Analysis of the cellular functions of PTEN using catalytic domain and C-terminal mutations: differential effects of C-terminal deletion on signalling pathways downstream of phosphoinositide 3-kinase

Nick R. LESLIE^{1,2}, Alex GRAY¹, Ian PASS, Elaine A. ORCHISTON and C. Peter DOWNES

Division of Signal Transduction Therapy, Department of Biochemistry, University of Dundee, Dundee DD1 5EH, U.K.

The tumour suppressor protein, PTEN (phosphatase and tensin homolog deleted on chromosome 10), is a phosphatase that can dephosphorylate tyrosine-containing peptides, Shc, focal adhesion kinase and phosphoinositide substrates. In cellular assays, PTEN has been shown to antagonize the PI-3K-dependent activation of protein kinase B (PKB) and to inhibit cell spreading and motility. It is currently unclear, however, whether PTEN accomplishes these effects through its lipid- or protein-phosphatase activity, although strong evidence has demonstrated the importance of the latter for tumour suppression by PTEN. By using a PTEN G129E (Gly¹²⁹ → Glu) mutant that has lost its lipid phosphatase activity, while retaining protein phosphatase activity, we demonstrated a requirement for the lipid phosphatase activity of PTEN in the regulation of PKB activity, cell viability and membrane ruffling. We also made a small C-terminal deletion of PTEN, removing a putative PDZ (PSD95, Dlg and ZO1)-binding motif, with no detectable effect on the

phosphatase activity of the protein expressed in HEK293 cells (human embryonic kidney 293 cells) assayed *in vitro*. Surprisingly, expression of this mutant revealed differential requirements for the C-terminus in the different functional assays. Wild-type and C-terminally deleted PTEN appeared to be equally active in down-regulating PKB activity, but this mutant enzyme had no effect on platelet-derived growth factor (PDGF)-induced membrane ruffling and was only partially active in a cell viability assay. These results stress the importance of the lipid phosphatase activity of PTEN in the regulation of several signalling pathways. They also identify a mutation, similar to mutations that occur in some human tumours, which removes the effect of PTEN on membrane ruffling but not that on PKB.

Key words: phosphoinositide signalling, phosphatase, tumour suppressor, PDZ domain.

INTRODUCTION

PTEN (phosphatase and tensin homolog deleted on chromosome 10; also known as MMAC1 and TEP1) is the product of a tumour suppressor gene that is mutated in a wide range of sporadic human tumour types, particularly glioblastomas and cancers of the prostate and endometrium [1–3], and in the familial hamartoma syndromes Cowden disease [4,5] and Bannayan–Zonana syndrome [6]. Analysis of *PTEN* mutant mice confirms this tumour suppressor status, since homozygous mice exhibit early embryonic lethality, while heterozygotes have a greatly increased incidence of tumours [7–9].

PTEN is a member of the protein tyrosine phosphatase family. Although PTEN has detectable tyrosine phosphatase activity *in vitro*, particularly against acidic synthetic peptide substrates [10,11], it has also been shown to dephosphorylate phosphoinositide signalling molecules, including PtdIns(3,4,5)*P*₃, specifically dephosphorylating the 3 position of the inositol ring of [12,13]. In accordance with this role as a lipid 3-phosphatase, several studies have shown that PTEN can negatively regulate cellular levels of PtdIns(3,4,5)*P*₃, and the activity of the well characterized phosphoinositide-regulated protein serine/threonine kinase, protein kinase B (PKB), inducing apoptosis or cell cycle arrest [12–17]. Using tumour-derived PTEN mutants

lacking lipid phosphatase activity, but retaining protein phosphatase activity, it has also been shown that the lipid phosphatase activity of PTEN is required for tumour suppression [13,18]. Strong evidence that PTEN antagonizes phosphoinositide 3-kinase (PI-3K) functions *in vivo* also comes from genetic studies of the *Caenorhabditis elegans* PI-3K/PKB pathway [19]. Several recent reviews have highlighted the critical functional role of the lipid phosphatase activity of PTEN [20,21]. However, it is also possible that PTEN has cellular protein substrates; indeed there are precedents for other enzymes and proteins that utilize both lipid and protein targets, such as PI-3Ks [22]. PTEN appears to dephosphorylate focal adhesion kinase and Shc *in vitro* [23,24]. However, whether these proteins represent physiological targets remains to be determined.

