The tumour-suppressor function of PTEN requires an N-terminal lipid-binding motif

Steven M. WALKER¹, Nick R. LESLIE², Nevin M. PERERA, Ian H. BATTY and C. Peter DOWNES

Division of Cell Signalling, School of Life Sciences, University of Dundee, MSI/WTB Complex, Dow Street, Dundee DD1 5EH, U.K.

INTRODUCTION

PTEN (phosphatase and tensin homologue deleted on chromosome 10) is a tumour suppressor and lipid phosphatase which antagonizes phosphoinositide 3-kinase-dependent signalling through metabolism of the membrane-localized lipid second messenger PtdIns(3,4,5)P³ [1–3]. PTEN is largely cytosolic (and nuclear in some cells) and interacts with membranes in order to access its lipid substrate. Although PTEN activity can also be regulated transcriptionally [4,5] and through oxidation [6,7], evidence indicates that the recruitment of the enzyme to its membranous substrate is regulated in several ways. Recent data have shown that the phosphatase and C2 domain, but not the C-terminal tail, are involved in electrostatic binding of PTEN to membranes and that this membrane interaction is required for efficient catalysis [8–10]. However, most cellular PTEN molecules are phosphorylated upon residues within the C-terminal tail, which appears to maintain the protein in a conformation that blocks membrane association, suggesting an important mechanism that could regulate the biological activity of PTEN in vivo [9,11].

Our previous work studying interfacial catalysis by PTEN showed that anionic lipids, the most potent of which was PtdIns(4,5)P2, greatly stimulate PTEN activity against vesicular substrates in vitro [10]. From bioinformatic analysis of PTEN, Maehama et al. [1] recently proposed a putative PtdIns(4,5)P2-binding motif at the N-terminus of PTEN, through similarity with a number of actin regulatory proteins. This polybasic motif is highly conserved within PTEN proteins from humans through to Xenopus, Drosophila, Dictyostelium and Arabidopsis, and is largely conserved in the human PTEN homologue, TPPIP [TPTE (transmembrane phosphatase with tensin homology)]- and PTEN-homologous inositol lipid phosphatase}. For example, either Lys-6 (human PTEN numbering), Arg-11, Lys-13 and Arg-14 were completely conserved or the charge was conserved (the only exception being Arg-14, where the corresponding Drosophila amino acid is Ile). Interestingly a mutation of Lys-13 to glumatic acid (K13E) has been identified in a sporadic human glioblastoma, and a Ser-10 to asparagine (S10N) mutation has been identified in a case of non-Hodgkins malignant lymphoma [12–14]. In contrast with most tumour-derived mutations investigated, PTEN S10N is known to retain substantial catalytic activity in vitro [15], whereas the effect of the K13E mutation has not been analysed. Although this N-terminal motif has been proposed to be important for regulating membrane targeting of Dictyostelium discoideum PTEN [16], and be required for optimal activity [17], its affinity for any lipids, precise mechanism of action and effect on biological function have not been addressed. Using a mutagenesis strategy, we have therefore analysed the properties of this motif in regulating lipid binding and the activity of the tumour suppressor in vitro and in cells.

EXPERIMENTAL

Mutagenesis and construction of expression vectors

The construction of pGEX6P1 PTEN wt has been described previously [10,18]. Mutagenesis of PTEN was performed by PCR mutagenesis of pGEX6P1 PTEN wt using the following oligonucleotides: K13E sense (5′-ATC GTT AGC AGA AAC GAG AGG AGA TAT CAA GAG GAT-3′), K13E antisense (5′-ATC CTC TTT GTA TCT CCT CCT GTT GCT AAT AAC GAT-3′), S10N sense (5′-ATC ATC AAA GAG ATC GTT AAC AGA AAC-3′) and PTEN sense (5′-ATC ATC AAA GAG ATC GTT AAC AGA AAC-3′). The vector was linearized with HindIII before transformation of BL21 DE3 chemically competent cells (Novagen). The construction of pGEX6P1 PTEN K13E, a deletion mutant of PTEN lacking any N-terminal sequence containing the polybasic lipid-binding motif, has been described previously [19].

