Targeting mutants of PTEN reveal distinct subsets of tumour suppressor functions

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The tumour suppressor protein PTEN (phosphatase and tensin homolog deleted on chromosome 10) is a lipid phosphatase which can antagonize the phosphoinositide 3-kinase (PI 3-kinase) signalling pathway, promoting apoptosis and inhibiting cell-cycle progression and cell motility. We show that very little cellular PTEN is associated with the plasma membrane, but that artificial membrane-targeting of PTEN enhances its inhibition of signalling to protein kinase B (PKB). Evidence for potential targeting of PTEN to the membrane through PDZ domain-mediated protein–protein interactions led us to use a PTEN enzyme with a deletion of the C-terminal PDZ-binding sequence, that retains full phosphatase activity against soluble substrates, and to analyse the efficiency of this mutant in different cellular assays. The extreme C-terminal PDZ-binding sequence was dispensable for the efficient down-regulation of cellular PtdIns(3,4,5)P₃ levels and a number of PI 3-kinase-dependent signalling activities, including PKB and p70S6K. However, the

PDZ-binding sequence was required for the efficient inhibition of cell spreading. The data show that a PTEN mutation, similar to those found in some tumours, affects some functions of the protein but not others, and implicate the deregulation of PTEN-dependent processes other than PKB activation in the development of some tumours. Significantly, this hypothesis is supported by data showing low levels of PKB phosphorylation in a glioblastoma sample carrying a mutation in the extreme C-terminus of PTEN compared with tumours carrying phosphatase-inactivating mutations of the enzyme. Our data show that deregulation of PKB is not a universal feature of tumours carrying PTEN mutations and implicate other processes that may be deregulated in these tumours.

Key words: glioblastoma, PDZ domains, phosphatase, phosphoinositide, protein kinase B.

INTRODUCTION

It is widely accepted that the development of human cancers requires the functional inactivation of tumour suppressor proteins. The PTEN (phosphatase and tensin homolog deleted on chromosome 10) tumour suppressor is mutated in a wide range of sporadic tumour types, particularly glioblastomas and endometrial carcinomas [1,2]. This tumour suppressor status is confirmed by knockout studies in mice, in which heterozygotes are highly susceptible to a range of tumour types, and by the association of germline mutations in PTEN with the inherited human hyperproliferative disorders, Cowden's disease and Bannayan–Riley–Ruvalcaba syndrome [3–5].

PTEN is a member of the protein tyrosine phosphatase family, with activity *in vitro* against both peptide and phosphoinositide substrates, specifically dephosphorylating the 3-position of the inositol ring in the latter [6–9]. Substantial evidence suggests that the tumour suppressor status of PTEN relies on its normal cellular activity as a lipid phosphatase directly dephosphorylating the phosphoinositide signalling molecule PtdIns(3,4,5)P₃ and possibly PtdIns(3,4)P₂ ([8,10,11] and reviewed in [3,4]). This activity reduces the cellular levels of PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂, and thus directly antagonizes the action of phosphoinositide 3-kinase (PI 3-kinase) enzymes and inhibits a number of signalling pathways, including protein kinase B (PKB)/Akt [10–13], Rac and cdc42 [14], and cellular processes including proliferation [15,16], cell survival [10], cell spreading, motility [17] and membrane ruffling [18]. Several studies have shown that both the N-terminal phosphatase domain and the

adjacent C2 domain are required for enzyme activity, since any truncation mutations N-terminal to the end of the C2 domain at approximately amino acid 351 led to a loss of all detectable phosphatase activity [19,20].

In order to access its substrates (and possibly for additional reasons), it seems likely that PTEN must be targeted to cell membranes, and two potential mechanisms of membrane targeting have been identified. It has recently been shown that PTEN has a calcium-independent C2 domain which is able to bind to lipid vesicles *in vitro* [21]. Furthermore, the extreme C-terminus of PTEN can bind to a number of other proteins through interactions mediated by PDZ domains within these proteins. PTEN can thus interact with the membrane-associated guanylate kinase ('MAGUK') proteins DLG, MAGI-1, -2 and -3, the microtubule-associated serine/threonine protein kinase MAST-205, and multi-PDZ-domain protein 1 ('MUPP1') [3,22–24]. Interestingly, transforming oncoproteins from several tumour viruses have also been shown to bind to the second PDZ domain of DLG, itself a homologue of the discs of large tumour suppressor in flies [25]. Evidence that lipid signalling molecules are not uniformly distributed throughout the cell membrane and for the functional significance of this specificity [26,27] stresses the importance of correctly localizing molecules that play roles in lipid signalling.