In addition to the N-terminal phosphatase domain and a C2 domain [25], the amino acid sequence of the PTEN C-terminus corresponds closely to the consensus sequence bound by one class of PDZ (PSD95, Dlg and ZO1) domains [3,26]. Strong evidence suggests that PDZ domains mediate protein–protein interactions that can result in the formation of localized multi-protein complexes [27,28], including the recruitment of components of lipid signalling pathways to particular membranes [29,30]. Although most pathological mutations of PTEN reside in the phosphatase domain of the enzyme, C-terminal frameshifts

Abbreviations: PTEN, phosphatase and tensin homolog deleted on chromosome 10; GFP, green fluorescent protein; DMEM, Dulbecco's modified Eagle's medium; HEK293 cells, human embryonic kidney 293 cells; PKB, protein kinase B; PDZ, acronym of PSD95, Dlg and ZO1; FBS, foetal bovine serum; PI-3K, phosphoinositide 3-kinase; PDGF, platelet-derived growth factor; G129E etc., (Gly¹²⁹ → Glu) etc.

¹ These authors contributed equally to this work.

² To whom correspondence should be addressed (e-mail n.r.leslie@dundee.ac.uk).

and missense mutations have also been identified within the last 30 amino acids of the protein [31]. It has been proposed that PTEN may interact with the multi-PDZ domain protein 1, MUPP1 [20]. Although there are few studies that address the biological significance of the C-terminus of PTEN, deletion of the putative PDZ-targeting sequence partially interferes with the inhibitory effect of stably expressed PTEN on colony formation [32].

Although *C. elegans* PTEN (DAF-18) lacks C-terminal sequence conservation with human PTEN and PDZ-binding sequences [19], database searches reveal a PTEN homologue in *Xenopus laevis* and two ESTs from the zebrafish *Danio rerio* (AI437378 and AI416006), with very strong sequence homology to the C-terminus of PTEN. Taken together with analysis of mutations found in tumour samples, this suggests that a C-terminal domain in PTEN that is likely to interact with PDZ domains is conserved amongst vertebrates, and that PTEN–PDZ protein interactions may be both functionally conserved and pathologically significant.

EXPERIMENTAL

Cells

Tissue culture media and additives were provided by Gibco BRL. U87MG cells were obtained from the European Cell Culture Collection and were maintained in minimal essential medium/2mM glutamine/1 × non-essential amino acids (Life Technologies)/1mM sodium pyruvate/10% foetal bovine serum (FBS). Human embryonic kidney 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/10% FBS. Swiss 3T3 cells were maintained in DMEM/2mM glutamine/10% FBS. Cells were transfected using Fugene-6 (Roche Diagnostics) according to the manufacturer's recommendations.

Plasmids

A human PTEN cDNA was obtained by PCR from a human brain cDNA library and cloned into pCR2.1-TOPO (Invitrogen). Mutations were introduced into PTEN using the QuikChange[®] site-directed mutagenesis kit (Stratagene) with the following mutagenic oligonucleotides: C124S-S 5' GTTGCAGCAATT-CACAGTAAAGCTGGAAAGGGACG, C124S-A 5' CGTC-CCTTTCCAGCTTTACTGTGAATTGCTGCAAC, G129E-S 5' CTGTAAAGCTGGAAAGGAACGAACTGGTGTAAATG, G129E-A 5' CATTACACCAGTTCGTTCCCTTTCCAGCTTT-ACAG, STOP-S 5' GATGAAGATCAGCATACATGAAAT-TACAAAAGTCTGAAAGG, STOP-A 5' CCTTTCAGACT-TTTGTAATTTTCATGTATGCTGATCTTCATC. For expression in mammalian cells, PTEN cDNAs were cloned into the G418-resistant green fluorescent protein (GFP) fusion vector, pEGFP-C2, (ClonTech) as *Eco*RI restriction enzyme fragments, resulting in expression of fusion proteins with GFP being N-terminal. All PTEN expression constructs have been fully sequenced. An expression vector for haemagglutinin-tagged PKB in pCMV5 was kindly provided by Anu Balendran and Dario Alessi (Biochemistry Department, University of Dundee, Dundee, U.K.), and has been described previously in [33].