Abbreviations used: PtdCho, phosphatidylcholine; PtdSer, phosphatidylserine; PKB, protein kinase B; PTEN, phosphatase and tensin homologue deleted on chromosome 10; TPPIP, TPTE (transmembrane phosphatase with tensin homology); and PTEN-homologous inositol lipid phosphatase.

¹ Present address: Cancer Research UK Beatson Laboratories, Garscuche Estate, Switchback Road, Glasgow G61 1BD, U.K.
² To whom correspondence should be addressed (e-mail n.r.leslie@dundee.ac.uk).
phorylation was performed as described previously [19,24]. Untagged PTEN expression constructs were generated by PCR with a primer containing a translation initiation sequence and Xhol restriction site into the full-length PTEN (mutants) required, by use of the XhoKozakPTENs’ primer (5’-CTC GAG GCC ACC ATG ACA GCC ATC ATC-3’). PCR products were inserted into the cloning vector pCR2.1 TOPO (Invitrogen) before sequencing. These PTEN mutant cDNAs were then subcloned using EcoRI non-directional restriction enzyme cloning into pCDNA3.1+ (Invitrogen) and sequenced fully.

Production of PTEN protein from Escherichia coli and 3-[32P]phosphoinositide substrates

Wild-type human PTEN and mutants were expressed as glutathione S-transferase fusion proteins and purified and tag-free as described previously [10,19]. The production of 32P-labelled substrates with phosphoinositide 3-kinase γ has also been described previously [10,19].

Phosphatase assays

Ins(1,3,4,5)P3 3-phosphatase activity was measured as described previously [20]. Phosphoinositide 3-phosphatase assays were performed using unilamellar lipid vesicles containing the requisite molar fraction of dipalmitoyl 3-phosphoinositide and 3-[32P]phosphoinositides. 3-Phosphoinositide-containing phospholipid vesicles were prepared as described previously [10,21]. The assay buffer was as described in [20] with the modification that lipid assays contained 150 mM NaCl. Reactions were terminated directly by the addition of 500 µl of ice-cold 1 M perchloric acid and 100 µg/ml BSA, left on ice for 30 min and spun at 15 000 g at 4°C for 5 min. The supernatant was removed, and mixed with 10% (v/v) of a 100 mg/ml ammonium molybdate solution. After extraction with 2 vol. of toluene/isobutyl alcohol (1:1, v/v), the upper phase was removed and radioactivity was determined by scintillation counting.

Proliferation assays and analysis of PKB (protein kinase B)/Akt phosphorylation

Anchorage-independent colony assays were performed by a method adapted from [22]. Briefly, U87MG cells were transiently transfected with pCDNA3.1+ alone, or PTEN expression constructs. Following transfection (24 h), cells were suspended in 15% (v/v) serum-containing media with 0.5 mg/ml G-418 and 0.1% (v/v) of a 100 mg/ml ammonium molybdate solution. After extraction with 2 vol. of toluene/isobutyl alcohol (1:1, v/v), the upper phase was removed and radioactivity was determined by scintillation counting.

