Assaying PTEN catalysis in vitro

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Abstract

PTEN is a major tumour suppressor protein and a regulator of numerous diverse biological processes. It has an evolutionarily conserved role as a phosphoinositide lipid phosphatase, regulating the PI3K signalling pathway, but also has catalytic phosphatase activity against protein substrates, although the significance of this latter activity is less well understood. Unlike many tumour suppressors, even modest changes in PTEN activity can have strong effects on phenotypes, including tumour formation. Due to this recognised functional significance, several experimental platforms have been developed to assay the catalytic activity of PTEN against different substrates and are being applied to understand this cellular substrate diversity and the regulation of PTEN. Here we present and discuss methods to assay the phosphatase activity of PTEN in vitro.
Highlights

- The lipid phosphatase activity of PTEN is critical for its tumour suppressor function
- Colorimetric method for free phosphate detection is simple and well established
- Radioactivity-based assay system allows the greatest assay sensitivity

Keywords

Phosphatase, Tumour suppressor, Phosphoinositide, Vesicle, Enzyme assay, Phosphate.

Introduction

The PTEN phosphatase plays decisive roles in the regulation of many diverse physiological processes and its loss or deregulation causes important pathologies, in particular many sporadic cancers [1]. The amino acid sequence and 3D structure of PTEN define it as a member of the protein tyrosine phosphatase (PTP) superfamily which rely on a conserved catalytic cysteine nucleophile and mechanism, acting as phosphomonoester hydrolases [2, 3].

The human PTEN gene at chromosome 10q23 encodes a heavily studied protein of 403 amino acids (approx. 50kD) localised in the cytosol and usually nucleus. PTEN also encodes a recently characterised longer form (PTEN-L or PTEN-Long), of 576 amino acids and approximately 75kD, that appears to be secreted and may be able to enter other cells [1, 4, 5]. The canonical PTEN protein comprises an N-terminal
PTP catalytic domain, a more C-terminal C2 domain which contributes to its transient interactions with membrane surfaces and a less highly structured C-terminal tail, which includes two characterised clusters of phosphorylated residues and a C-terminal PDZ-binding sequence [1, 6]. In addition to phosphorylation, PTEN can be regulated by post-translational ubiquitination, SUMOylation, oxidation and acetylation and its expression controlled at many steps including promoter methylation, transcriptional control and by miRNAs [1, 6-8].

It is clear that many of the functions of PTEN are mediated by its catalytic activity but with only very limited published data addressing this question in vivo, the significance of phosphatase independent mechanisms of action is currently unclear. Several phosphatase independent functions have been proposed for PTEN, but the dominant role for catalysis in mediating PTEN function is supported by the phenotypic similarity between mice carrying alleles which cause a complete loss of PTEN expression and mice expressing catalytically inactive stable mutant PTEN proteins [9, 10].

PTEN has an evolutionarily conserved role in many lineages of eukaryotes, including metazoan and many protozoa, dephosphorylating the D3 position of phosphatidylinositol 3,4,5-trisphosphate (commonly abbreviated either to PtdInsP₃ or PIP₃), the primary lipid product of the class I PI 3-kinases (PI3Ks), making PTEN a core inhibitory component of the PI3K signalling pathway [11]. One long standing uncertainty regarding PTEN is the significance of alternate substrates [12]. In vitro, PTEN has robust phosphatase activity against acidic phospho-tyrosine substrates, although substantially weaker than that observed with dedicated protein tyrosine phosphatases, and an even weaker activity against phospho-serine and phospho-threonine [13]. PTEN is also able to dephosphorylate the inositol phosphate, inositol
1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P$_4$), the soluble headgroup of PtdInsP$_3$, and also several related inositol phosphates [14-16]. Whether alternate protein or inositol phosphate substrates are dephosphorylated physiologically has been a matter of great speculation but given the limitations of current experimental approaches, conclusive evidence has been hard to generate. These efforts to determine the substrate selectivity of PTEN have involved the development of a range of different assays for PTEN’s phosphatase activities against both lipid and soluble substrates and which are described and discussed here.