Assay of U87MG cell numbers expressing PTEN

U87MG cells and human embryonic kidney 293 cells (HEK293 cells) cells growing in 6-well plates were simultaneously transfected with 1 µg of various GFP-PTEN expression constructs, also encoding G418 resistance. Cells were maintained in 10%

serum and initial transfected cell numbers were calculated after 12 h and 20 h by fluorescence microscopy. Transfected cell numbers were measured as the number of visibly green cells per microscope field. Total cell numbers did not vary significantly between samples and transfection efficiency with GFP alone or PTEN mutants was always between 5% and 10% into U87MG cells. 20 h after transfection, U87MG cells were incubated in fresh medium with 10% serum and 400 µg/ml G418 (Life Technologies) and transfected cell numbers counted again at 4 days and 7 days post-transfection.

Analysis of PKB phosphorylation

U87MG cells, growing at approx. 20% confluence in 15 cm dishes, were co-transfected with 100 ng of HA-PKB expression vector and 200 ng of GFP or GFP-PTEN vectors. Cells were maintained in 10% FBS-containing medium for 15 h before preparation of cell lysates. The lysis buffer consisted of 50 mM Tris (pH 7.4), 150 mM sodium chloride, 1% Nonidet P40, 0.5% deoxycholate, 1 mM sodium orthovanadate, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 10 µg/ml leupeptin, 100 µM PMSF and 1 mM benzamidin. Lysates were precleared by centrifugation at 15000g and 4 °C for 30 min and by incubation with Protein-G Sepharose for 1 h at 4 °C. Cleared lysates were then incubated at 4 °C for 1 h with 2 µg of anti-HA antibody (Roche Diagnostics) pre-bound to Protein-G–Sepharose. Immune complexes were washed twice in lysis buffer, and twice in lysis buffer with 500 mM lithium chloride, before analysis by SDS/PAGE and Western blotting with antibodies against phosphoserine-473 PKB. Antibodies against total PKB were used to verify the efficiency of expression and immunoprecipitation. SDS/PAGE and Western blotting with PolyScreen PVDF membrane (NEN, Boston, MA, U.S.A.) followed standard protocols and manufacturer's instructions. Co-expression of GFP-PTEN was determined by Western blotting of cell lysates with anti-PTEN antibodies (Santa Cruz). Antibodies against phospho-PKB and total PKB have been described previously [34].

Immunoprecipitate phosphoinositide phosphatase assay

HEK293 cells were transfected with 1 µg of GFP-PTEN expression vectors and cell lysates prepared after 24 h. Lysis buffer and preclearing were as described above. Cleared lysates were then incubated at 4 °C for 1 h with 2 µg anti-GFP antibody (Roche Diagnostics) pre-bound to Protein-G–Sepharose. Immune complexes were washed twice in lysis buffer, twice in lysis buffer with 500 mM lithium chloride, and twice in react/wash buffer [10 mM Tris (pH 8.0), 50 mM sodium chloride]. Samples were then divided for assay of phosphatase activity against 3-³³P-labelled Ins(1,3,4,5)P₄, and for electrophoresis and Western blotting analysis against PTEN. PTEN activity against Ins(1,3,4,5)P₄ was assayed over 1 h at 37 °C in 50 mM Tris (pH 8.0), 10 mM dithiothreitol, 1 mM EGTA, and 1 µM unlabelled Ins(1,3,4,5)P₄, in a final volume of 50 µl with shaking. Reactions were terminated with 1 M perchloric acid, and released ³³P-labelled inorganic phosphate was analysed as described previously [13]. Antibodies against PTEN [used at 1:1000 (v/v) dilution] and GFP [used at 1:5000 (v/v) dilution] were provided by Santa Cruz and Roche Diagnostics respectively.