The results of PTEN’s polybasic N-terminal motif in vitro and in cells, a series of point mutations was generated, singly and in combination. These focused on conserved basic residues likely to be involved in anionic lipid binding and the tumour-derived mutations noted above (Figure 1). We avoided Arg-15, since mutation of this residue has previously been shown to compromise catalytic activity (I. Pass, N. R. Leslie and C. P. Downes, unpublished work, and [17,23]). All nine mutant proteins were initially expressed in bacteria and purified (Figure 2), although the quadruple point mutant K6A R11A K13A R14A was expressed rather poorly, probably due to effects on protein stability. PTEN protein expressed in, and purified from, bacteria should be in its non-phosphorylated, and hence active/open, state, allowing analysis of its association with membranes and its activity state, which would not be possible in cellular experiments. Each mutant was assayed against Ins(1,3,4,5)P3 in solution and PtdIns(3,4,5)P3, in neutral PtdCho vesicles. All the mutants retained robust phosphatase activity against both substrates, indicating that the targeted residues of this motif do not play important structural or catalytic roles (Figure 2B, and results not shown). Significantly, subsequent analysis indicated that many of these catalytically competent mutants were affected in the degree of activation by anionic lipids in vitro and displayed impaired function in cells. These effects upon PTEN function were most evident with the quadruple point mutant K6A R11A K13A R14A, and with the tumour-derived mutant K13E (see below, and results not shown). Because of this, and the noted effects of the quadruple mutation on protein stability, subsequent analyses concentrated upon the effects of the K13E mutation upon PTEN function. Importantly, PTEN K13E displays similar activity to the wild-type enzyme in vitro against the soluble substrate Ins(1,3,4,5)P3, indicating that the enzyme is not generally catalytically impaired (Figure 2C, and results not shown).

PTEN PtdIns(3,4,5)P3 3-phosphatase activity is stimulated by increasing the bulk concentration of acidic PtdIns(4,5)P2 within technology. PtdCho (phosphatidylcholine) vesicles consisting of a fixed mole fraction of PtdSer (phosphatidylserine; 0.1) or PtdIns(4,5)P2 (0.05) were prepared by sonication, containing 100 000 d.p.m. of [3H]PtdCho. Immobilized wild-type PTEN and PTEN K13E were incubated with vesicles in binding buffer (10 mM Hepes, pH 7.4, 1 mM EGTA and 10 mM dithiothreitol) for 30 min at 4°C. Bound vesicles were separated from free ones via filtration through 10 µm polypropylene filters (Millipore), washed twice on the filter with two rapid passages of binding buffer, and vesicles associated with the immobilized PTEN determined by scintillation counting of the filter.

RESULTS AND DISCUSSION

To analyse the possible role of PTEN’s polybasic N-terminal motif in vitro and in cells, a series of point mutations was generated, singly and in combination. These focused on conserved basic residues likely to be involved in anionic lipid binding and the tumour-derived mutations noted above (Figure 1). We avoided Arg-15, since mutation of this residue has previously been shown to compromise catalytic activity (I. Pass, N. R. Leslie and C. P. Downes, unpublished work, and [17,23]). All nine mutant proteins were initially expressed in bacteria and purified (Figure 2), although the quadruple point mutant K6A R11A K13A R14A was expressed rather poorly, probably due to effects on protein stability. PTEN protein expressed in, and purified from, bacteria should be in its non-phosphorylated, and hence active/open, state, allowing analysis of its association with membranes and its activity state, which would not be possible in cellular experiments. Each mutant was assayed against Ins(1,3,4,5)P3 in solution and PtdIns(3,4,5)P3, in neutral PtdCho vesicles. All the mutants retained robust phosphatase activity against both substrates, indicating that the targeted residues of this motif do not play important structural or catalytic roles (Figure 2B, and results not shown). Significantly, subsequent analysis indicated that many of these catalytically competent mutants were affected in the degree of activation by anionic lipids in vitro and displayed impaired function in cells. These effects upon PTEN function were most evident with the quadruple point mutant K6A R11A K13A R14A, and with the tumour-derived mutant K13E (see below, and results not shown). Because of this, and the noted effects of the quadruple mutation on protein stability, subsequent analyses concentrated upon the effects of the K13E mutation upon PTEN function. Importantly, PTEN K13E displays similar activity to the wild-type enzyme in vitro against the soluble substrate Ins(1,3,4,5)P3, indicating that the enzyme is not generally catalytically impaired (Figure 2C, and results not shown).
neutral PtdCho vesicles that also contain a low molar fraction of substrate phosphoinositide (below the interfacial $K_m$) [10]. PtdIns(4,5)$P_2$ vesicles also inhibit PTEN activity in vitro against its soluble substrate Ins(1,3,4,5)$P_4$ [the headgroup of PtdIns(3,4,5)$P_3$]. However, given the size of the N-terminal motif (less than 10 amino acids), and the promiscuous binding exhibited by similar basic motifs in other proteins [25], it seemed unlikely that an effect mediated through this small motif would display strong selectivity for PtdIns(4,5)$P_2$ over other acidic phospholipids. Therefore we tested whether another abundant cellular anionic phospholipid, PtdSer, could also elicit these effects. We found that increasing PtdSer concentration in PtdCho vesicles stimulated PTEN activity towards PtdIns(3,4,5)$P_3$ (Figure 3A); however, in comparison with PtdIns(4,5)$P_2$, the potency of response was significantly lower, with the maximal activation being in the order of 5-fold of the control at a mole fraction of 0.1, whereas PtdIns(4,5)$P_2$ generated a maximal 7-fold activation of the control at a mole fraction of 0.01. This seems likely to reflect the less acidic nature of the PtdSer headgroup relative to PtdIns(4,5)$P_2$. If the two lipids were incorporated into vesicles together, a response was obtained that was similar to that to PtdSer alone (Figure 3B), but interestingly lower than that to the corresponding concentration of PtdIns(4,5)$P_2$ alone. This suggests firstly that both lipids act through a common mechanism. Secondly, this small reduction in activity, along with the decreasing activity seen when each anionic lipid is increased well above the optimal concentration, is consistent with previous studies of other lipid-metabolizing enzymes. This suggests such a strong association with individual vesicles that the enzyme is not free to ‘hop’ between vesicles, but remains vesicle bound, acting in a ‘scooting’ mode of catalysis, as seen with some phospholipases and lipid kinases [26–28]. By contrast, in the absence of anionic lipids, PTEN displays relatively low affinity for PtdCho vesicles and has been shown clearly to ‘hop’ under these circumstances [10].