In the present study we present evidence for the functional significance of PTEN membrane targeting and, in one experimental system, derive data that show a requirement for the extreme C-terminus in the regulation by PTEN of cell spreading, but not PKB activation. Since similar mutations are found in

Abbreviations used: PTEN, phosphatase and tensin homolog deleted on chromosome 10; GFP, green fluorescent protein; PKB, protein kinase B; PI 3-kinase, phosphoinositide 3-kinase; GSK3 α , glycogen synthase kinase-3 α ; FKHR, forkhead in rhabdomyosarcoma; PDGF, platelet-derived growth factor; C124S etc., (Cys¹²⁴ $\rightarrow$ Ser).

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tumours, this implicates processes other than PKB deregulation, that may include cytoskeletal rearrangements characterized by cell spreading, in the development of this subset of tumours. Finally, we present data directly from glioblastoma tumour samples supporting this hypothesis that different PTEN mutations can lead to the deregulation of subsets of signalling pathways predicted to have different pathological significance.

EXPERIMENTAL

Cell culture

Tissue culture media and additives were provided by Gibco BRL Life Technologies. U87MG cells, obtained from the European Collection of Animal Cell Cultures, were maintained in minimal essential medium, plus 2 mM glutamine, 1 × non-essential amino acids, 1 mM sodium pyruvate and 10% fetal bovine serum. HEK-293 cells were maintained in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum. Recombinant rat platelet-derived growth factor (PDGF-BB) was purchased from Sigma. Fibronectin and collagen S were purchased from Life Technologies and Roche respectively. Fibronectin-coated tissue culture dishes, purchased from Becton Dickinson, were used in cell-spreading assays. Expression vectors were introduced into U87MG cells using either FuGENE-6 (Roche), according to manufacturer's recommendations, or a novel baculoviral delivery system adapted for mammalian expression (A. Gray, unpublished work).

Antibodies

Mouse monoclonal antibodies against the C-terminus of PTEN (A2B1) and green fluorescent protein (GFP) were purchased from Santa Cruz Biotechnology and Roche respectively. Antibodies raised against the PH domain of PKB, phosphoserine-473 PKB, glycogen synthase kinase-3 α (GSK3 α), phosphoserine-21 GSK3 α and p70S6K have been described previously [28,29]. Antibodies to the forkhead in rhabdomyosarcoma (FKHR)L1 transcription factor (total and phosphothreonine-32) were purchased from Upstate Biotechnology, Lake Placid, NY, U.S.A. An antibody recognizing phosphothreonine-389 p70S6K was purchased from New England Biolabs (Hitchin, Herts., U.K.). Immunoprecipitation experiments from tumour samples used a rabbit polyclonal antibody against PKB purchased from Santa Cruz Biotechnology.

Mutagenesis and construction of expression vectors

GFP-PTEN wild-type, C124S, G129E and 399Stop constructs were based on pEGFP-C2 (Clontech Laboratories) and have been described previously [18]. The M-CBR3 mutation was as first described in [21], and was produced by PCR mutagenesis of wild-type GFP-PTEN using the mutagenic primers CBR3-S (5'-CTTCCACAAACAGAACGCAGCAGGAGCCGCAGACGCAATGTTTCACTTTTGGG-3') and CBR3-A (5'-CCCAAAAGTGAACATTGCGTCTGCGGCTCCTGCTGCGTTCTGTTTGTGGAAG-3'). The N-terminal myristoylation and palmitoylation signal from Lck, as described in [30], was added to the N-terminus of untagged PTEN using the primers Myr-S (5'-TCGAGCCATGGGCTGCGGTTGTTCTTCCACCTGAGGATTACCCATACGACGTCCACGAC-TACGCTTCA-3') and Myr-A (5'-AGCTTGAAGCGTAGTCTGGGACGTCGTATGGGTAATCCTCAGGGTGGGAA-GAACAACCGCAGCCCATGGC-3'). The PTEN X404L mutation, as described in [31], was produced as a PCR product from a wild-type PTEN cDNA using an upstream primer and X404L-A (5'-CCTTATCCCTCTTGATAAAAAAATTAA-

GACTTTTGTAAATTTG-3') and introduced into the cloning vector pCR2.1 TOPO (Invitrogen) following the manufacturer's instructions. Vectors for bacterial expression as GST fusion proteins were produced from pGEX2T-PTEN wild-type [7] by restriction endonuclease cloning.