Membrane ruffling

For the membrane ruffling assay, Swiss 3T3 cells were grown to 60% confluence on glass coverslips in DMEM supplemented

with 10% FCS. Cells were transfected with 1 μ g of construct DNA per coverslip using Eugene-6 in accordance with the manufacturer's instructions (Roche Diagnostics). After 12 h the cells were serum starved for 4 h in DMEM supplemented with 0.5% BSA and subsequently transferred to modified Krebs buffer [25 mM Hepes (pH 7.4), 118.4 mM NaCl, 4.7 mM KCl, 1.18 mM MgSO_4 , 1.29 mM CaCl_2 , 1.18 mM K_2HPO_4 , 11.7 mM glucose], before stimulation with 10 ng/ml platelet-derived growth factor (PDGF) for 10 min at 37 °C. Cells were then fixed in 3% paraformaldehyde solution in PBS for 10 min, washed three times in PBS, permeabilized with 1% (w/v) Triton X-100 in PBS for 5 min, washed three times in PBS, before staining polymerized actin with 0.1 μ g/ml Rhodamine Phalloidin (Sigma) in PBS for 5 min. Cells were then washed three times in PBS and mounted for confocal microscopy. Confocal images of the cells were collected using a Bio-Rad confocal microscope system.

RESULTS

Three PTEN mutants were used to analyse the requirements for the lipid phosphatase activity and C-terminus of PTEN in different cellular assays. The C124S (Cys124 $\rightarrow$ Ser) mutation of the catalytic cysteine eradicates all detectable phosphatase activity [11–13], whilst the G129E (Gly¹²⁹ $\rightarrow$ Glu) mutation sub-

stantially depletes the lipid phosphatase activity without affecting protein phosphatase activity, as indicated by assays utilizing $\text{PtdIns}(3,4,5)\text{P}_3$ or poly(Glu,Tyr-P) respectively [13,18]. The Q399STOP mutation results in the deletion of the last five amino acids of the protein, which is predicted to abolish any PDZ domain-mediated protein–protein interactions.

We have used mutant PTEN proteins tagged at their N-termini with green fluorescent protein (GFP) to analyse the effects of removing the lipid phosphatase activity and the C-terminus of the protein on cell survival, regulation of PKB activity and growth factor-induced membrane ruffling responses. To assess their inositol 3-phosphatase activities, these proteins were transiently expressed in HEK293 cells, immunoprecipitated with anti-GFP antibodies, and assayed using $3\text{-}^{32}\text{P}$ -labelled $\text{Ins}(1,3,4,5)\text{P}_4$ as the substrate. The results in Figure 1 show that expression of equivalent levels of the different PTEN constructs resulted in comparable levels of activity in immunoprecipitates from wild-type and PTEN 399Stop transfected cells, indicating that the GFP tag does not ablate 3-phosphatase activity and that the C-terminal truncation has no effect on this activity. As expected, no detectable activity was recovered from cells expressing the C124S (Figure 1) or G129E (results not shown) mutations.