We also found that whereas neutral PtdCho vesicles had little effect upon PTEN activity towards Ins(1,3,4,5)$P_4$ (results not...
Figure 3 Anionic lipids modulate wild-type PTEN activity, but not that of PTEN K13E

(A, B) PTEN activity was measured using PtdCho (PC) vesicles containing a fixed mole fraction of PtdIns(3,4,5)P3 \(0.0001\) with 100,000 c.p.m. of \[^{32}P\]PtdIns(3,4,5)P3 and various mole fractions of PtdSer (PS; A) and/or PtdIns(4,5)P2 (PIP2; B). Assays were performed at 30 °C using 50 ng of wild-type (WT) PTEN or PTEN K13E for 10 min. Data are means ± S.E.M from three experiments performed in triplicate. (C, D) 50 ng of wild-type PTEN or PTEN K13E was assayed against PtdCho vesicles consisting of a fixed mole fraction of PtdIns(3,4,5)P3 (0.0001) and of PtdSer (0.1) or PtdIns(4,5)P2 (0.05) (C) or against 1 µM Ins(1,3,4,5)P4 (D). Data are means ± S.E.M from three experiments performed in triplicate.

shown), incorporation of PtdIns(4,5)P2 or PtdSer into these neutral vesicles caused a dramatic inhibition of PTEN activity towards the soluble substrate Ins(1,3,4,5)P4. Most significantly, the K13E mutation almost completely prevented both the enhancement of enzyme activity towards PtdIns(3,4,5)P2 and the inhibition of activity towards Ins(1,3,4,5)P4 caused by either PtdIns(4,5)P2 or PtdSer seen with wild-type PTEN (Figures 3C and 3D). These results strongly indicate a role for the N-terminal motif in mediating the effects on PTEN activity of anionic lipids, and that the inhibition of soluble substrate hydrolysis and the stimulation of vesicular substrate hydrolysis are likely to represent distinct manifestations of the same effect. The data also indicate that the inhibition of PTEN activity towards Ins(1,3,4,5)P4 by anionic lipids is not mediated by competition for active-site binding, as activity towards lipid substrates is enhanced under the same conditions. This inhibition of PTEN activity towards soluble substrates is quite striking (Figure 3D, [10], and results not shown) and suggests that the active site of PTEN may be oriented towards lipid substrates [8,29], or otherwise segregated from the aqueous environment, when bound to membrane surfaces.

