Interfacial kinetic analysis of the tumour suppressor phosphatase, PTEN: evidence for activation by anionic phospholipids

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We investigated the kinetic behaviour and substrate specificity of PTEN (phosphatase and tensin homologue deleted on chromosome 10) using unilamellar vesicles containing substrate lipids in a background of phosphatidylcholine. PTEN displays the characteristics expected of an interfacial enzyme, since the rate of enzyme activity is dependent on the surface concentration of the substrate lipids used (mol fraction), as well as the bulk concentration. Surface-dilution analysis revealed the catalytic efficiency of PTEN for PtdIns(3,4,5)P_3 to be 200-fold greater than for either PtdIns(3,4)P_2 or PtdIns(3,5)P_2, and 1000-fold greater than for PtdIns3P. The interfacial K_m value of PTEN for PtdIns(3,4,5)P_3 was very low, reflecting the small proportions of this lipid that are present in cellular membranes. The catalytic-centre activity (k_{cat}) for PtdIns(3,4,5)P_3 was at least 200-fold greater than that for the water-soluble substrate Ins(1,3,4,5)P_4. The preference for lipid substrates may result from an interfacial activation of the enzyme, rather than processive catalysis of vesicular substrates. Moreover, both PtdIns(4,5)P_2 and univalent salts stimulated the activity of PTEN for PtdIns(3,4,5)P_3, but profoundly inhibited activity against Ins(1,3,4,5)P_4. The stimulatory effect of PtdIns(4,5)P_2 was greater in magnitude and more potent in comparison with other anionic phospholipid species. A mutation in the lipid-binding C2 domain (M-CBR3) that is biologically inactive did not alter overall catalytic efficiency in this model, but decreased the efficiency of the interfacial binding step, demonstrating its importance in the catalytic mechanism of PTEN.

Key words: phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase, surface dilution analysis.

INTRODUCTION

Mutations in the tumour suppressor gene *PTEN* (phosphatase and tensin homologue deleted on chromosome 10) are frequent in sporadic glioblastomas, prostate and breast cancers, as well as in the autosomal dominant disorders, Cowden's disease and Bannayan–Ruvaba–Rily syndrome [1–3]. PTEN is a mixed-function phosphatase that can dephosphorylate the D-3 position of inositol phospholipids *in vitro* and in cell lines [4,5]. The Gly^{129}→Glu mutation, identified in sufferers of Cowden's disease, has wild-type activity against the standard synthetic phosphotyrosine peptide substrate, poly(GluTyr^p), but undetectable activity against 3-phosphorylated inositol phosphates, suggesting that the inositol phosphatase activity of PTEN is required for its biological function [5–7]. PTEN null cell lines have elevated levels of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2, as well as elevated protein kinase B activity, relative to cells expressing wild-type PTEN. Re-expression of PTEN in these cell lines reverses these defects, suggesting that PTEN may exert its tumour suppressor function by antagonizing the activity, and hence the cell growth and anti-apoptotic signals downstream, of phosphoinositide 3-kinase (PI 3-kinase) [2,3,7,8].

PTEN has several structural features, not seen in other dual-specificity protein phosphatases, which enable it to metabolize inositol phosphate groups, including a highly basic active-site pocket that is both wider and deeper than those of dual-specificity and tyrosine phosphatases [9]. PTEN also contains a Ca^{2+}-independent C2 domain that can bind to phospholipid vesicles *in vitro*, and which resembles the Ca^{2+}-dependent C2 domains of other lipid-metabolizing enzymes, such as phospholipase A_2, phospholipase $C_{\delta 1}$ and the conventional protein kinase Cs [10–12]. A C2 domain mutant (M-CBR3) that has a decreased ability to bind phospholipid vesicles *in vitro* retains wild-type activity against a water-soluble PtdIns(3,4,5)P_3 derivative, but when expressed in PTEN null cell lines has decreased activity towards PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2, and a diminished ability to suppress cell growth compared with wild-type PTEN. Therefore the membrane-binding function of the C2 domain is important for the tumour suppressor activity of PTEN [9,13,14].

Lipid-metabolizing enzymes, such as phospholipases A_2/C and lipid kinases, act on substrates that form a lipid/water interface [15,16]. Such enzymes have therefore adapted catalytic mechanisms that involve the initial binding of the usually cytosolic protein to the surface of the lipid bilayer via a non-catalytic interfacial binding site, which is believed to precede, and hence facilitate, the binding of substrate. Once the substrate is hydrolysed, the enzyme can either return to the aqueous phase or it may remain attached to the membrane, during which time multiple catalytic cycles can occur. This mechanism is thought to aid catalysis: (i) by enhancing proximity and the effective concentration of substrate; and/or (ii) via a conformational change that results in the productive orientation of the active site towards the lipid substrate.

To date there have been no detailed studies of the kinetic properties of PTEN when hydrolysing phospholipid substrates under conditions that mimic those in biological membranes. We

Abbreviations used: DTT, dithiothreitol; GST, glutathione S-transferase; iK_m, interfacial K_m; PI 3-kinase, phosphoinositide 3-kinase; PtdCho, phosphatidylcholine; PtdSer, phosphatidylserine; PTEN, phosphatase and tensin homologue deleted on chromosome 10.

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developed assay procedures for PTEN on the basis of substrate presentation in phospholipid vesicles of defined composition and size. PTEN was found to display classic features of interfacial catalysis, with the rate of enzyme-catalysed metabolism of lipid substrates being heavily dependent on both bulk substrate concentration and the surface concentration of substrate (mole fraction) in vesicles. Under our conditions, PtdIns(3,4,5)P_3 was a substantially better substrate than other physiologically significant 3-phosphoinositides, and water-soluble substrates were found to be comparatively much weaker than previous reports had suggested. We also present evidence in support of an interfacial activation model for hydrolysis of vesicular substrates by PTEN and for modulation of activity by anionic phospholipids, especially PtdIns(4,5)P_2.

EXPERIMENTAL

Expression, cleavage and purification of enzyme preparations

Wild-type human PTEN and M-CBR3 PTEN were expressed as glutathione S-transferase (GST)-fusion proteins and purified as described previously [5]. Cleavage of the GST tag was initiated by the addition of cleavage buffer [50 mM Tris/HCl (pH 7.0)/150 mM NaCl/1 mM EGTA/1 mM dithiothreitol (DTT)] containing 40 units of PreScission (Amersham Biosciences) protease® to the GST–PTEN (or GST–M-CBR3) on packed glutathione–Sepharose beads. The reaction was performed for 16 h at 4 °C, after which cleaved PTEN was collected by centrifugation and purified further by FPLC using a HiTrap Q-Sepharose HP IEX column eluted with a gradient of 150–500 mM NaCl in buffer A [20 mM Tris/HCl (pH 8)/2 mM EDTA/2 mM DTT/150 mM NaCl] and buffer B [20 mM Tris/HCl (pH 8.0)/2 mM EDTA/2 mM DTT/500 mM NaCl]. The purity of the final preparation was determined to be greater than 90 % by SDS/PAGE, before the addition of 100 mg/ml BSA (to give a final BSA concentration of 1 mg/ml) and storage at −80 °C.