It has previously been shown that introduction of PTEN into certain tumour cells lacking expression of the protein results in inhibition of cell proliferation through induction of either apoptosis or cell cycle arrest, while introduction into wild-type cells did not [13–15,18,35–37]. We transiently expressed GFP–PTEN fusion proteins in the previously described glioblastoma-derived PTEN null cell line U87MG [1,2] and in HEK293 cells, which express endogenous wild-type PTEN. Transfected 293 cell numbers were counted after 20 h to ensure even transfection efficiency with each construct (Figure 2). U87MG cell numbers expressing GFP–PTEN fusion proteins were counted after 12 h, 20 h, 4 days and 7 days. To allow expansion of transfected cell numbers, untransfected cells were selected against by addition of 400 μ g/ml of G418 20 h after transfection. Confirming previous data, expression of wild-type PTEN caused a dramatic reduction in the number of detectable transfected cells from 20 h onwards (Figure 2), compared with the expression of GFP alone or GFP–PTEN C124S. Expression of the G129E mutation of PTEN did not lead to a reduction in the survival of transfected cells, indicating that this effect of PTEN requires the lipid phosphatase, but not the tyrosine phosphatase activity. Although the initial transfection efficiency with the C-terminally truncated construct was similar to that with GFP alone and the active-site PTEN mutants, C124S and G129E, the number of cells expressing GFP–PTEN 399Stop consistently fell sharply over a period of several days. These results suggest that C-terminal deletion substantially reduces, but does not remove, the ability of PTEN to prevent the survival of U87MG cells.

In accordance with its activity *in vitro* as a lipid 3-phosphatase-metabolizing $\text{PtdIns}(3,4,5)\text{P}_3$ and $\text{PtdIns}(3,4)\text{P}_2$, PTEN can down-regulate the cellular activity of PKB/Akt, and cells lacking PTEN have a constitutively high level of PKB activity [13,15–17,38]. We therefore investigated the phosphorylation of PKB in unstimulated U87MG cells transfected with expression vectors for wild-type PTEN and C124S, G129E and 399Stop mutants. A previously characterized phospho-specific antibody against phosphoserine 473 PKB was used as an indication of PKB activity [34]. As described by other workers, U87MG cells exhibited a high basal level of PKB phosphorylation that was increased only slightly by stimulation with either serum or insulin (results not shown; [13,17]). The expression of wild-type PTEN dramatically reduced the level of PKB phosphorylation,

Figure 1 PTEN 399Stop retains 3-phosphatase activity against $\text{Ins}(1,3,4,5)\text{P}_4$

HEK293 cells were transfected in duplicate with expression vectors for GFP or GFP-PTEN proteins. 24 h post-transfection, lysates were prepared and expressed proteins immunoprecipitated with anti-GFP antibodies. Immune complexes were divided then assayed for phosphatase activity against $\text{Ins}(1,3,4,5)\text{P}_4$ and analysed by anti-GFP Western blotting (WB). Lanes 1 and 2, GFP; 3 and 4, GFP-PTEN wild-type; 5 and 6, GFP-PTEN C124S; 7 and 8, GFP-PTEN 399Stop; 9 and 10, no enzyme through assay.

Figure 2 Effect of PTEN genotype on U87MG transfected cell numbers

Expression vectors for GFP and GFP-PTEN fusion constructs were transfected into U87MG and HEK293 cells, and transfected cell numbers expressing GFP and GFP-PTEN were measured by fluorescence microscopy. Transfected cell numbers were determined for U87MG cells after 12 h, 20 h, 4 days and 7 days. After 20 h post-transfection, these cells were incubated in the presence of 400 μ g/ml G418. Data are presented as the mean GFP-expressing cell number relative to the GFP 20 h data point with standard errors. Each data point shown for U87MG cells is from three pooled experiments, representing at least five transfections and 40 measurements. HEK293 cells were counted after 20 h and data points are presented as mean GFP-expressing cell number per microscope field from 10 measurements from one representative experiment.

whilst expression of PTEN G129E lacking lipid phosphatase but retaining tyrosine phosphatase activity, and phosphatase dead PTEN C124S, did not (Figure 3A). Surprisingly, however, in this assay, expression of PTEN 399Stop reduced the level of PKB phosphorylation to the same degree as that of the wild-type enzyme. Experiments in which reduced amounts of expression vector for PTEN wild-type and PTEN 399Stop were used still showed no difference in the regulation of PKB between these two PTEN proteins; only when greatly reduced quantities of either construct was used, did PKB phosphorylation decrease and approach that in control cells (Figure 3B).