It seems possible that anionic lipids could enhance the interfacial activity of PTEN through several mechanisms, including enhanced recruitment on to membrane surfaces and allosteric activation of the enzyme [9,10,17]. In order to assess whether the inclusion of anionic lipids enhanced the binding of PTEN to lipid vesicles, and whether this requires the N-terminal basic motif, we used a vesicle association assay. PtdCho vesicles were bound rather weakly by both wild-type and K13E PTEN, but for the wild-type enzyme only this binding was significantly enhanced by incorporation of PtdSer and PtdIns(4,5)P2 into the vesicles (Figure 4). Anionic lipid content had little effect upon the vesicle binding of PTEN K13E. However, in contrast with the phosphatase activity assays, the binding to vesicles containing PtdSer was greater than that to vesicles containing PtdIns(4,5)P2 (Figure 4). These data strongly support a model in which PTEN activity is enhanced towards substrates contained within vesicle or membrane surfaces that have a significant anionic content through greater association of the enzyme with the vesicle/membrane surface. Using rather different methods, data have been presented recently that also support this model [9]. However, it is still possible that PTEN is also allosterically activated at the membrane through interaction with acidic lipids.

The activation of PTEN by anionic lipids has also been addressed in vitro by Campbell et al. [17], based on the use of medium-chain-length (diC8) synthetic substrates and potential activators. However, these authors attributed the observed effects of diC8 PtdIns(4,5)P2 to a pure allosteric activation of PTEN, as they assumed the medium-chain lipids would be monomeric in solution. Our data, on the other hand, support a model in which non-substrate binding to vesicle/membrane surfaces favourably orients the active site for interaction with substrates that are endogenous components of the membrane. A diagnostic feature of this mechanism is that binding to vesicles concomitantly stimulates metabolism of vesicular substrates, but profoundly...
Functional significance of the PTEN N-terminus

Figure 4 PTEN K13E is impaired in its ability to bind to anionic lipid vesicles

PTEN K13E is impaired in its ability to bind to anionic lipid vesicles. Functional significance of the PTEN N-terminus

Figure 5 Effects of acyl-chain length on inhibition of PTEN Ins(1,3,4,5)P₄ phosphatase activity by PtdIns(4,5)P₂

Wild-type and K13E PTEN (250 ng) were assayed against 1 µM Ins(1,3,4,5)P₄ at 37 °C for 30 min in the presence of a range of concentrations of PtdIns(4,5)P₂ of the indicated acyl-chain length, or the corresponding head group Ins(1,4,5)P₃. PtdIns(4,5)P₂ lipids were sonicated prior to assay preparation. Each data point represents the mean ± S.E.M. from two experiments each performed in triplicate.

inhibits metabolism of a genuinely monodispersed substrate such as Ins(1,3,4,5)P₄. To test whether diC8 PtdIns(4,5)P₂ behaves as a pure allosteric activator or in an aggregated form as for long-chain lipids, its effects on Ins(1,3,4,5)P₄ metabolism by PTEN were analysed. The data in Figure 5 show that diC8 PtdIns(4,5)P₂ completely inhibited the Ins(1,3,4,5)P₄ 3-phosphatase activity of PTEN with an IC₅₀ of approx. 0.5 µM. For comparison we show inhibition by diC16 PtdIns(4,5)P₂, which gave an IC₅₀ of 0.1 µM, similar to values we reported previously [10]. By contrast, diC4 PtdIns(4,5)P₂ was an even weaker inhibitor. The difference in the observed potencies of diC8 and diC16 lipids is compatible with the probable greater solubility, and greater proportion in the monomeric form, of the former. In all of these experiments, K13E PTEN was far less sensitive to inhibition by PtdIns(4,5)P₂ than the wild-type enzyme. These data strongly support the regulation of PTEN activity by anionic lipids in these in vitro systems by enhanced recruitment and orientation to substrate-containing vesicles or micelles, rather than allosteric activation.