Production of [3-^{33}P]phosphoinositides

Samples containing a final concentration of 100 μM phosphatidylserine (PtdSer) and 100 μM of substrate lipid (dipalmitoyl phosphoinositides) were prepared by drying under vacuum and resuspending by sonication (three cycles of 15 s) in 25 mM Hepes/HCl, pH 7.6, 100 mM NaCl and 1 mM EGTA.

PI 3-kinase γ (6 μg; supplied by Dr A. Gray, University of Dundee, Dundee, Scotland, U.K.), was incubated at 37 °C for 30 min with sonicated phospholipid vesicles (prepared as described above) in the presence of 250 μCi of [γ-^{32}P]ATP (specific radioactivity 3000 Ci/mmol), 25 mM Hepes/HCl, pH 7.6, 120 mM NaCl, 1 mM EGTA, 0.2 mM EDTA, 2.5 mM $MgCl_2$, 1 mM DTT, 1 mM vanadate, and PMSF, leupeptin and benzamidine all at a concentration of 10 μg/ml. Reactions were terminated by the addition of chloroform/methanol/HCl (40:80:1, by vol.), and lipids were extracted as described previously [5]. The lower phase was then dried down *in vacuo* and resuspended in chloroform/methanol (2:1, v/v).

Preparation of 3-phosphoinositide-containing phospholipid vesicles

The lipid mix of dipalmitoyl 3-phosphoinositide substrate, 3-[3-^{33}P]phosphoinositide substrate and the stated carrier lipid [PtdSer or phosphatidylcholine (PtdCho)] was prepared by drying under vacuum and resuspending in 10 mM Hepes, pH 7.4 and 1 mM EGTA. To form a multilamellar lipid suspension that can be extruded to a suspension of small unilamellar vesicles of defined and uniform size, the lipid mixture was freeze–thawed 5 times. Small unilamellar vesicles were prepared by extrusion through a polycarbonate filter of fixed pore size (0.1 μm) 10 times (as described previously in [17]). Vesicles prepared in this way exhibited a narrow size distribution (0.1–0.2 μm), and there was insignificant vesicle fusion when the mixture was analysed by electron microscopy. Radiolabelled PtdIns(3,4,5)P_3 inserted efficiently and remained associated with vesicles as assessed by pull-down experiments using sucrose-loaded vesicles. Phospholipid exchange between unilamellar vesicles of this type is reported to be very slow, of the order of several hours [18], making them ideally suited to studies of interfacial catalysis. The vesicles also exhibited negligible substrate exchange between the inner and outer leaflets of the bilayer, as judged from the extent of substrate availability during prolonged incubations with PTEN (see the Results section).

Phosphatase assays

Ins(1,3,4,5)P_4 3-phosphatase activity was measured as described previously [13]. Phosphoinositide 3-phosphatase assays were performed using unilamellar lipid vesicles containing the requisite mole fraction of dipalmitoyl 3-phosphoinositide and 3-[3-^{33}P]-phosphoinositides. The assay buffer was as described in [13], with the modification that assays contained 150 mM NaCl except where stated otherwise. Reactions were terminated by the addition of inorganic solvents, and the measurement of [^{33}P]P_i was performed as described previously [13].

Calculation of vesicle/enzyme stoichiometry

The number of vesicles in the reaction mixtures was estimated on the basis of the following assumptions: the vesicle is a sphere with a bilayer thickness of 4 nm and average surface area of a phospholipid in the bilayer of 0.6 nm^2 [19]. Knowing the radius of the vesicle, the surface areas of the outer and inner monolayers were determined utilizing the geometrical equation for the surface area of a sphere, and hence the number of phospholipid molecules in both the inner and outer monolayers of one vesicle could be predicted. With this value and the total phospholipid concentration, the number of vesicles per assay was calculated. To determine enzyme molarity, the preparation was assumed to be pure with a molecular mass of 47 kDa.

Analysis of surface dilution kinetics

The kinetic properties of PTEN when utilizing lipid substrates were analysed according to procedures developed by Dennis and co-workers [20,21] and were based on the following equation

$$V_0 = \frac{V_{max} \cdot X_s \cdot [S_0]}{i k_m \cdot K_s + i k_m \cdot [S_0] + X_s \cdot [S_0]} \quad (1)$$

where V_0 is initial velocity, V_{max} is the maximal velocity, K_s is the dissociation constant for enzyme from the vesicle surface, X_s is the surface concentration (molar fraction) of the lipid substrate, $[S_0]$ is the bulk substrate concentration and iK_m is the interfacial K_m (which is unitless, i.e. in terms of surface concentration of substrate or molar fraction).

In 'Case 1' experiments, bulk substrate concentrations were varied at a fixed surface concentration by varying the concentrations of the substrate and carrier lipid proportionately. Plots of V_0 against $[S_0]$ result in a rectangular hyperbola with:

$$V_{max}\ (\text{apparent}) = V_{max}/(iK_m/X_s + 1) \quad (2)$$

and

$$K_m\ (\text{apparent}) = K_s/(X_s/iK_m + 1) \quad (3)$$

where V_{max} (apparent) is the maximal rate achieved at a given value of X_s and K_m (apparent) is the value of $[S_0]$ giving 0.5 V_{max} (apparent) for a given value of X_s.

In practice, V_{max} (apparent) and K_m (apparent) values were determined for several different surface concentrations of each substrate by fitting to a single-rectangular, two-parameter hyperbola. In addition, double-reciprocal plots of the primary data were analysed by linear regression. Both procedures yielded similar values for the relevant parameters.

In 'Case 2' experiments, the surface concentrations of substrate lipids were varied while maintaining the bulk concentration constant. Plots of V_0 against X_s give rectangular hyperbolae, which define the true V_{max}, and where:

$$iK_m \text{ (apparent)} = iK_m(K_s/[S_0]+1) \quad (4)$$

iK_m (apparent) and V_{max} values were determined from the above 'Case 2' data by linear-regression analysis of double-reciprocal plots.

To determine the value of iK_m for each substrate, V_{max} values obtained from 'Case 2' experiments were fitted to eqn (2), along with the data from the 'Case 1' experiments. To calculate K_s, iK_m and K_m (apparent), values obtained from 'Case 1' experiments were fitted to eqn (3). In practice, K_s values were obtained for each substrate across a range of surface concentrations for comparison.

Data analysis

The data obtained from kinetic experiments were fitted as described above using the Sigma-Plot program.

RESULTS

PTEN catalyses PtdIns(3,4,5)P_3 hydrolysis in the 'hopping' mode

Characterization of the kinetic parameters of a lipid-metabolizing enzyme, such as PTEN, requires the presentation of substrate lipid to the enzyme in a form that resembles the aggregated structure of a lipid bilayer. Phospholipid/detergent-mixed micelles, carrier phospholipid vesicles and lipid monolayers at the water/air interface are systems that have been used previously to achieve this. In the present study, we investigated the use of phospholipid/detergent-mixed micelles and phospholipid vesicles as possible substrate vehicles to analyse the kinetic properties of PTEN. However, phospholipid/detergent-mixed micelles proved unsuitable for the kinetic analysis of PTEN, because all the detergents investigated (dodecylmaltoside, Genapol X-80, Triton X-100 and Zwittergent 3-14) affected catalytic activity (results not shown).