The lipid products of PI-3Ks have been shown to regulate a wide variety of cellular enzymes and processes, and although attention has thus far focussed on PKB, it is important to establish whether PTEN can inhibit other PI-3K-dependent responses, especially as some functions of PI-3K appear to result from its protein kinase activity [22]. Membrane ruffling is a response of fibroblasts and epithelial cells to growth factors that reflects a coordinated polymerization of actin filaments at the cell periphery. This response has been analysed in most detail in Swiss 3T3 cells responding to PDGF and is thought to require the sequential involvement of PI-3K and activation of the Rho family GTPase, Rac1 (via a PI-3K-dependent guanine nucleotide exchange protein), but not PKB [39,40]. We therefore

Figure 3 Regulation of PKB activity by PTEN

(A) U87MG cells were co-transfected with expression vectors for haemagglutinin (HA)-tagged PKB and each GFP-PTEN protein. 18 h after transfection unstimulated cells were lysed and transfected PKB was immunoprecipitated (IP) with anti-HA antibodies. This HA-PKB was then analysed by Western blotting (WB) with an anti-phosphoserine-473 antibody and an antibody against total PKB. Expression of GFP-PTEN proteins was confirmed by anti-PTEN Western blot analysis of cell lysates. (B) Cells were similarly co-transfected with HA-PKB and with GFP or GFP-PTEN expression vectors. Cells were transfected with 200 ng, 40 ng, 8 ng and 1.6 ng of expression vector for PTEN wild-type (wt) and PTEN399Stop and the total quantity of expression vector was maintained at 200 ng by addition of GFP vector. One dish of GFP-transfected cells was treated with the PI-3K inhibitor wortmannin (100 nM) for 30 min before lysis.

investigated the sensitivity of this response to the over-expression of PTEN and PTEN mutants as GFP fusion proteins.

Membrane ruffling was assessed by confocal microscopy of fixed cells, labelled for filamentous actin with Rhodamine-conjugated Phalloidin, as shown in Figure 4 (red channel). Stimulation with PDGF resulted in the appearance of extensive ruffles defined as areas of the plasma membrane which stain intensely for polymerized actin and which detach from the surface. Transient expression of GFP and GFP-tagged proteins resulted in about 5–10% of the cells expressing significant amounts of protein as indicated by fluorescence intensity in the green channel. The effects of PTEN expression on the ruffling response could then be determined by comparing the morphologies of expressing and non-expressing cells in the same field of view. Thus the expression of wild-type GFP-PTEN ablated the ruffling response to PDGF as seen by comparing the polymerized

Figure 4 Effect of PTEN on PDGF stimulated membrane ruffling

Green (GFP) and red (actin Phalloidin-stained) images of the same field of view for unstimulated cells (control) and cells stimulated with PDGF (PDGF) in cells transfected with wild-type PTEN and the three mutants C124S, G129E and 399Stop. The GFP-transfected cells in the red images are marked * and membrane ruffles are arrowed.

actin distribution in expressing cells (showing green) versus non-expressing cells (Figure 4). By making similar comparisons it can be seen that catalytically inactive inositol 3-phosphatase, but not tyrosine phosphatase inactive PTEN mutants, lack this inhibitory effect on membrane ruffling. GFP-PTEN 399Stop,

when over-expressed at comparable levels to that of wild-type transfectants, had no apparent effect on the ruffling response. In order to provide a more quantitative assessment of the effects of the PTEN constructs, the percentage of transfected cells which were observed to be ruffling 10 min after addition of PDGF was

Table 1 Percentage of transfected cells ruffling in response to PDGF stimulation for each of the GFP-PTEN fusion proteins used in this study

In each case the data are shown as mean $\pm$ S.E.M. of triplicate independent experiments, in which 100 transfected cells were scored for membrane ruffling.

