both lipid and protein substrates, a partially dephosphorylated state (lacking phosphates on Ser366 and Thr370), active only against soluble substrates including phosphoproteins, and a fully dephosphorylated state that is active against both soluble and liposome-incorporated substrates. The activation states are mediated by intramolecular inhibitory interactions between the tail and the C2 and phosphatase domains that occlude both the membrane-binding region as well as the active site.

Whether these three activation states are applicable to PTEN-L is not yet clear. PTEN-L is an N-terminally extended translational variant of PTEN, which was shown to be capable of exiting the cell and entering a neighbouring cell, down-regulating their p-Akt levels [29] and possibly localizing to mitochondria to regulate cytochrome c oxidase activity [30]. We have found that the N-terminal elongation of PTEN-L is not entirely devoid of secondary structure and that an ordered region, most likely an α-helix,
PTEN-L has a disordered C-terminal tail that can interact with the membrane and its N-terminal extension. The MBH of PTEN-L may interact with the membrane surface or may be a transmembrane helix. This interaction changes the interface catalytic mechanism from a ‘hopping’ mode, characteristic of PTEN to a ‘scooting’ mode, characteristic of PTEN-L.

Figure 5  Possible membrane-binding mode of PTEN-L

The MBH of PTEN-L may interact with the membrane surface or may be a transmembrane helix. This interaction changes the interface catalytic mechanism from a ‘hopping’ mode, characteristic of PTEN to a ‘scooting’ mode, characteristic of PTEN-L.

complexity of regulation of PTEN and PTEN-L. Further work on the mechanistic aspects that underpin PTEN and PTEN-L function is essential to enable their use as a therapeutic agent.

AUTHOR CONTRIBUTION

Glenn Masson designed and performed experiments and wrote the manuscript. Olga Perisic also performed experiments, constructed plasmids and helped to write the manuscript. John Burke aided in experiment design. Roger Williams designed experiments and helped to write the manuscript.

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REFERENCES

1 Liaw, D., Marsh, D.J., Li, J., Dahia, P.L.M., Wang, S.I., Zheng, Z., Bose, S., Call, K.M., Tsou, H.C., Peacocke, M. et al. (1997) Germ-line mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. Nat. Genet. 16, 64–67 CrossRef PubMed

2 Steck, P.A., Pershouse, M.A., Jasser, S.A., Yang, W.K.A., Lin, H., Ligon, A.H., Angard, L.A., Baumgard, M.L., Hattie, T., Davis, T. et al. (1997) Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. Nat. Genet. 15, 356–362 CrossRef PubMed

3 Yuan, T.L. and Cantley, L.C. (2008) PK3 kinase pathways alter cancer: variations on a theme. Oncogene 27, 5497–5510 CrossRef PubMed

4 Myers, M.P., Stolarov, J.P., Eng, C., Li, J., Wang, S.I., Wigler, M.H., Parsons, R. and Tonks, N.K. (1997) P-TEN, the tumor suppressor from human chromosome 10q23, is a dual-specificity phosphatase. Proc. Natl. Acad. Sci. U.S.A. 94, 9052–9057 CrossRef PubMed

5 Myers, M.P., Pass, I., Battly, I.H., Van der Kaay, J., Stolarov, J.P., Hemnings, B.A., Wigler, M.H., Downes, C.P. and Tonks, N.K. (1998) The lipid phosphatase activity of PTEN is critical for its tumor suppressor function. Proc. Natl. Acad. Sci. U.S.A. 95, 13513–13518 CrossRef PubMed

6 Maehama, T. and Dixon, J.E. (1998) The tumor suppressor, Pten/MMAC1, dephosphorylates the Lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. J. Biol. Chem. 273, 13375–13378 CrossRef PubMed

7 Cantley, L.C. and Neel, B.G. (1999) New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. Proc. Natl. Acad. Sci. U.S.A. 96, 4240–4245 CrossRef PubMed

8 Shi, Y., Wang, J., Chandraratna, S., Cross, J., Thompson, C., Rosen, N. and Jiang, X. (2014) PTEN is a protein tyrosine phosphatase for IRS1. Nat. Struct. Mol. Biol. 21, 522–527 CrossRef PubMed