We therefore chose to make use of unilamellar phospholipid vesicles as carriers of substrate lipids in PTEN assays. Initially, phospholipid vesicles utilizing anionic lipids such as PtdSer or phosphatidylglycerol as carriers were found to inhibit PTEN activity profoundly against water-soluble substrates, such as Ins(1,3,4,5)P_4 (results not shown). By contrast, the neutral phospholipid PtdCho proved to be a neutral diluent, free of inhibitory or stimulatory effects on PTEN activity (results not shown). Consequently, PtdCho vesicles were used for all subsequent experiments.

The 'scooting' and 'hopping' models of interfacial catalysis were developed in order to explain the kinetic behaviour of phospholipases [18,22]. In the scooting mode, the enzyme binds irreversibly to the first phospholipid vesicle that it encounters and hydrolyses all of the substrate present in the outer monolayer of that vesicle only. In the hopping mode, the enzyme binds relatively briefly to vesicle surfaces. Depending on the catalytic-centre activity (k_{cat}) of the enzyme and the relative rates of binding and desorption from vesicle surfaces, the enzyme may carry out one or more catalytic cycles each time it encounters a substrate-containing vesicle. Catalysis in the hopping mode is characterized by the hydrolysis of all the substrate present in the outer monolayer of all the vesicles present, even when the number of vesicles greatly exceeds the number of enzyme molecules present in the assay.

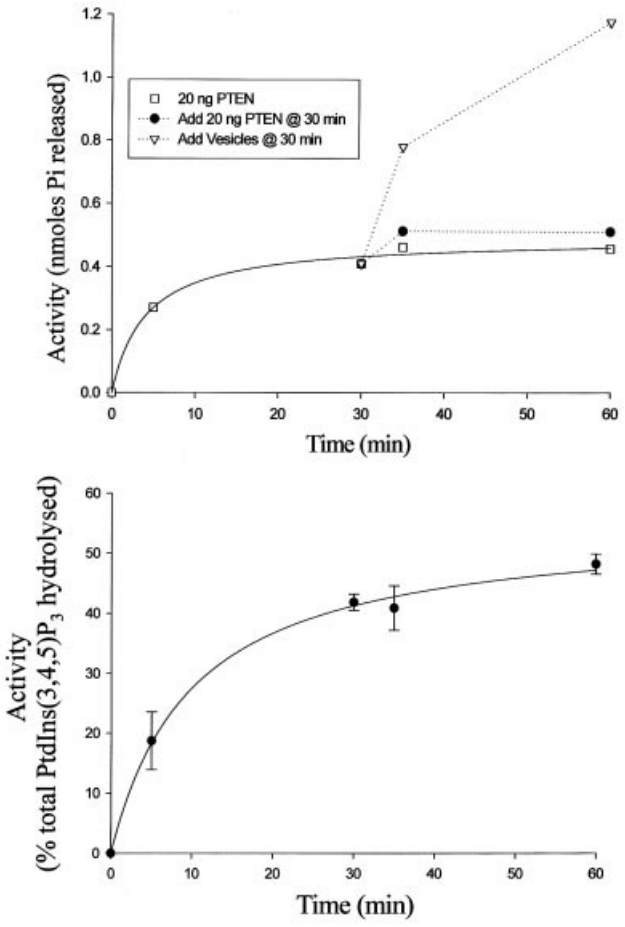

Figure 1 Hydrolysis of PTEN vesicular lipid substrates in the 'hopping' mode (upper panel) and metabolism of all of the available substrate in PtdCho vesicles by PTEN (lower panel)

Upper panel: PTEN activity towards 10 μM PtdIns(3,4,5)P_3 at 10 mol% in PtdCho phospholipid vesicles extruded to 30 nm in diameter as a function of time is shown. Another aliquot of 20 ng of PTEN, or another aliquot of vesicles, was added to the reaction at the 30 min time point. Assays were performed as described in the Experimental section and the data are representative of three experiments performed in triplicate. Lower panel: the assay was performed using vesicles as described above. The data are expressed in terms of the percentage of total substrate hydrolysed, and are the means $\pm$ range/$\sqrt{2}$ of duplicate incubations.

To determine whether PTEN was operating in the scooting or hopping mode under the chosen conditions, a second aliquot of either enzyme- or substrate-containing vesicles was added to reaction mixtures containing an estimated 3–4-fold excess of vesicles to enzyme molecules after the initial completion of the reaction (Figure 1, upper panel). The addition of a second aliquot of substrate vesicles resulted in a recapitulation of the reaction progress curve that was observed initially (Figure 1, upper panel), consistent with hopping behaviour. Also consistent with this interpretation, the addition of another aliquot of PTEN

Table 1 Summary of the kinetic analysis of PTEN 3-phosphatase activity for Ins(1,3,4,5)P_4 (a) and for 3-phosphoinositides (b)

Summarized are the kinetic analyses of PTEN and M-CBR3 towards the full range of phosphoinositide substrates, for comparison with Ins(1,3,4,5)P_4. The results are shown as the means $\pm$ range/$\sqrt{2}$ (where $n = 2$), or otherwise, as the mean $\pm$ S.E.M. for the stated number (n) of independent experiments. K_s values reported in (b) are the means for two values determined at different substrate mole fractions [for PtdIns(3,4,5)P_3, 0.001 and 0.002; for PtdIns(3,4)P_2, 0.001 and 0.01; for PtdIns(3,5)P_2, 0.02 and 0.05; and for PtdIns3P, 0.05 and 0.1].

(a)

Kinetic parameter	Substrate ...	PTEN Ins(1,3,4,5)P_4	M-CBR3 Ins(1,3,4,5)P_4
k_{cat} (min^{-1})		0.37 ± 0.02 ($n = 3$)	1.35 ± 0.32 ($n = 3$)
K_m (mM)		0.005 ± 0.0009 ($n = 3$)	0.0067 ± 0.0009 ($n = 3$)
k_{cat}/K_m ($mM^{-1} \cdot min^{-1}$)		73	202

(b)

Kinetic parameter	Substrate ...	PTEN PtdIns(3,4,5)P_3	PTEN PtdIns(3,4)P_2	PTEN PtdIns(3,5)P_2	PTEN PtdIns3P	M-CBR3 PtdIns(3,4)P_2
k_{cat} (min^{-1})		73 ± 4.4 ($n = 2$)	7 ± 0.3 ($n = 3$)	22 ± 1.6 ($n = 3$)	9 ± 0.3 ($n = 3$)	9 ± 0.7 (n = 3)
iK_m (X_s; mol%)		0.04 ± 0.005 ($n = 6$)	0.72 ± 0.05 ($n = 4$)	1.6 ± 0.16 ($n = 5$)	6 ± 1 ($n = 3$)	0.3 ± 0.02 (n = 4)
k_{cat}/iK_m ($X_s^{-1} \cdot min^{-1}$)		182 500	972	1375	150	2999
K_s (mM; surface binding model)		84 ± 9.0 ($n = 6$)	20 ± 4.9 ($n = 4$)	1 ± 0.3 ($n = 5$)	0.2 ± 0.1 ($n = 3$)	115 ± 9.0 ($n = 4$)

at the end of the reaction progress curve did not result in another burst of activity, presumably because all of the PtdIns(3,4,5)P_3 molecules predicted to be available for hydrolysis in the outer monolayers of the vesicles in the assay population (between 50 and 60% of the total substrate lipid in the assay) had already been metabolized (Figure 1, lower panel). In theory, these results could be explained by inter-vesicle exchange of substrate and/or product. Whereas transfer of phospholipids in mixed micellar assays can be very rapid, exchange between unilamellar vesicles is generally considered to occur with a half-time of several hours, well beyond the scope of our experiments [18].