Vector	% of transfected cells ruffling
Wild-type	20.5 $\pm$ 0.4
C124S	78.0 $\pm$ 0.8
G129E	74.0 $\pm$ 0.9
Q399STOP	79.0 $\pm$ 1.6
GFP control	76.0 $\pm$ 1.4

determined. The results in Table 1 clearly establish the significant impact of wild-type PTEN on ruffling and the lack of any discernible effect of any of the mutant PTEN proteins used in this study.

DISCUSSION

PTEN is a member of the protein tyrosine phosphatase superfamily which exhibits phosphoinositide 3-phosphatase and phosphotyrosine phosphatase activities, the latter being directed towards highly acidic peptides such as poly(Glu₄-Tyr-P). We, and others, have identified point mutations that selectively abrogate the lipid phosphatase activity without detectable effect upon the tyrosine phosphatase activity of PTEN. Although expression of PTEN in a variety of cell lines has been shown to antagonize PKB signalling and cell motility that both require the activity of PI-3K [16,17,24], it has not been established that these effects result exclusively from the lipid phosphatase activity of PTEN. We have now made use of three cellular assay systems and a range of PTEN mutants to establish firmly that PTEN antagonizes PI-3K signalling primarily via expression of phosphoinositide 3-phosphatase activity. Conversely, the results establish the importance of the lipid products of PI-3K, as opposed to protein substrates, in these responses. Thus PTEN expression drastically reduced the viability of U87MG cells which lack endogenous PTEN, decreased the activity state of PKB in these cells, and blocked membrane ruffling in Swiss 3T3 cells upon exposure to PDGF. In each case, PTEN was rendered ineffective both by mutation of the active-site cysteine residue to serine (C124S) or by mutation of glycine-129 to glutamic acid (G129E), the latter blocking the lipid phosphatase, but not the tyrosine phosphatase activity *in vitro*.

We have also analysed the effects in these assays of making a small deletion of the C-terminal, putative PDZ domain targeting sequence and found that this deletion effected the function of PTEN in these cellular assays in a differential manner. Since evidence strongly suggests that small deletions of the PTEN C-terminus do not affect the activity of the enzyme *in vitro* ([41]; Figure 1), it seems likely that in these cellular assays the effects of this deletion are caused by a lack of enzyme targeting through PDZ domain-mediated interactions. At one extreme, the PTEN399Stop mutation had no detectable effect on the ability of the expressed protein to reduce the activity state of PKB in U87MG cells. This might indicate that PDZ-mediated targeting of PTEN is not required to dephosphorylate the PtdIns(3,4,5)P₃ and/or PtdIns(3,4)P₂ that is required to maintain PKB in an activated state in these cells.

The results obtained analysing PTEN-transfected cell numbers might support the above proposal. We analysed the numbers of

visibly green cells expressing GFP fusion proteins from 12 h to 7 days after transfection with different expression constructs. There were dramatically fewer green cells after expression of wild-type PTEN compared with each of the mutant PTEN proteins. As this difference is clear 20 h after transfection, it could not be caused only by reduced cellular proliferation caused by cell cycle arrest, as shown by other studies using longer times. It would seem that this difference must be due either to apoptosis of cells expressing high levels of wild-type PTEN or other factors affecting the expression of GFP-PTEN in cells transfected with high levels of expression vector. Previous studies have demonstrated that PTEN can both induce apoptosis and interfere with gene expression; indeed it is possible that the transfection process or G418 treatment could act as apoptotic stimuli [15,35,38].