To address the role of the N-terminal motif in the cellular metabolism of PtdIns(3,4,5)P₃ by PTEN, we analysed the biological function of PTEN K13E in a number of established cellular assays. The PTEN-null cell line, U87MG, displays high basal levels of PKB activity and phosphorylation that can be reversed by exogenous expression of active PTEN. Basal PKB phosphorylation at Ser-473 is clearly detectable in control cells transfected with phosphatase-dead PTEN (C124S; Figure 6A). Although wild-type PTEN ablated PKB phosphorylation in this assay, PTEN K13E did not prevent PKB phosphorylation, even though it was expressed at a similar level to the wild-type protein. In a similar vein, we analysed the ability of several PTEN constructs to inhibit both anchorage-dependent and -independent proliferation of U87MG cells. In both of these assays, expression of wild-type PTEN reduced cellular proliferation, but phosphatase-dead PTEN did not. In each case, the ability of PTEN K13E was greatly impaired relative to the wild-type enzyme, having only a modest effect, if any, upon cell number (Figures 6B and 6C).

As discussed previously [25], relative to other phosphoinositides, due to the abundance and charge of PtdIns(4,5)P₂, it is a likely binding target of a short basic motif such as that found in PTEN. However, given the relative strength as activators of the
Figure 6 PTEN K13E is significantly impaired in its biological activity, yet targeting to the plasma membrane restores function

(A) U87MG cells were co-transfected with expression vectors for HA (haemagglutinin)-PKB and the indicated PTEN proteins. HA-PKB was immunoprecipitated and phosphorylation was assessed using antibodies specific for phospho-Ser-473 PKB (pS-PKB). IP, immunoprecipitation; WB, Western blot; Wt, wild-type. Similar results were obtained in two separate experiments. (B, C) The PTEN N-terminal motif is required for the inhibition of proliferation in soft agar (B) and in adherent conditions (C). U87MG cells were transfected with expression vectors for the indicated PTEN proteins, selected in G-418 and seeded either suspended in soft agar (B) or in 96-well plates (C). Cells were then incubated for 3 weeks (B) or 5 or 9 days (C) as shown. Data are presented as means ± S.E.M. of colony number from six replicates (B) and mean ± S.D. of cell number from 12 replicates (C) respectively. These experiments were performed twice with similar results. (D) U87MG cells were co-transfected with expression vectors for HA-PKB and for the indicated PTEN proteins, before PKB phosphorylation was determined as in (A). Myr is used to indicate PTEN proteins targeted to the plasma membrane by N-terminal myristoylation.

anionic lipids studied (Figure 3 and [10]), and their relative abundance in cells, it is very difficult to conclude whether the physiological ligand for the N-terminal basic motif is PtdIns(4,5)P_2 or a less charged, but more abundant, phospholipid such as PtdSer or PtdIns.

Our data indicate that the N-terminal basic motif of PTEN is required for its efficient recruitment to and activity on anionic lipid surfaces. A previous study found that PTEN proteins with mutations in this motif displayed generally reduced phosphatase activity [17]. In contrast, our experimental system indicates a role for this motif specifically in the enhancement of activity by anionic lipids. This supports a model in which PTEN activity is restricted to pools of substrate located in cell membrane compartments or subdomains that display an acidic character. This conclusion is supported by recent evidence that PTEN expression reduces substrate levels in the plasma membrane, but not the endoplasmic reticulum [31].

We thank Yvonne Lindsay for production of radiolabelled Ins(1,3,4,5)P_4. This work was supported by a studentship from the Medical Research Council (U.K.) to S.M.W. Work in C.P.D.'s laboratory is supported by the MRC (grant no. G9403619), Astra Zeneca, Boehringer Ingelheim, GlaxoSmithKline, Novo Nordisk and Pfizer.

REFERENCES


Received 1 December 2003/6 January 2004; accepted 7 January 2004
Published as BJ Immediate Publication 7 January 2004, DOI 10.1042/BJ20031839

© 2004 Biochemical Society