Univalent salts differentially affect enzyme activity towards vesicular and soluble substrates respectively

Table 1(a) shows data confirming that the soluble head group of PtdIns(3,4,5)P_3, Ins(1,3,4,5)P_4, is an effective substrate of PTEN. The K_m of 5 μM is similar, although the V_{max} (and hence k_{cat}) values are approx. 10–15-fold higher than results published previously [4,5]. Physiologically relevant univalent-metal salts, such as NaCl, were found to inhibit activity profoundly against soluble substrates such as Ins(1,3,4,5)P_4 (Figure 2a). At physiological salt concentrations (150 mM), Ins(1,3,4,5)P_4 3-phosphatase activity was less than 10% of that observed in the presence of buffer salts alone. This effect was found to result primarily from a large decrease in K_m with little effect on V_{max} (Figure 2b), suggesting that simple salts decrease the affinity of substrate binding at the active site. This is compatible with structural data showing that the active site of PTEN contains several positively charged residues positioned to form salt links with non-scissile phosphates of the inositol phosphate [9].

This behaviour, however, contrasts markedly with the effects of salt on the hydrolysis of lipid substrates by PTEN. In the absence of added salt, hydrolysis of PtdIns(3,4,5)P_3 in PtdCho vesicles was slow (Figure 2c), but the addition of 150 mM NaCl immediately enhanced the rate by approx. 10-fold. In the absence of salt, the overall time course of hydrolysis was linear, since less than one-third of available substrate was hydrolysed over 60 min under the conditions depicted. In the presence of salt, however, the time course was hyperbolic, because most of the available substrate had been metabolized within 30 min (Figure 1, lower panel).

NaCl was found to promote the hopping behaviour of phospholipase A_2 [18,22–24], but this does not explain our data which show a clear enhancement of initial rate. The combined effects of NaCl on lipid and soluble substrates are predicted to select in favour of the former by a factor of approx. 100. Since it is likely that the head group of PtdIns(3,4,5)P_3 interacts with the active-site pocket of PTEN in a similar manner to that of Ins(1,3,4,5)P_4, the differential effects of salt are intriguing. Perhaps salt overcomes the decreased affinity for binding at the active site by promoting non-catalytic binding to vesicular substrates, or by favouring interfacial activation of PTEN brought about by such non-catalytic interactions.

Kinetic analysis of PTEN in the hopping mode

Further characterization of the properties and substrate specificity of PTEN was undertaken by analysing the enzyme in the hopping mode in the presence of 150 mM NaCl. 'Case 1' and 'Case 2' experiments were performed for each of the known naturally occurring 3-phosphoinositides that are potential PTEN substrates. Representative data for PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 are shown respectively in Figures 3 and 4, and the combined calculated data (determined as described in the Experimental section) for the range of kinetic parameters and substrates are summarized in Tables 1(a) and 1(b).

The 'Case 1' analysis, comparing PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2, is shown in Figure 3. Apparent V_{max} values decreased markedly as the surface concentration of each substrate was decreased, providing the first clear-cut data establishing that PTEN functions as an interfacial enzyme, with V_0 being a function of both bulk and surface concentrations of each substrate. The results in Figure 3 also provide a qualitative comparison of these two substrates, and show that PtdIns(3,4,5)P_3 is utilized more efficiently than PtdIns(3,4)P_2. The 'Case 2' analysis confirms this qualitative conclusion, and defines more precisely the relationship between reaction rate and surface concentration of the substrate. Qualitatively similar, but quantitatively distinct, results were obtained also for PtdIns3P and PtdIns(3,5)P_2 (summary data are shown in Table 1b).

The quantitative analyses, summarized in Table 1(b), clearly indicate that PtdIns(3,4,5)P_3 is superior to PtdIns(3,4)P_2 as a substrate, with a 10-fold enhancement in k_{cat} and an almost-20-fold enhancement of iK_m. Overall, PTEN's catalytic efficiency is almost 200-fold greater for PtdIns(3,4,5)P_3 than for PtdIns(3,4)P_2. Extending this comparison to other substrates,

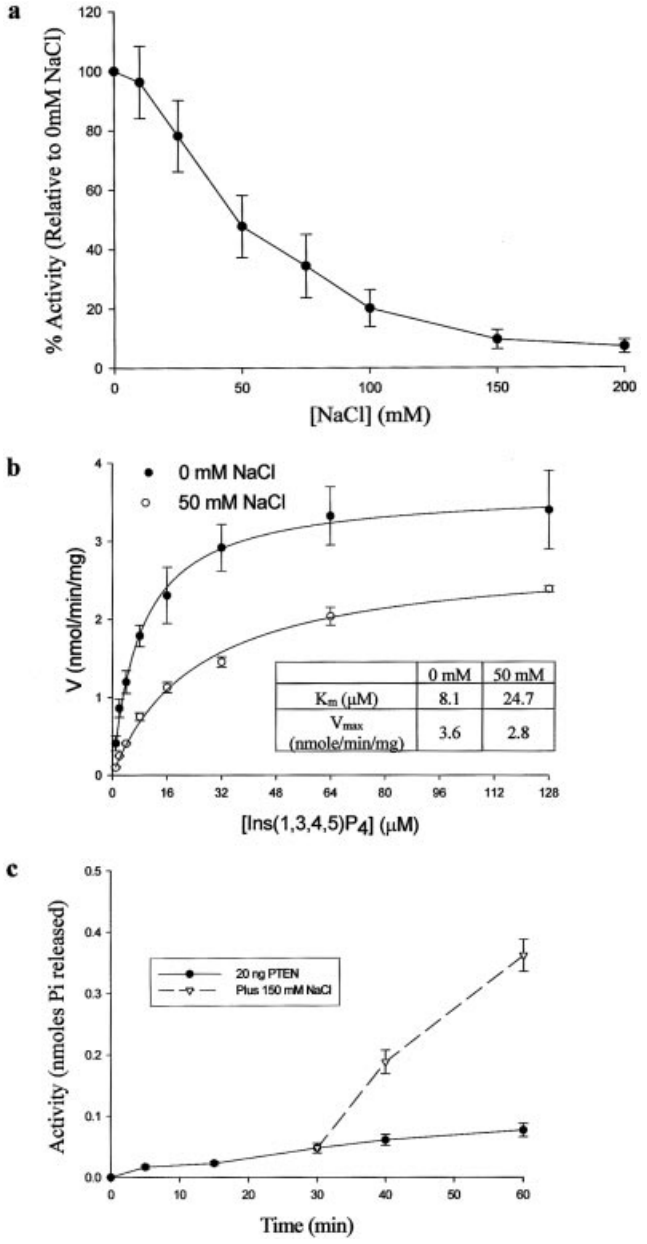

Figure 2 Inhibition of the Ins(1,3,4,5)P_4 3-phosphatase activity of PTEN by NaCl (a), alteration of the kinetic parameters of PTEN Ins(1,3,4,5)P_4 3-phosphatase activity by NaCl (b), and promotion of PTEN activity against a vesicular substrate by NaCl (c)