2. Materials and Methods

2.1. Samples preparation for phosphatase assays

2.1.1. Expression and purification of recombinant proteins

To express and purify recombinant proteins to be used in an in vitro assay, cDNAs encoding the target protein, in this case PTEN WT or mutants were expressed individually in *E. coli* BL21 cells as fusion proteins with glutathione S-transferase (GST). DNA of vectors based upon pGEX 6P1 (GE Healthcare, Amersham, UK) was transformed in BL21 cells and a single transformed colony was amplified in 10 ml of LB medium with 50 µl/ml of ampicillin overnight. This 10 ml stationary phase culture was added to 500 ml of LB medium with 50 µl/ml of ampicillin and grown at 37 ºC until 0.6 OD at 596 nm was reached (approx. 3 hours) before protein expression was induced with 100 µM IPTG (Melford Laboratories Ltd, Ipswich, Suffolk, UK) for a further 18 hours at 18ºC. The bacterial pellet was recovered by centrifugation at 1800xg for 20 minutes at 4ºC and resuspended in ice cold lysis buffer with protease inhibitors (50 mM Tris-HCl pH 7.4; 150 mM sodium chloride, 1%
Triton X-100; 1 mM EDTA, 1 mM EGTA, 0.1% β-mercaptoethanol (v/v), 0.2 mM PMSF, 1 mM benzamidine, 100 μg/ml leupeptin, 1 mg/ml lysozyme). The suspension was transferred to a 50 ml Falcon tube and sonicated on ice for 5 15-second bursts (70% amplitude). The lysate was pre-cleared by centrifugation (10000xg) for 45 minutes at 4°C. -Glutathione Sepharose 4B (GSH-sepharose) (GE Healthcare, Amersham, UK) was pre-washed in buffer A (50 mM Tris-HCl pH 7.4, 0.03% Triton X-100, 150 mM sodium chloride, 0.1 mM EGTA, 1 mM EDTA, 0.2 mM PMSF, 0.1% (v/v) β-mercaptoethanol, 1 mM benzamidine, 100 μg/ml leupeptin) and added to the pre-cleared supernatant. After binding for 1 hour at 4°C, the GSH-sepharose was recovered by centrifugation at 450xg for 1 minute at 4°C. The GSH-sepharose and bound proteins were batch-washed twice with 20 ml equilibrium buffer before packing them into a column and washing with a further 20 ml of buffer A. For purification of GST-tagged proteins, these were then eluted using 0.5 ml quantities of elution buffer (50 mM Tris-HCl pH 7.4, 250 mM sodium chloride, 0.1 mM EGTA, 20 mM reduced L-Glutathione, 0.1% (v/v) β-mercaptoethanol, 1 mM benzamidine, 0.2 mM PMSF, 100 μg/ml leupeptin, adjusted to pH 7.4 at 4°C). Fractions of 700 µl were collected and the protein concentration of the fractions was determined using Bradford method. High protein concentration fractions were pooled together and snap frozen in liquid nitrogen before long-term storage at -80°C, giving a yield of approximately 1.5 mg GST-PTEN per litre of E. coli culture.

It is also possible to purify untagged protein via proteolytic removal of the GST tag. In this case, proteins were not eluted, but the GSH-sepharose bound proteins were incubated overnight with 15 μg of Prescision protease enzyme (DSTT, University of Dundee, UK), in 2 ml of cleavage buffer (50 mM Tris-HCl pH 7.4, 150 mM sodium chloride, 1 mM EGTA, 1 mM DTT). After overnight incubation another 15 μg of
Prescission protease were added for an extra hour and then after centrifugation the untagged proteins were collected in the supernatant.

2.1.2. Preparation of immune precipitates for phosphatase assays

Immunoprecipitation of PTEN, usually from mammalian cell lysates, was carried out using either A2B1 antibody (Santa Cruz Biotechnology) pre-coupled to protein G sepharose beads (GE Healthcare Life Sciences) or an affinity purified polyclonal sheep antibody raised against the N-terminal PTEN peptide, MTAIIKEIVSRNKRKY [17]. Briefly, the antibody was added to the protein G sepharose and incubated for 1 hour on a shaker. After 2 minutes of centrifugation, the supernatant was discarded and the beads washed twice with lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM sodium chloride, 1 mM EGTA, 1 mM EDTA, 10 mM β-glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 100 μM PMSF, 1 mM benzamidine, 0.1% β-mercaptoethanol, 1% NP-40). 1 ml of cell lysate was added to the beads/antibody conjugate mixture and left 1 hour under rotary agitation at 4°C. After 1 hour of incubation immune complexes were washed and assayed against either lipid vesicles containing $^{33}$P-PtdIns(3,4,5)P$_3$ or $^{33}$P-Poly(Glu,Tyr)-P as described in the following sections.