9 Suzuki, A., Te Pompa, D.L., Stambolic, V., Elia, A.J., Sasaki, T., Barrantes, I.D.B., Ho, A., Wakeham, A., Itie, A., Khou, W. et al. (1998) High cancer susceptibility and embryonic lethality associated with mutation of the PTEN tumor suppressor gene in mice. Curr. Biol. 8, 1169–1178 CrossRef PubMed

10 Salmina, L., Caracciolo, A. and Pandolfi, P.P. (2008) Tenets of PTEN tumor suppression. Cell 133, 403–414 CrossRef PubMed

11 Berger, A.H., Kneudson, A.G. and Pandolfi, P.P. (2011) A continuum model for tumor suppression. Nature 476, 163–169 CrossRef PubMed

12 Papa, A., Wan, L., Bonora, M., Salmina, L., Song, M.S., Hobbs, R.M., Lunardi, A., Webster, K., Ng, C., Newton, R.H. et al. (2014) Cancer-associated PTEN mutants act in a dominant-negative manner to suppress PTEN protein function. Cell 157, 595–610 CrossRef PubMed

13 Lee, J.-O., Yang, H., Georgescu, M.-M., Di Cristofano, A., Maehama, T., Shi, Y., Dixon, J.E., Pandolfo, P. and Pavelich, N.P. (1999) Crystal structure of the PTEN tumor suppressor: Cell 99, 323–334 CrossRef PubMed

14 Walker, S.M., Leslie, N.R., Pereira, N.M., BATTY, I.H. and Downes, C.P. (2004) The tumour-suppressor function of PTEN requires an N-terminal lipid-binding motif. Biochem. J. 379, 301–307 CrossRef PubMed
Bolduc, D., Rahdar, M., Tu-Sekine, B., Sivakumaren, S.C., Raben, D., Amzel, L.M., Cordier, F., Chaffotte, A., Terrien, E., Préhaud, C., Theillet, F.-X., Delepierre, M., Lafon, M., Johnston, S.B. and Raines, R.T. (2015) Catalysis by the tumor-suppressor enzymes PTEN and PTEN-L. PLoS One 4, e5577 CrossRef PubMed

Vazquez, F., Grossman, S.R., Takahashi, Y., Rokas, M.V., Nakamura, N. and Sellers, W.R. (2001) Phosphorylation of the PTEN tail acts as an inhibitory switch by preventing its recruitment into a protein complex. J. Biol. Chem. 276, 48627–48630 CrossRef PubMed

Rahdar, M., Inoue, T., Meyer, T., Zhang, J., Vazquez, F. and Devreotes, P.N. (2009) A phosphorylation-dependent intramolecular interaction regulates the membrane association and activity of the tumor suppressor PTEN. Proc. Natl. Acad. Sci. U.S.A. 106, 480–485 CrossRef PubMed

Okumura, K. (2006) PCAF modulates PTEN activity. J. Biol. Chem. 281, 26562–26568 CrossRef PubMed

Lee, S.R., Yang, K.S., Kwon, J., Lee, C., Jeong, W. and Rheg, S.G. (2002) Reversible phosphorylation of the PTEN tail acts as an inhibitory switch by preventing its association with the plasma membrane. Nat. Commun. 3, 911 CrossRef PubMed

Putz, U., Höfft, J., Doan, A., Goh, C.P., Low, L.H., Silke, J. and Tan, S.S. (2012) The tumor suppressor PTEN is exported in exosomes and has phosphatase activity in recipient cells. Sci. Signal. 5, ra70 CrossRef PubMed

Pullido, L., Baker, S.J., Barata, J.T., Carracedo, A., Cid, V.J., Chinh-Sang, D., Dave, V., Herold, J., Devreotes, P. and Eckhardt, B.J. et al. (2014) A unified nomenclature and amino acid numbering for human PTEN. Sci. Signal. 7, pe15 CrossRef PubMed

Hopkins, B.D., Fine, B., Steinbach, N., Denly, M., Rapp, Z., Shaw, J., Papas, K., Yu, J.S., Hodakoski, C., Mense, S. et al. (2013) A secreted PTEN phosphatase that enters cells to alter signaling and survival. Science 341, 399–402 CrossRef PubMed

Li, Q., Wanderling, S., Paduch, M., Medovoy, D., Singharoy, A., McGreevy, R., Lumb, C.N. and Sansom, M.S.P. (2013) Defining the membrane-associated state of the tumor suppressor phosphatase, PTEN. Proc. Natl. Acad. Sci. U.S.A. 110, 15259–15264 CrossRef PubMed