Cellular fractionation

For fractionation, adherent cells were washed in PBS before removal from plates with a rubber cell scraper. Cells were pelleted at 1000 *g* for 20 s, before resuspension in hypotonic buffer [20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA and 'complete' protease inhibitor cocktail (Roche)] and disruption by 12 passages through a gauge-25 syringe needle. Unlysed cells and nuclei were removed by centrifugation at 1000 *g* for 10 min at 4 °C, and the supernatant was further centrifuged at 100 000 *g* and 4 °C for 45 min. Both supernatant and pellet were made up with SDS-containing sample buffer for electrophoresis of both fractions derived from the same original quantity of total cellular protein, and analysis by Western blotting. The efficiency of the fractionation process was verified by Western blotting with antibodies raised against the endogenous epidermal growth factor receptor (Upstate Biotechnology) and glyceraldehyde-3-phosphate dehydrogenase (Biogenesis, Poole, Dorset, U.K.) and transfected GFP in parallel samples. Finally, membranes were stained with Coomassie Blue to verify the relative protein content of each fraction.

Phosphoinositide phosphatase assays

GST-tagged PTEN proteins were expressed in *Escherichia coli* strain BL21 and purified as previously described [7]. Assays were conducted at 37 °C in 50 mM Tris, pH 8.0, 10 mM dithiothreitol, 1 mM EGTA and 1 μ M unlabelled Ins(1,3,4,5) P_4 using 3-[32 P]Ins(1,3,4,5) P_4 (prepared according to the method described in [32]) as a substrate. After 20 min, assays were terminated in 1 M HClO $_4$ /1% ammonium molybdate, mixed with 2 vol. of toluene/isobutanol (1:1), and the aqueous phase was counted for released [32 P]phosphate.

Analysis of lipid levels

Cells were labelled with [3 H]inositol in inositol-free medium (NEN Life Science Products) for 48 h, prior to lysis in 0.5 M HClO $_4$ and extraction of phospholipids with methanol/chloroform/12 M HCl (80:40:1). The lower phase was dried and de-acylated in methylamine for 30 min at 53 °C, and the glycerophosphoinositol phosphates then resolved on an anion-exchange column (25 cm Partisphere, 5 μ m, SAX; Whatman), with an increasing gradient of NH $_4$ H $_2$ PO $_4$ (pH 3.8) from 0 to 1.0 M over 105 min [33]. Fractions were collected and counted for radioactivity.

Immunoprecipitation, Western blotting and PKB assays

Cells were lysed in 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Nonidet P40, 0.5% deoxycholate, 0.1% 2-mercaptoethanol, 1 mM sodium orthovanadate, 1 mM EDTA, 1 mM EGTA, 10 mM β -glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 10 μ g/ml leupeptin, 100 μ M PMSF and 1 mM benzimidazole. Lysates were pre-cleared by centrifugation at 15000 *g* and 4 °C for 30 min. Tumour samples were similarly lysed with additional disruption in a Dounce homogenizer and additional pre-clearing with Protein G-Sepharose. PAGE used gels and reagents purchased from Novex, San Diego, CA, U.S.A. Western blotting, using PolyScreen PVDF membrane (NEN Life Science Products) and ECL $^{\text{®}}$ detection reagents (Amersham),

followed standard protocols and manufacturer's instructions. For immunoprecipitation, cleared lysates were incubated at 4 °C for 1 h with 5 µg of anti-PKB antibody pre-bound to Protein G-Sepharose. For kinase assays, immune complexes were washed and assayed against the peptide substrate Crosstide (Gly-Arg-Pro-Arg-Thr-Ser-Ser-Phe-Ala-Glu-Gly), as previously described [29]. For blotting with phospho-specific antibodies, immune complexes were washed twice in lysis buffer and twice in lysis buffer with 500 mM LiCl before electrophoresis and Western blotting with antibodies against phosphoserine-473 PKB. Antibodies against total PKB were used to verify the efficiency of expression and immunoprecipitation.