2.2. Phosphatase assays

The lipid phosphatase activity of PTEN can be determined either using water soluble substrates or lipid vesicles, using prepared radiolabelled substrates or unlabelled substrates and detecting free phosphate release using malachite green reagents.
2.2.1. Malachite Green Phosphate Assay – PtdInsP₃, InsP₄ or protein substrates

The assay is based on the formation of a complex between malachite green molybdate and free orthophosphate that absorbs at 620-640 nm. A number of PTEN substrates have been successfully used, including water soluble diC₈ phosphoinositides (Cell Signals, Lexington, KY, USA), inositol phosphates including InsP₄ and InsP₅ and peptide substrates. For a 96 well plate assay format, 1 µg of recombinant PTEN protein was incubated with 50 µM substrate in a final volume of 50 µl of buffer containing 50 mM Tris-HCl (pH 7.5) and 10 mM dithiothreitol (DTT). Plates were incubated for up to 1 hour at 37 ºC and the reaction was terminated by the addition of 100 µl of malachite green reagent (Biomol Green, AK-111). The colour developed after an incubation of 15 min at room temperature, was measured at 620 nm. The amount (nmol) of free phosphate released in each enzyme reaction was determined by linear regression analysis against a standard phosphate curve and the initial free phosphate concentration in all assay components was also determined.

2.2.2. Production of (³³P)-PtdIns(3,4,5)P₃ by labelling at the D3 position

3-[³³P] PtdIns(3,4,5)P₃ was produced using PtdIns(4,5)P₂ as the substrate for PI 3-kinase [18, 19]. A final concentration of 100 µM phosphatidylserine (PtdSer) and 100 µM of PtdIns(4,5)P₂ were dried under vacuum and re-suspended in 25 mM Hepes pH 7.4, 100 mM sodium chloride and 1 mM EGTA by three cycles of 15 seconds bursts of sonication. Lipid vesicles were then incubated, at 37 ºC for 45 minutes, with 18.5 MBq of (γ-³³P) ATP in the presence of 10 µg of PI3-Kinases α (supplied by James Hastie and Hilary McLaughlin, University of Dundee), 25 mM Hepes pH 7.4,
100 mM NaCl, 1 mM EGTA, 0.2 mM EDTA, 2.5 mM MgCl$_2$, 1 mM dithiothreitol (DTT), 1 mM sodium orthovanadate, 0.2 mM PMSF, 1 mM benzamidine. After this, a second aliquot of PI3-Kinase enzyme was added and left at 37 ºC for further 45 minutes. The reaction was then terminated by the addition of 750 µl of chloroform/methanol/HCl (40:80:1) followed by 250 µl CHCl$_3$ and 0.1 M HCl to make the ratio of CHCl$_3$ (1): CH$_3$OH (1): Aqueous (0.9). After the formation of the two layers, the upper phase was removed and the lower organic phase washed three times with synthetic upper phase. The lower phase was then dried down under vacuum and the dried substrate re-suspended in 500 µl of CHCl$_3$:CH$_3$OH (2:1 v/v) for storage at -20 ºC.

2.2.3. Production of ($^{33}$P)-Poly(Glu,Tyr)-P

1 mg of the synthetic acidic peptide polymer poly(Glu,Tyr) 4:1 was dissolved in 100 µl of 50 mM Hepes pH 7.4 and then phosphorylated with 18.5 MBq of ($^{33}$P) ATP in the presence of 1µg of Insulin receptor kinase in a 50 mM buffer containing 12 mM MgCl$_2$, 1 mM β-mercaptoethanol, 10% glycerol, 0.02% Triton X-100, 1 mM EGTA. The reaction was incubated for 1 hour at 32 ºC, then a further 0.5 µg of Insulin receptor kinase was added for a further 1 hour at 32ºC. The reaction was stopped by adding 20 % cold trichloroacetic acid (TCA) (1M). After 15 minutes of incubation on ice, samples were centrifuged for 2 minutes and the pellet, including the denatured, phosphorylated polymer, washed with 10 % TCA (0.5 M). The pellet was then redissolved in 500 µl of 1 M Tris-HCl (pH 7.5), dialysed against 10 mM Tris-HCl (pH 7) at 4ºC for 2 hours and then overnight, using a 3500 MW-CO slide-A-lyzer dialysis cassette (Pierce Biotechnology Inc, Rockford, IL, USA). The dialysed
\(^{(3^3P)}\)poly(Glu,Tyr)-P was then stored and the concentration of the \(^{33}P\) labelled substrate determined using a Beckman scintillation counter.