(**a**) Reactions were performed with 500 ng of PTEN with the stated final concentrations of NaCl, as described in the Experimental section. The data shown are means ± S.D. ($n = 4$). (**b**) Increasing concentrations of Ins[3-^{33}P](1,3,4,5)P_4 were incubated with 250 ng of PTEN for 10 min at 37 °C in the presence or absence of 50 mM NaCl. Activity was determined as described in the Experimental section. Results for 0 mM NaCl are shown as the means ± S.D. for four experiments performed in triplicate; results for 50 mM NaCl are means ± S.D. for two experiments performed in triplicate. (**c**) The assay was performed using vesicles as described in the legend to Figure 1. An aliquot of NaCl was added to the assay at the 30 min time point, making the final concentration of NaCl 150 mM. The data are expressed as means ± S.E.M. for three experiments performed in duplicate.

PtdIns(3,5)P_2 is similar to PtdIns(3,4)P_2, since its somewhat higher iK_m is balanced by a higher value for k_{cat}. PtdIns3P, however, appears to be a much weaker substrate than any of the other lipids studied.

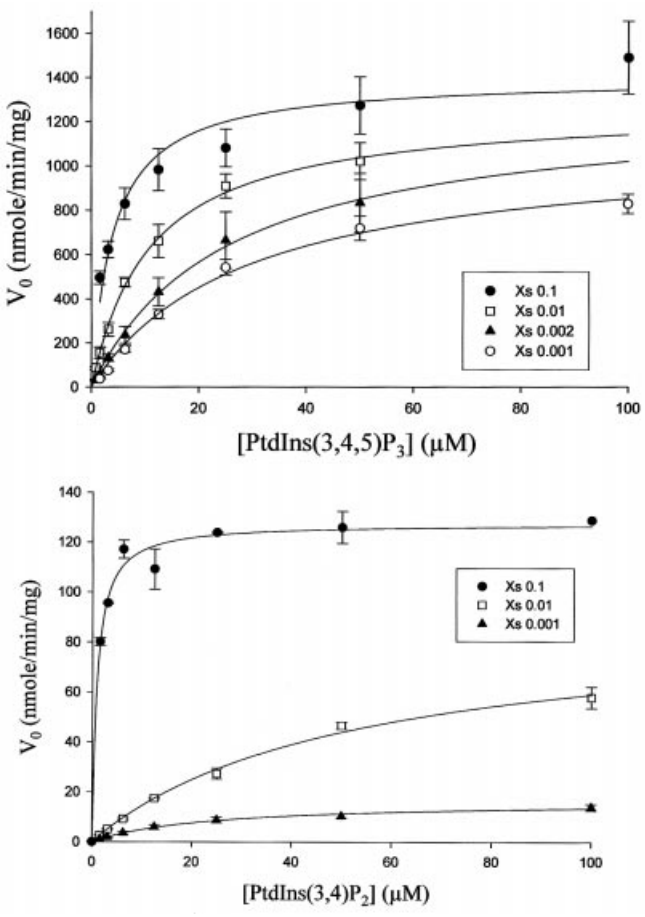

Figure 3 'Case 1' analysis of PTEN activity in PtdCho vesicles against PtdIns(3,4,5)P_3 (upper panel) or PtdIns(3,4)P_2 (lower panel)

Upper panel: PTEN activity towards PtdIns(3,4,5)P_3 was measured over a range of PtdIns(3,4,5)P_3 concentrations at a set of fixed molar fractions (X_s values of 0.1, 0.01, 0.002 and 0.001). The assays were performed as described in the Experimental section. The data are plotted as a function of the bulk concentration of the substrate phospholipid, as defined by the 'substrate/phospholipid-binding' model. The data are the means ± S.E.M. for three experiments performed in triplicate. Lower panel: PTEN activity towards PtdIns(3,4)P_2 was measured over a range of PtdIns(3,4)P_2 concentrations at a set of fixed molar fractions (X_s values of 0.1, 0.01 and 0.001). The assays were performed as described in the Experimental section. The data are plotted as a function of the concentration of the substrate phospholipid, PtdIns(3,4)P_2, as defined by the 'substrate/phospholipid-binding' model. The data represent the means ± range/$\sqrt{2}$ for two experiments performed in triplicate.

Factors affecting K_s

K_s is a measure of the interaction between the enzyme and the vesicle surface and, according to models of interfacial catalysis, reflects interactions at non-catalytic interfacial binding sites. The values reported in Table 1 are the means for values determined independently at two different surface concentrations for each substrate. For example, calculated values for K_s using PtdIns(3,4,5)P_3 as substrate were 96 and 71 mM respectively when analysed at surface concentrations of 0.001 and 0.002. Moreover, as Table 1 shows, K_s values varied substantially between substrates. This suggests either that the substrate itself binds at a putative non-catalytic site, or that anionic phospholipids, in general, influence the binding affinity of PTEN for vesicle, and presumably membrane, surfaces. This may have important implications for the physiological functions of PTEN,

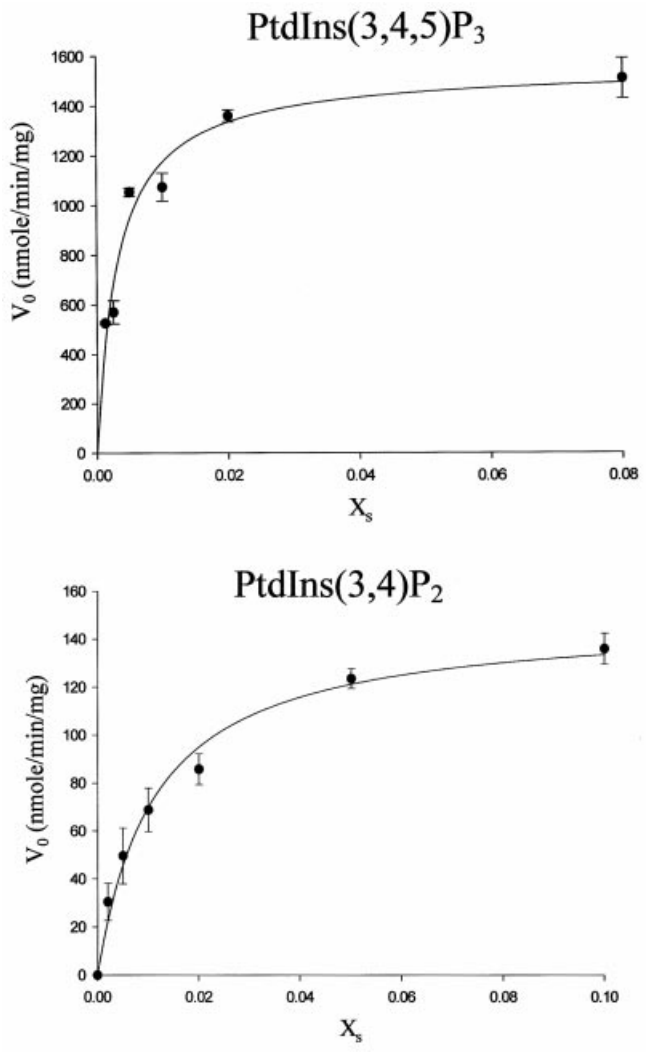

Figure 4 'Case 2' analysis of PTEN activity in PtdCho vesicles against PtdIns(3,4,5)P_3 (upper panel) or PtdIns(3,4)P_2 (lower panel)