Cell-spreading assay

Cell spreading was assayed according to the method described in [17]. Briefly, U87MG cells were infected with viruses encoding GFP or GFP-PTEN proteins for 24 h. Cells were then trypsin-treated thoroughly for 15 min and then seeded in serum-containing medium on to prepared substrates. After 20 min, cells were fixed and the fraction of cells spread was assessed by blind counting. Duplicate samples were allowed to adhere fully, lysed, and expression of PTEN was analysed by Western blotting.

RESULTS

Several lines of evidence identify the membrane phospholipids PtdIns(3,4,5) P_3 , and possibly PtdIns(3,4) P_2 , as cellular substrates of PTEN [8–10]. However, several reports show PTEN as mostly cytosolic [6,17]. Since PTEN has also been identified in the nucleus of some cell types [34] and associated with the plasma membrane [23], we analysed the cellular localization of PTEN in several cell types by cellular fractionation and fluorescence light microscopy. Our data, using a number of epithelial and fibroblast cell types, also showed a largely cytosolic/soluble distribution, with little or no PTEN being in the membrane/pellet fraction or visibly membrane associated (results not shown). In these experiments, overexpressed GFP-tagged PTEN protein was more strongly membrane-associated than endogenous protein (results not shown).

Since most cellular PTEN protein does not appear to be associated with cellular membranes, it seems likely that the colocalization of PTEN with substrates in cellular membranes would have a significant effect on the regulation of downstream signalling by PTEN. Therefore we engineered an artificially membrane-targeted form of PTEN by addition of the N-terminal myristoylation and palmitoylation signal from Lck [30]. This myristoyl-PTEN has an increased membrane association, as judged by cellular fractionation, and has an enhanced inhibitory effect on the activation of cellular PKB both in unstimulated and PDGF-stimulated cells (Figure 1). This supports the hypothesis that targeting of PTEN to cellular membranes can be functionally significant. In these experiments myristoyl-PTEN appeared as a doublet when analysed by Western blotting (Figures 1B and 1C). Although the reason for this is unclear, the slower migrating of the two forms appears to be relatively more abundant in the cellular membrane fraction than the faster-migrating form (Figure 1C). These data (Figure 1A, untransfected cells) also reflect the approximately maximal activation state of PKB in unstarved U87MG cells in these conditions, as in similar experiments, PDGF stimulation does not increase PKB activity, but still increases cellular PtdIns(3,4,5) P_3 levels severalfold (results not shown).

Two potential mechanisms of membrane association have been identified that would allow access of PTEN to phospholipid

Figure 1 N-terminal acylation signal enhances the inhibition of PKB activity by PTEN

(A) The N-terminal myristoylation and palmitoylation signal from the Src family kinase, Lck, was added to the PTEN N-terminus to make Myr-PTEN. U87MG cells were infected with viruses encoding untagged wild-type (WT) PTEN and Myr-PTEN for 24 h before stimulation of some cells with PDGF at the concentrations shown for 10 min. Cells were then lysed and endogenous PKB activity was immunoprecipitated (IP) and assayed *in vitro*. Activity is presented as c.p.m. of [32 P]phosphate incorporated into the peptide substrate. Each data point is presented as the mean $\pm$ S.E.M. from triplicate determinations in two independent infections. In this experiment, the mean PKB activity in unstimulated untransfected (UT) U87MG cells was $69\,653 \pm 5\,729$ c.p.m. (B) Expression of WT-PTEN and Myr-PTEN in the first lysate of each experimental set from (A) including unstimulated untransfected cells, was determined by Western blotting with antibodies against PTEN. (C) U87MG cells were infected with viruses encoding untagged wild-type and Myr-PTEN for 24 h and then fractionated to give membrane (m) and soluble (s) protein. These fractions were analysed by Western blotting with antibodies raised against PTEN.

substrates: through lipid binding of the C2 domain [21] or through interaction of the C-terminus with membrane-bound PDZ domain-containing proteins [22–24]. In order to analyse the role of these membrane-association mechanisms in PTEN function, we mutated the two targeting domains and investigated the