### 2.2.4. \(^{33}P\)-PtdIns(3,4,5)\(_3\) phosphatase assay

To test the lipid phosphatase activity of PTEN against PtdIns(3,4,5)P\(_3\), lipid vesicles were prepared mixing 100 µM of phosphatidylcholine (PC), 1 µM of unlabelled diC\(_{16}\) PtdIns(3,4,5)P\(_3\) and a volume of \(^{33}P\)-PtdIns(3,4,5)P\(_3\) to give 100.000 counts per assay. Lipid were dried down under vacuum, resuspended in a buffer containing 10 mM Hepes pH 7.4 and 1 mM EGTA, and then sonicated in a cup horn sonicator for three 15 seconds bursts. To start the reaction, the enzymes (usually 100 ng (2pmol) of recombinant PTEN per assay) were diluted in 50 µl of H\(_2\)O, then 25 µl of the substrate vesicles and 25 µl of assay buffer containing 50 mM Hepes pH 7.4, 1 mM EGTA, 10 mM dithiothreitol (DTT) and 150 mM sodium chloride (NaCl) were added. After 1 hour of incubation at 37ºC, the reactions were stopped through the addition of 10 µl of bovine serum albumin (10 mg/ml) and 500 µl of 1M ice cold perchloric acid (PCA). The samples were mixed and incubated for 30 minutes on ice, followed by centrifugation at 6000xg for 10 minutes at 4ºC to remove precipitated lipid and protein. 50µl of 10% (w/v) ammonium molybdate was added to the supernatant to allow the inorganic phosphate to become soluble in the organic phase. After incubation of 10 minutes at room temperature, 1 ml Toluene:Isobutyl alcohol (1:1 v/v) was added. A two-phase mix was formed, and the upper organic phase containing inorganic phosphate, was then removed, mixed with scintillation cocktail (Scint Safe 3, Fisher Chemical) and radioactivity was counted by liquid scintillation counting.

### 2.2.5. \(^{33}P\)-Poly(Glu,Tyr)-P phosphatase assay
To test the protein phosphatase activity of PTEN, usually 500 ng (10 pmol) of recombinant PTEN proteins, diluted in 50 µl H₂O, was mixed with a volume of ³²P-Poly(Glu,Tyr)-P substrate to give final 100,000 dpm counts per assay in a buffer containing 25 mM Hepes pH 7.4, 1 mM EGTA and 10 mM dithiothreitol (DTT). After up to 90 minutes of incubation at 37ºC, the reactions were stopped through the addition of 10 µl of bovine serum albumin (10 mg/ml) and 500 µl of 1M ice cold perchloric acid (PCA). The samples were mixed and incubated for 30 minutes on ice, followed by centrifugation at 10000xg for 10 minutes at 4ºC to remove precipitated lipid and protein. 50µl of 10% (w/v) ammonium molybdate was added to the supernatant to increase the partitioning of inorganic phosphate into the organic phase. After incubation of 10 minutes at room temperature, 1 ml Toluene:Isobutyl alcohol (1:1 v/v) was added. A two-phase mix was formed, and the upper organic phase, containing inorganic phosphate, was then removed and radioactivity was counted by liquid scintillation counting.

3. Results and Discussion

3.1. Assay format considerations

PTEN requires no co-factors for activity so is generally assayed in simple buffers, although a reducing environment is needed. A number of different PTEN assay formats have been described, each with their own advantages and limitations. A very commonly used platform for the analysis of many phosphatase enzymes relies upon the colorimetric detection of free phosphate using malachite green reagents. For PTEN as with many other phosphatases, these assays provide relatively cheap, simple and established methods that are well suited to the characterisation of recombinant purified PTEN and can also be used with (relatively large scale)
immunopurified material and are amenable to high throughput analysis. Alternatively, the preparation of specific substrates labelled with radioisotopes of phosphorus ($^{32}$P or $^{33}$P) is time consuming and also brings additional safety and waste disposal considerations, but allows the greatest assay sensitivity and signal to noise. This allows the robust analysis of activity using small quantities of PTEN in the low nanomolar enzyme concentration range, allowing for example, analysis of protein immunoprecipitated from 100µg-500µg soluble protein extracted from cells or tissues. Attempts to develop assays with more complex detection platforms, but with high throughput, specifically suited to screening for inhibitors of PTEN (and other lipid metabolising enzymes) has also been successful [20, 21]. Progressing on this front, most recently, a novel gold nanoparticle based assay format presenting PtdInsP$_3$ in a membrane-like lipid surface with good sensitivity have also been described [22].