Upper panel: PTEN activity towards PtdIns(3,4,5)P_3 was measured as a function of the PtdIns(3,4,5)P_3 surface concentration. PtdIns(3,4,5)P_3 bulk concentration was 50 μM. The experiment was performed as otherwise described in the Experimental section. Data are the means $\pm$ range/$\sqrt{2}$ for two experiments performed in triplicate. Lower panel: PTEN activity towards PtdIns(3,4)P_2 as a function of the PtdIns(3,4)P_2 surface concentration. PtdIns(3,4)P_2 bulk concentration was 100 μM. The experiments were performed as described in the Experimental section, and data are means $\pm$ S.E.M. for three experiments performed in triplicate.

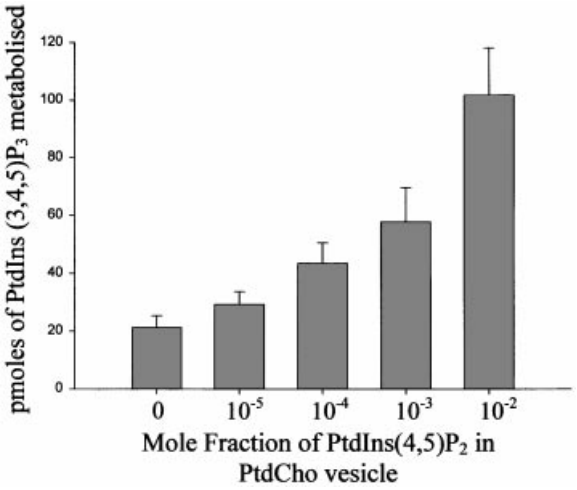

Figure 5 Activation of PTEN activity against PtdIns(3,4,5)P_3 by PtdIns(4,5)P_2

PTEN activity was measured against PtdCho vesicles consisting of a fixed molar fraction of X_s 0.0001 PtdIns(3,4,5)P_3 (bulk concentration 10 μM), and 0, 0.00001, 0.0001, 0.001 and 0.01 molar fraction of PtdIns(4,5)P_2. The data are means $\pm$ S.E.M. for three experiments performed in triplicate.

and is the subject of further, more detailed studies (see the Discussion).

To test whether the C2 domain of PTEN acts as a non-catalytic interfacial binding site, and therefore whether it has a role in PTEN's activity against its lipid substrates, surface dilution kinetics were performed using the M-CBR3 mutant, in which the highly basic sequence in the C2 domain (K^{263}MLKKDK^{269}) is replaced with a more neutral sequence (A^{263}AGAADA^{269}). Initial experiments established that the M-CBR3 mutation exerts a substantial effect on the value of K_s when using PtdIns(3,4,5)P_3 as substrate. However, it proved impossible to complete the data set with this substrate, since the combination of very low iK_m and apparently high K_s meant that very high concentrations of bulk lipid would need to be attained. Such high concentrations were found to block the polycarbonate extrusion membrane used to prepare vesicular substrates. This suggested that it should, however, be possible to define K_s for the mutant enzyme when using a substrate with a higher iK_m than that for PtdIns(3,4,5)P_3. This proved to be the case, and explains why Table 1(b) provides a comparison of the K_s values obtained for wild-type PTEN compared with M-CBR3 PTEN using PtdIns(3,4)P_2 as the substrate. Interestingly, the principal effect of the M-CBR3 mutation is an increase in the value of K_s of approx. 6-fold, although its overall catalytic efficiency proved to be at least as great as that for the wild-type enzyme. This explains why soluble substrates were found to be metabolized efficiently by M-CBR3 PTEN *in vitro*.

Interfacial activation of PTEN by anionic phospholipids

The inner leaflet of the plasma membrane contains in the region of a 100–1000-fold excess of PtdIns(4,5)P_2 over PtdIns(3,4,5)P_3. Interestingly, increasing the mole fraction of PtdIns(4,5)P_2 in PtdCho vesicles (below the K_s) containing PtdIns(3,4,5)P_3 at levels below its iK_m value stimulated the activity of PTEN for this lipid substrate (Figure 5). The catalytic activity of PTEN was stimulated 5-fold in the presence of PtdIns(4,5)P_2 at levels 100-fold greater than those of PtdIns(3,4,5)P_3. The effect of PtdIns(4,5)P_2 was not reproduced by a similar concentration of the anionic phospholipid, PtdSer. Moreover, PtdIns4P gave a much weaker activation in comparison with PtdIns(4,5)P_2 (Figure 6a). Inclusion of a much higher concentration of PtdSer in substrate-containing vesicles, however, did stimulate PTEN activity slightly, but this was always enhanced further by the addition of a lower molar fraction of PtdIns(4,5)P_2 (Figure 6b). This suggests that PTEN is likely to be more efficient at hydrolysing PtdIns(3,4,5)P_3 at the inner leaflet of the plasma membrane, where anionic phospholipids and, in particular, PtdIns(4,5)P_2, are concentrated. Whether PtdIns(4,5)P_2 affects iK_m, K_s or V_{max} is not known at present, but given the effects of substrate lipids described above, a good possibility is likely to be an effect on K_s. This is the subject of ongoing investigation.

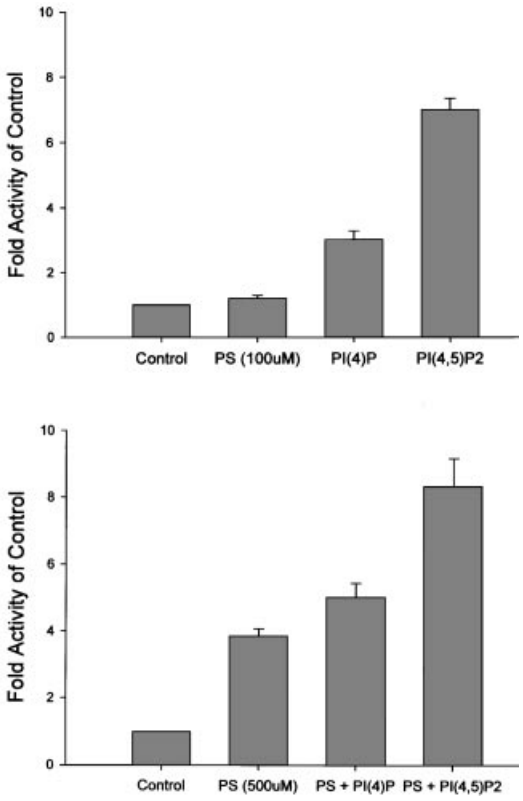

Figure 6 Specificity of PtdIns(4,5)P_2 effects on PTEN activity against PtdIns(3,4,5)P_3

Upper panel: PTEN activity was measured against PtdCho vesicles consisting of a fixed molar fraction of PtdIns(3,4,5)P_3 (X_s 0.0001; 'Control') or PtdCho plus substrate and either PtdSer (PS), PtdIns4P [PI(4)P] or PtdIns(4,5)P_2 [PI(4,5)P2], each at a molar fraction (X_s) of 0.01. Lower panel: as above, but with PtdCho plus substrate alone or PtdCho plus PS (X_s 0.05) with substrate and no further additions, or PtdCho plus PS and substrate with either PI(4)P (X_s 0.01) or PI(4,5)P2 (X_s 0.01). The data are means ± S.E.M. for three experiments performed in triplicate.