Jain, M.K. and Berg, O.G. (1989) The kinetics of interfacial catalysis by phosphatase A2 and regulation of interfacial catalysis: hopping versus scouting. Biochim. Biophys. Acta 1002, 127–156 CrossRef PubMed

McComas, G., Pass, I., Walker, S.M. and Downes, C.P. (2003) Interfacial kinetic analysis of the tumour suppressor phosphatase, PTEN: evidence for activation by anionic phospholipids. Biochem. J. 371, 947–955 CrossRef PubMed

Vanhaven, V.B. (1999) Autophosphorylation of pT105 and shifting of the phosphatase activity of pTEN. A new paradigm for the regulation of lipid kinases in vitro and in vivo. EMBO J. 18, 1292–1302 CrossRef PubMed

Dey, N., Crosswell, H.E., De, P., Parsons, R., Peng, D., Su, J.D. and Burden, D.L. (2008) The protein phosphatase activity of PTEN regulates Src family kinases and controls glioma migration. Cancer Res. 68, 1862–1871 CrossRef PubMed

Gu, T., Zhang, Z., Wang, J., Guo, J., Shen, W.H. and Yin, Y. (2011) CREB is a novel nuclear target of PTEN phosphatase. Cancer Res. 71, 2821–2825 CrossRef PubMed

Schneider, E., Kippier, R., Prawitt, D., Steinwender, C., Roos, F.C., Thirstrup, J.W., Lausch, E. and Brenner, W. (2011) Migration of renal tumor cells depends on dephosphorylation of Shc by PTEN. Int. J. Oncol. 38, 823–831 PubMed

Nakdimon, I., Walser, M., Fröhli, E. and Hajnal, A. (2012) PTEN Negatively Regulates MAPK Signaling during C. elegans vulval development. PLoS Genet. 8, e1002881 CrossRef PubMed

Shnaitat, I., Bashkerov, M., Masson, G.R., Ogumiji, A.A., Moseisian, S., Cabeza, E.A., Hirsch, C.L., Treka, D., Gish, G. Jiao, J. et al. (2015) PTEN regulates cilia through Dishevelled. Nat. Commun. 6, 6338 CrossRef PubMed

Blind, R.D., Suzawa, M. and Inghram, H.A. (2012) Direct modification and activation of a nuclear receptor-RIP2 complex by the inositol lipid kinase IPMK. Sci. Signal. 5, ra44 CrossRef PubMed

Nguyen, H.H., Aikari, Y., Senoh, S., Sesaki, H., Devreotes, P.N. and Iijima, M. (2013) Mechanism of human PTEN localization revealed by heterologous expression in Dicyostelium. Oncogene 32, 5898–5906 CrossRef PubMed

Yasui, M., Matsuka, S. and Ueda, M. (2014) PTEN hopping on the cell membrane is regulated via a positively-charged C2 domain. PLoS Comput. Biol. 10, e1003817 CrossRef PubMed

Lumb, D.N. and Sansom, M.S.P. (2013) Defining the membrane-associated state of the PTEN tumor suppressor protein. Biophys. J. 104, 613–621 CrossRef PubMed

Li, Q., Wheeler, S., Paduch, M., Medovoy, D., Singharyo, A., McGreavy, R., Villalta-Galaia, C.A., Hulse, R.E., Roux, B. and Schullten, K. et al. (2014) Structural mechanism of voltage-dependent gating in an isolated voltage-sensing domain. Nat. Struct. Mol. Biol. 21, 244–252 CrossRef PubMed

Hobiger, K., Utesch, T., Mroginski, M.A., Seeboth, G. and Friedrich, T. (2013) The linker pivot in Ci-VSP: the key to unlock catalysis. PLoS One 8, e60722 CrossRef PubMed

Wei, Y., Stoe, B., Redfield, A.G., Weerapanana, E. and Roberts, M.F. (2015) Phospholipid-binding sites of phosphatase and tensin homolog (PTEN). J. Biol. Chem. 290, 1592–1606 CrossRef PubMed

Dowler, S., Kular, G. and Alessi, D.R. (2002) Protein lipid overlay assay. Sci. Signal. 5, p6 CrossRef PubMed