Unlike many lipid-metabolising enzymes, PTEN has good activity against several soluble substrates. Therefore, the other key variable in assay formats using lipid substrates is their presentation either with normal long-chain fatty acids within a membrane-like vesicle surface or in solution using short fatty acid chain synthetic lipids. The use of lipid/detergent micelles as PTEN substrates has been limited [18, 23, 24]. The use of soluble lipid substrates with a single phase assay brings advantages in terms of speed and potential assay throughput. However, many of the mechanisms regulating PTEN activity in cells appear to act through modulation of its non-substrate mediated association with membrane surfaces and components [1, 6, 25]. Therefore, assays using substrate vesicles provide a closer approximation to physiological PTEN function and allow researchers to investigate more components of the regulation of the enzyme.
3.1.1 Lipid Substrates

In vitro, PTEN will robustly dephosphorylate any of the 3-phosphorylated phosphoinositide lipids, in the approximate order of efficiency PtdIns(3,4,5)P3>PtdIns(3,5)P2; PtdIns(3,4)P2>PtdIns3P. In cells, the lipid signal PtdIns(3,4,5)P3 is a key substrate of PTEN and is present at low concentrations (very approximately in the range of 1/1000 to 1/50000th of cellular lipid depending on cell type and context) in the inner leaflet of the plasma membrane of most mammalian cells, and potentially in other cellular compartments [26-28]. Although strong evidence that other lipids are dephosphorylated physiologically by PTEN is lacking, it has been hard to firmly exclude PtdIns(3,4)P2 as a PTEN substrate because in most cells almost all cellular PtdIns(3,4)P2 appears to be formed by dephosphorylation of PtdInsP3 and therefore changes in PTEN activity usually affect both lipids. Significantly, the inositol ring of PtdInsP3 including the targeted substrate phosphate at the 3 position, is presented at the membrane/cytosol interface. Many lipid metabolising enzymes fit poorly to standard Michaelis-Menten models of enzyme kinetics, as their activity is affected greatly both by their affinity for their substrate lipid, but also by any non-substrate affinity for membrane surfaces [29] and such considerations should not be overlooked when designing an assay for PTEN. In vitro, the only extensive analysis of PTEN using surface dilution kinetics against different phosphoinositides found a 200 fold preference for PtdInsP3 over other lipids including PtdIns(3,4)P2 [18]. Very little is known regarding the effect of substrate fatty acid chain variation on PTEN activity, although both synthetic PtdInsP3 and natural PtdInsP3, either partially purified or generated by phosphorylation of natural purified PI(4,5)P2, have been used successfully. It could be mentioned that although
3.1.2. Soluble substrates: proteins, peptides and inositol phosphates

Although several model phosphatase substrates, such as para-nitrophenylphosphate (pNPP) commonly used for other related enzymes are very poor substrates for PTEN, a range of protein peptide and inositol phosphates have been used in the laboratory.

No protein has been conclusively identified as a substrate for PTEN, so it is not clear that optimal characterisation of this activity has been performed, but it generally appears weak relative to other dedicated protein phosphatases. PTEN will dephosphorylate several proteins in vitro including IRS1, focal adhesion kinase and the PDGF receptor [31-33]. However, in vitro experiments are more commonly performed using synthetic acidic tyrosine phosphopeptides, such as EEEY<sup>phos</sup>EEE and occasionally similar phospho-serine and phospho-threonine peptides against which PTEN has weaker activity [13]. Also commonly used are acidic tyrosine containing peptide polymers such as phosphorylated poly(Glu<sub>4</sub>:Tyr<sub>1</sub>) although the heterogeneity of this substrate makes some aspects of kinetic quantitation problematic.
Several soluble inositol phosphates are efficiently dephosphorylated by PTEN in vitro, particularly Ins(1,3,4,5)P$_4$ (which represents the headgroup of PtdInsP$_3$) and similar molecules [14-16]. Small changes in the abundance of several inositol phosphate pools (several isoforms with the same molecular mass) have been observed in response to the re-expression of PTEN in cells lacking the enzymes, supporting the hypothesis that some may represent additional physiological substrates for the tumour suppressor [14, 15]. However, many metabolites show similar small changes during PTEN induced growth arrest and it is not currently clear whether these changes are direct or indirect. It should also be noted that the activity of PTEN against inositol phosphates is dramatically inhibited in vitro by physiological salt concentrations. As methods to measure individual inositol phosphate species improve it should be possible to determine whether some examples of these molecules are specifically regulated, perhaps directly, by PTEN.