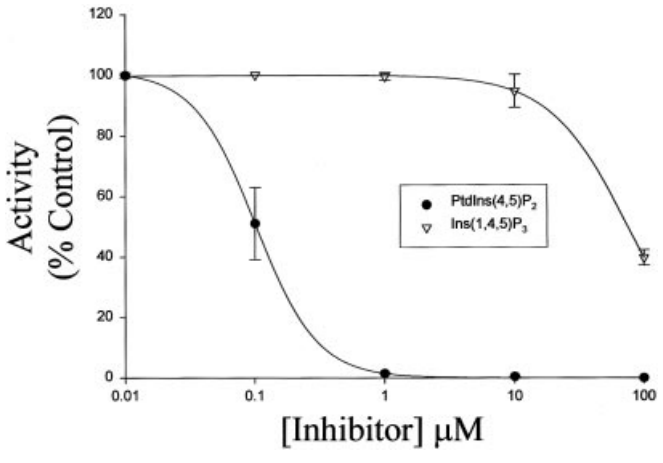

Figure 7 Inhibition of PTEN activity against Ins(1,3,4,5)P_4 with PtdIns(4,5)P_2 and Ins(1,4,5)P_3

Assays were performed as described in the Experimental section. PtdIns(4,5)P_2 was added as a sonicated suspension to give the stated final concentrations. The data are means ± range/$\sqrt{2}$ for two experiments performed in triplicate.

Surprisingly, and in contrast with its effects on PtdIns(3,4,5)P_3 hydrolysis, PtdIns(4,5)P_2 potently inhibited the activity of PTEN for the water-soluble substrate Ins(1,3,4,5)P_4 with half-maximal activity occurring at 0.2 μM PtdIns(4,5)P_2 (Figure 7). The water-soluble head group of PtdIns(4,5)P_2, Ins(1,4,5)P_3, also inhibited the activity of PTEN, but much less potently, with an IC_{50} of 100 μM. The opposing effects of PtdIns(4,5)P_2 on water-soluble compared with lipid substrates establish that this lipid is unlikely to be acting as a competitor for the active site of the enzyme, and thus provide the first evidence for potential interfacial regulation of PTEN.

DISCUSSION

We have analysed the kinetic basis of the ability of PTEN to metabolize its preferred phosphoinositide substrates. PTEN clearly has many of the mechanistic characteristics expected for an interfacial enzyme. The observation that the apparent V_{max} depends upon the molar fraction of substrate in PtdCho carrier lipid vesicles is compatible with the idea that the enzyme mechanism involves an interfacial binding step, and provides the basis for a thorough analysis of substrate specificity that has not previously been possible.

Water-soluble versus lipid substrates

The catalytic efficiency of PTEN towards PtdIns(3,4,5)P_3 is much greater than that of its water-soluble head group, Ins(1,3,4,5)P_4, under the conditions described here. Although the K_m values of PTEN towards these two substrates cannot be compared directly, since they have different units, a comparison of the apparent K_m of PTEN towards PtdIns(3,4,5)P_3, calculated from the 'Case 1' experiment at a high fixed mole fraction (X_s 0.1), suggested that the k_{cat}/K_m (apparent) of PTEN towards PtdIns(3,4,5)P_3 was 3000-fold greater than that of the previously published value for Ins(1,3,4,5)P_4 [4] and 200-fold greater than the value obtained in the present study. This is primarily a k_{cat} effect, since the K_m (apparent) for PTEN towards PtdIns(3,4,5)P_3 and the K_m for Ins(1,3,4,5)P_4 are both approx. 5 μM. Our data strongly suggest that this difference would be amplified under physiological conditions, since 150 mM NaCl inhibited Ins(1,3,4,5)P_4 metabolism by at least a factor of 10. Moreover, an excess of PtdIns(4,5)P_2 and/or other anionic phospholipids potentiated PtdIns(3,4,5)P_3 phosphatase activity, but profoundly inhibited Ins(1,3,4,5)P_4 hydrolysis. If these results are relevant to the cellular environment, PtdIns(3,4,5)P_3 metabolism might exceed that of Ins(1,3,4,5)P_4 by a factor of between 10^3 and 10^4. We therefore conclude that cellular Ins(1,3,4,5)P_4 metabolism by PTEN is likely to be negligible, in line with the results of recent overexpression studies [25].

The preferential metabolism of substrate lipid at the lipid/water interface over water-soluble substrates has been thoroughly established for several lipid-metabolizing enzymes [18,22,26], and, for the first time, for PTEN in the present study. It has proved difficult in many of the above cases to distinguish between the scooting and conformational activation models of interfacial catalysis. The increase in the k_{cat} value of PTEN towards PtdIns(3,4,5)P_3 in the lipid bilayer compared with that for its water-soluble head group is compatible with either mechanism. Irrespective of its physiological relevance, analysis of PTEN using PtdCho-based unilamellar vesicles has the experimental advantage of observing activity in a pure hopping mode. Consistent with this, the relatively high K_s value of PTEN for PtdIns(3,4,5)P_3-containing PtdCho vesicles suggests that the

enzyme associates only briefly with the lipid bilayer. We attempted to measure binding of PTEN directly using pull-down assays with sucrose-loaded vesicles, but these proved unsuccessful, compatible with the low affinities predicted from the kinetic data. Direct binding measurements reported by others were obtained using vesicles with compositions different than those reported here [9]. Given the value of k_{cat} of approx. 1 s^{-1}, it seems unlikely that PTEN can perform more than one or, at least, only a few catalytic cycles each time it encounters a PtdCho vesicle. This would not be compatible with the minimum value of 200-fold for the difference in k_{cat} between PtdIns(3,4,5)P_3 and its soluble head group. We therefore propose that interfacial binding of PTEN productively orientates the active site of the enzyme to lipid substrate, leading to greatly enhanced rates of hydrolysis even in the absence of scooting behaviour.

This conclusion is reinforced by our observation of the effects of PtdIns(4,5)P_2 on enzyme activity. Because this lipid enhanced PtdIns(3,4,5)P_3 phosphatase activity, but profoundly inhibited Ins(1,3,4,5)P_4 metabolism by PTEN, its effects cannot be directed at the active site of the enzyme. This suggests that productive orientation of the active site, via PtdIns(4,5)P_2 binding specifically or via charge-enhanced interfacial binding, excludes water-soluble substrates either through steric hindrance or via a conformational change. Our data do not allow us to distinguish between these possibilities at present. PTEN contains a consensus binding site for PtdIns(4,5)P_2 at its extreme N-terminus [27], which might account for the effects we observed and which is required for chemotaxis and membrane localization of PTEN in *Dictyostelium* [28]. Preliminary experiments to investigate this, however, by making mutations in this region of the protein have not yet been informative, because they have generated proteins with greatly decreased activity which have not allowed an assessment of the effects of PtdIns(4,5)P_2. This therefore remains a goal for future work.