Although expression of GFP-PTEN399Stop had little effect on the number of green cells observed after 12 or 20 h, it progressively reduced the recovery of such cells, compared with GFP-expressing controls, so that after 7 days this value was reduced to only 40%. This suggests that truncation of the C-terminal PDZ domain-binding sequence substantially, but not completely, inhibits PTEN function in this assay. This result is interesting in view of the effect of PTEN399Stop on PKB activity, suggesting that an elevated level of PKB activity is not required for cell viability in these cells in the first 20 h of this experiment. As PDZ domain interactions have been shown to result in the recruitment of proteins to specific cellular locations, it might be expected that the subcellular distribution of GFP-PTEN would be altered by deletion of the C-terminus. That no evidence for this prediction was observed may reflect the masking of any localization by the relatively high levels of protein expression that are obtained in these transient expression studies.

At the other extreme were the effects of expression of GFP-PTENStop on membrane ruffling in response to stimulation of Swiss 3T3 cells with PDGF. In this assay, which is thought to reflect a pathway involving PI-3K-dependent activation of Rac that is independent of PKB [39,42], the effects of PTEN were completely blocked by deletion of the C-terminus. This result, together with the similar lack of effect of the G129E mutant, implies that the C-terminus of PTEN via its presumed interaction with PDZ domain-containing protein(s) is required for efficient access to activators or its signalling phospholipid substrates.

There are several possible explanations for the efficacy of PTEN399Stop in the PKB assay but not the ruffling assay. Membrane ruffling responses appear to be significantly more sensitive to agonists acting via PI-3K than the activation of PKB [39,42–44]. Thus if PTEN399Stop is less efficient than wild-type, it might reduce PtdIns(3,4,5)P₃ levels sufficiently to inhibit PKB phosphorylation, but not membrane ruffling. Although titration of the quantity of PTEN expression vector and analysis of PKB phosphorylation (Figure 3B) suggests that in this assay 399Stop is no less efficient than the wild-type enzyme, this effect could be due in part to protein expression in a lower fraction of cells rather than reduced expression per cell. This problem is currently being addressed by construction of stable, inducible PTEN-expressing cell lines.

It is also possible, however, that the different sensitivities to C-terminal deletion observed in the different assays reflect qualitative differences in the requirements for PDZ domain-mediated targeting to antagonize different cellular responses involving PI-3 kinases. We chose to analyse the effects of PTEN on membrane ruffling because PKB and Rac-induced responses are thought to represent distinct pathways downstream of PI-3K, and it seemed quite likely that the effects of PTEN on these responses might have different requirements for PDZ domain-dependent targeting. The apparently absolute requirement for an intact PTEN

C-terminus in the regulation of membrane ruffling is particularly interesting in view of the fact that this response is a highly spatially co-ordinated process that is likely to require tightly regulated localization of the participating components. Another possibility is that, as there are a growing number of PI-3K catalytic and regulatory subunits [45,46], several of which may be found in individual cell types, 3-phosphoinositides may be produced at distinct locations for different purposes, depending on the species of PI-3K involved. Thus PDZ domain-based targeting of PTEN might be required to antagonize some, but not all PI-3K dependent responses. In light of the occurrence of pathological C-terminal deletions of PTEN and our results showing that similar mutants retain the ability to down-regulate PKB activity, this raises the interesting possibility that in some tumour types regulation of PKB is not sufficient for full tumour suppression by PTEN. This would agree with the proposed role for Rac not PKB downstream of PI-3K in cellular invasion [47].

Some support for a requirement for targeting in some functions, but not others, comes from comparisons between PTEN and its homologues in vertebrate and non-vertebrate species. For example, *C. elegans* PTEN (DAF-18) lacks the C-terminal consensus PDZ domain-binding sequence, but has been shown genetically to act upstream of PKB in the PI-3K-dependent insulin receptor-like signalling pathway in this organism [19]. It is possible, therefore, that PDZ domain-based targeting is not required for down-regulation of PKB in mammalian cells as our results suggest, but has evolved to facilitate the regulation of other lipid signalling responses. The isolation of PTEN-binding proteins and analysis of their effects on targeting and the functional consequences of PTEN expression will contribute to this debate.

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