3.2. Malachite green assay with short and long chain lipids

A colorimetric assay for inorganic phosphate that relies upon the formation of a phosphomolybdate malachite green complex can be used in assays of many phosphatases including PTEN. This flexible assay format also allows the use of multiple substrates with relative ease. In 2000, Maehama and collaborators described a malachite green based PTEN assay using lipid vesicles as substrate, containing diC$_{16}$ PtdInsP$_3$ in the presence of PtdSer, and showing that the removal of the lipid substrate suspension by centrifugation before the detection of free phosphate drastically reduced the background absorbance. Indeed, one of the limitations of this original method was due to the increased absorbance due to the lipid suspension light scatter [34]. This very useful assay allows the surface based
assay of PTEN activity without the need to prepare labelled substrates. The alternative, as mentioned above, is to use soluble short fatty acid chain (usually diC8) synthetic phosphoinositide substrates, which do not form large stable micelles at the micromolar concentrations used [35]. It should be noted that many laboratory reagents are supplied or prepared in phosphate containing buffers, and the commercial malachite green reagents are sensitive to assay conditions such as redox potential, therefore it is important to test the phosphate content of all individual assay components to exclude interference from contaminating phosphate and to perform control assays to preclude confounding changes in phosphate detection sensitivity.

An example of a phosphatase assay using untagged recombinant human PTEN purified from bacteria against unlabelled water soluble phosphoinositides is shown in Figure 1A and 1B. Expression of PTEN and purification from bacteria has been used by many laboratories due to its ease and suitability for the comparison of multiple mutants. On the other hand, PTEN purified from bacteria generally contains some aggregated protein and for approaches such as crystallography, insect cell expression has been favoured [3]. In Figure 1B, the phosphate released was quantified by comparison to inorganic phosphate standards. This assay format can be easily applied for high-throughput screening (HTS) using either 384 well plate (5-100 µl volume) or 1536-wells (1-10 µl volume). In Figure 1C and 1D we have compared the phosphatase activity of PTEN against increasing doses of PtdIndP3 in a 96 well plate and the 384 well plate format assay and find that setting the assay in 25 µl volume rather than 50 µl had little effect on the results.

3.3. Radioactivity-based assay
In Figure 2 and 3 examples of PTEN lipid and protein catalytic activity against radiolabelled substrates are shown. As tools for cellular experiments to study the significance of PTEN activity against lipid and protein substrates, PTEN mutants have been developed with selective substrate selectivity. PTEN G129E is a mutant identified in the germline of two families, shown to have activity against protein/peptide substrates but not lipids or inositol phosphates [36, 37] and PTEN Y138L was developed in a laboratory based screen and displays wild-type lipid phosphatase activity whilst selectively lacking activity against soluble substrates including peptides/proteins and inositol phosphates [38]. The principal physiological substrate of PTEN is the lipid PtdIns(3,4,5)P$_3$ which it is known to specifically dephosphorylate at the D3 position. Therefore it is possible to synthesise radiolabelled substrate through phosphorylation of PtdIns(4,5)P$_2$ (and other phosphoinosotides) with class I PI3K. Dephosphorylation of PtdIns(3,4,5)P$_3$ and synthetic protein substrate Poly(Glu,Tyr)P catalysed by purified recombinant PTEN is shown in Figure 2A and B and in a time course format in Figure 2C. Lipid phosphatase activity can be also measured in immune complexes of endogenous PTEN purified from cultured human cells (Figure 3A) or PTEN protein expressed using lentiviral particles (Figure 3B). In all cases the catalytically inactive mutant C124S (which is expressed endogenously in the glioblastoma cell line U343MG), showed no detectable phosphate release compared to PTEN WT. For immune complexes assay, cells were lysed in the absence of protein tyrosine phosphatase inhibitor sodium orthovanadete, which would routinely be included in such lysis buffers.