Further evidence in support of an interfacial mechanism with distinct vesicle binding and substrate binding steps arises from our analysis of a C2 domain mutant (M-CBR3) on the activity of PTEN towards its lipid substrates. Although expression studies have shown that this mutation severely limits PTEN's biological activity, and *in vitro* studies have shown that it is defective in vesicle pull-down assays, there has been no direct demonstration of compromised catalytic activity. Our data show that the only measurable defect of M-CBR3 is a large increase in the value of K_s, and provide compelling evidence for the importance of an interfacial binding step in the enzymic actions of PTEN. Because the wild-type enzyme only associates with PtdCho vesicles for a very brief period, it is possible that the interaction of the C2 domain with the lipid bilayer, perhaps via the CBR3 loop, is necessary for the proposed productive orientation of the active site towards the substrate phospholipid. This model is supported by the observation that the artificial targeting of the M-CBR3 mutation to the membrane by a myristoylation signal only partially rescues its biological effects, suggesting that the function of the CBR3 loop is more than simply to target PTEN to the lipid bilayer [14]. In assays using wild-type PTEN, the value of K_s was also found to vary, depending on the overall lipid composition of the vesicles. A systematic study of the effects of vesicle composition is beyond the scope of the present paper, and is the subject of ongoing work.

Lipid substrates

Cells are known to contain at least four phosphoinositides possessing a phosphate group in the 3-position of the inositol ring, and each of these has been analysed in the present study. These studies suggest that the catalytic efficiency (k_{cat}/iK_m) of PTEN is 200-fold greater with PtdIns(3,4,5)P_3 as substrate than that for PtdIns(3,4)P_2 and PtdIns(3,5)P_2, and is 1000-fold greater than that for PtdIns3*P*. PTEN has a similar catalytic efficiency towards both PtdIns(3,4)P_2 and PtdIns(3,5)P_2, since although PtdIns(3,5)P_2 has a higher rate of turnover than PtdIns(3,4)P_2, its affinity for the active site of PTEN is less than that of PtdIns(3,4)P_2. These results suggest a much higher degree of substrate specificity than had been suggested previously, and emphasize the importance of lipid-bilayer structures to assay and examine the regulation of lipid-metabolizing enzymes.

Structural analysis of PTEN [9] suggested that His^{93} and Lys^{128} may interact with the D-5 phosphate group. In particular a mutation of $His^{93}\rightarrow$Ala, which occurs frequently in tumours, caused a 75% reduction in activity using PtdIns(3,4,5)P_3, but had no effect using PtdIns(3,4)P_2 as substrate. These results are compatible with our observation that PtdIns(3,4,5)P_3 has an iK_m value that is 10-fold lower than that for PtdIns(3,4)P_2, and implies that the former substrate is biologically more significant than the latter. Similar conclusions about the significance of the D-4 phosphate group can be drawn both from the further 10-fold increase in iK_m using PtdIns3*P* as substrate and from the 40-fold difference in iK_m between PtdIns(3,4,5)P_3 and PtdIns(3,5)P_2. These results are also compatible with the structural evidence for direct binding of the D-4 phosphate with side chains in the active site. In addition to its apparent role in substrate-binding affinity, the D-5 phosphate interaction appears to play a part in the productive orientation of the D-3 phosphate in the active-site pocket, since the k_{cat} for PtdIns(3,5)P_2 is 3 times greater than that for PtdIns(3,4)P_2. The kinetic analysis has thus provided evidence that is complementary to the structural data, demonstrating that PtdIns(3,4,5)P_3 is much the preferred phosphoinositide substrate of PTEN. The active-site pocket of PTEN has evolved structural features that interact with the phosphate groups, resulting in the tight binding of the head group and the productive orientation of the D-3 phosphate in the active site.

K_s is a measure of the bulk dissociation constant for enzyme binding to vesicles, and may reflect non-specific binding to the surface, binding of one or more specific components of vesicles to non-catalytic sites on the enzyme, or a combination of specific and non-specific components. A significant feature of the data presented in Table 1(b) is that the value of K_s was found to differ substantially with respect to different substrates, with K_s for PtdIns3*P*-containing vesicles being approx. 400-fold lower than for PtdIns(3,4,5)P_3. This might suggest the involvement of specific binding of substrates at non-catalytic sites, with an order of potency favouring PtdIns3*P*. As noted in the legend to Table 1, however, the determination of K_s values required different molar fractions for each of the substrates analysed because of the large differences in iK_m. K_s was determined using vesicles containing 0.001 and 0.002 molar fraction of PtdIns(3,4,5)P_3, but 0.05 and 0.1 molar fraction for PtdIns3*P*. This means that the overall negative charge of PtdIns3*P*-containing vesicles was very much greater than that of the PtdIns(3,4,5)P_3-containing vesicles. Given the stimulatory effects of anionic lipids on PTEN activity discussed above, it seems more likely that the differences in K_s result from these overall differences in charge, rather than differential specific binding of substrate lipids. This in turn suggests the possibility that more physiological membrane compositions might give lower K_s values, and that PTEN might operate in a mixed hopping/scooting mode under such conditions. A thorough analysis of the effects of lipid composition and surface charge on the kinetic behaviour of PTEN using its preferred substrate, PtdIns(3,4,5)P_3, is the subject of future work.

It would be useful to identify a mutant displaying an increased iK_m value for PtdIns(3,4,5)P_3 without affecting its K_s value. A possible candidate for this analysis would be an N-terminal mutation, $Arg^{15} \rightarrow Ile$, which has been shown to have an increased K_m for Ins(1,3,4,5)P_4 (I. Pass, unpublished work). A mutant form of PTEN which contains a non-active-site mutation and which affects the catalytic efficiency of PTEN would also be useful to confirm the proposed interfacial activation mechanism of PTEN. The basic patch of the Cα2 helix, which is adjacent to the CBR3 loop, and the hydrophobic patch of the CBR3 loop are potential candidate sites for such a mutational analysis [9].

PTEN is a very efficient interfacial enzyme

The catalytic efficiency of PTEN towards PtdIns(3,4,5)P_3 at the lipid/water interface is better than that of many other lipid-metabolizing enzymes. Although the k_{cat} for PTEN is relatively low, its very low iK_m value for PtdIns(3,4,5)P_3 (0.0004 or 0.04 mol%) makes it one of the most efficient interfacial enzymes studied to date. This result shows that PTEN is suited to metabolizing lipids that comprise a very small proportion of the total lipid content of the membrane. Unstimulated levels of PtdIns(3,4,5)P_3 are thought to be less than 0.001% of the total cellular phospholipid, although much higher concentrations are expected around signalling complexes containing activated PI 3-kinase. PTEN, therefore, appears to be well adapted to the efficient metabolism of such a scarce lipid substrate.

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