3.4 Future directions
Current assay methods have supported a large body of work which has greatly developed our understanding of PTEN and the regulatory mechanisms within which it functions. Since many experiments are performed using soluble lipid substrates, even simpler assay methods that allow the analysis of PTEN activity against surface incorporated lipids such as vesicles may encourage the analysis of PTEN activity in more physiological settings by small non-specialist laboratories. Similarly, the development of novel methods, or perhaps wider validation of recently described methods [22], for such surface based PTEN assays that can be applied with high throughput will support the wider investigation of PTEN as a target for therapeutic intervention.

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References


Figure legends

Fig. 1. Malachite green assay. (A) Coomassie blue stained gel of PTEN WT expressed in bacteria as GST-fusion proteins. (Left) Protein purified and eluted as GST-PTEN WT. (Right) GST tag was removed by proteolysis using a GST Prescission protease. (B) Purified recombinant untagged PTEN WT was assayed against short chain lipid substrates. 50 µM diC₈ PtdIns3P, PtdIns4P, PtdIns5P,
PtdIns(3,4)P₂, PtdIns(3,5)P₂, PtdIns(3,4,5)P₃ were used. SAP (shrimp alkaline phosphatase) was added as control in a sample lacking PTEN. After incubation for 1 hour at 37°C, phosphate released was determined by malachite green as described in Materials and Methods. Activity (nmol of phosphate released) is presented as the mean activity ± s.e.m from three experiments performed in duplicates. (C) Comparison of colorimetric phosphatase assay performed in 96 well plate (50 µl final volume) and 384 well plate (25 µl final volume). (D) Timecourse of untagged PTEN activity against soluble diC₈ PIP₃ performed in 384 well format.

**Fig. 2.** Radiometric assay. An example of PTEN lipid and protein catalytic activity against radiolabelled substrates. PTEN WT and mutants proteins were assayed against (A) ³³P radiolabelled PtdIns(3,4,5)P₃ and (B) ³³P phosphorylated PolyGluTyr, respectively for 60 and 90 minutes. The activity of untagged PTEN WT and mutants PTEN C124S and Y138L is presented as the mean activity ± s.e.m from three experiments performed in duplicates. (C) A time course of the activity of untagged PTEN WT and mutant PTEN Y138L assayed with radiolabelled PtdIns(3,4,5)P₃.

**Fig. 3.** Phosphatase assay on immune complexes. (A) Endogenous PTEN protein was immunoprecipitated, using A2B1 antibody pre-coupled to protein G sepharose from HEK293T and U343MG cells and divided for assaying against separate substrates and for immunoblotting. Immune complexes were assayed against radiolabelled PtdIns(3,4,5)P₃ vesicles and phosphorylated PolyGluTyr. The activity is shown as a mean activity ± s.e.m. from three experiments each performed in duplicate normalised to the activity of HEK293T. (B) PTEN null U87MG cells were transduced with lentiviruses encoding GFP, PTEN WT or PTEN C124S and lysed after 48 hours. An antibody raised against the N-terminus of PTEN was used to
immunoprecipitate PTEN protein or antibodies raised against GFP. Immune complexes were assayed against $^{33}$P radiolabelled PtdIns(3,4,5)P$_3$ in vesicles for 60 minutes at 37 ºC and PTEN expression investigated by Western blotting. The phosphatase activity is shown in dpm (decay per minute) as mean raw data ± s.e.m from two experiments.
Figure 1

A

GST-PTEN WT

KDa

64
51
39

Cleaved PTEN WT

B

PTEN WT

Phosphate released (nmol)

Phosphate released (nmol)

PtdIns(3,4,5)P

PtdIns(3,4,5)P

PtdIns(3,4)P

PtdIns(3,4)P

PtdIns(3,4)P

PtdIns(3,4)P

PtdIns(3,4,5)P + SAP

C

PTEN WT

Phosphate released (nmol)

10 μM
20 μM
30 μM
40 μM
50 μM

PtdIns(3,4,5)P (diC8) concentration

384 well plate
96 well plate

D

PI(3,4)P

Phosphate released (nmol)

T0
T2
T5
T10
T15
T30

(minutes)