Figure 2 *In vitro* analysis of PTEN mutants

(A) GST-PTEN proteins [wild-type (WT), 399Stop and M-CBR3] were expressed in bacteria and purified. These enzymes were then assayed *in vitro* for 20 min against the soluble substrate $\text{Ins}(1,3,4,5)\text{P}_4$, labelled in the 3-position with $[^{32}\text{P}]$ phosphate. Activity is displayed as c.p.m. released radioactive phosphate and as percentage release of the total phosphate label in the assay. Assays were performed in duplicate as shown for each mutant with 25 ng and 50 ng of enzyme. These data are representative of three experiments. (B) GFP-PTEN proteins (wild-type and 399Stop) were expressed in U87MG cells by viral infection for 24 h before lysis. Purified GST-hDlg (1 μg) was then incubated with 500 μg of U87MG cell protein and affinity purified with GSH-Sepharose. Proteins in this pull-down were analysed by Western blotting with PTEN antibodies and then by general protein stain. PTEN expression in the original lysate was also determined by immunoblotting.

effects of these mutations on cellular PTEN function. In the C2 domain we used a mutation described by Lee et al. [21], replacing a stretch of basic and hydrophobic residues in the CBR3 loop, PTEN M-CBR3. In the previous study [21], this mutation was found to reduce both the association of PTEN with lipid vesicles *in vitro* and the effect of PTEN on anchorage-independent growth and proliferation in cultured cells. To interfere with binding to PDZ domain-containing proteins, a previously described mutant lacking the C-terminal five amino acids was used, PTEN Q399Stop [18]. In addition, the previously described phosphatase dead mutant, C124S, and the lipid phosphatase dead protein phosphatase retained mutant, G129E, were used to implicate the lipid phosphatase activity in the cellular responses investigated [8,35].

A prerequisite for the use of these mutant enzymes to implicate the significance of targeting mechanisms is that these mutations do not affect the activity of the enzyme in other ways. Therefore the phosphatase activity of these mutants was assayed *in vitro* against the soluble substrate, $\text{Ins}(1,3,4,5)\text{P}_4$ (Figure 2A). Since neither mutation significantly affects the activity of the enzyme in these assays, it seems likely that any effects of these mutations on the activity of PTEN in cellular assays are caused by interfering

Figure 3 Effect of PTEN expression on cellular levels of $\text{PtdIns}(3,4,5)\text{P}_3$

U87MG cells were incubated for 48 h with labelled inositol and infected with viruses expressing PTEN proteins for the last 24 h before incubation with and without 50 ng/ml PDGF for 10 min. Cells were then lysed, lipids were extracted and deacylated, and the levels of $\text{PtdIns}(3,4,5)\text{P}_3$ determined by HPLC analysis. Data is presented as the quantity of label incorporated into $\text{PtdIns}(3,4,5)\text{P}_3$ as a percentage of the label in phosphatidylinositol. The percentage of label incorporated into $\text{PtdIns}(3,4,5)\text{P}_3$ in unstimulated untransfected cells (0.0227% of that in PI) corresponds to 1342 d.p.m. above background. Data are shown as the mean of two independent infections. This experiment was performed twice with similar results. WT, wild-type.

with its cellular co-localization with substrates and/or possibly activators. In order to verify that the Q399Stop mutation interferes with binding to PDZ domains, we analysed the interaction of PTEN with hDlg *in vitro*. It has been reported that PTEN can bind to hDlg in the yeast two-hybrid screen, and that the second PDZ domain of hDlg can bind to peptides corresponding to the C-terminus of PTEN *in vitro* [24]. We used GST-hDlg to pull down GFP-PTEN overexpressed in mammalian cells, and showed that GST-hDlg interacts strongly with GFP-PTEN, but not with PTEN399Stop (Figure 2B).

Considerable evidence supports the conclusion that the membrane-associated signalling lipid $\text{PtdIns}(3,4,5)\text{P}_3$ is a physiological substrate of PTEN, and that many of the cellular effects of PTEN are due to the dephosphorylation of this lipid [8–10,36]. Therefore we analysed the effects of expression of wild-type and mutant PTEN proteins on the cellular level of $\text{PtdIns}(3,4,5)\text{P}_3$ in U87MG PTEN-null glioblastoma cells incubated for 48 h with labelled inositol. We found that these cells have a very high basal level of $\text{PtdIns}(3,4,5)\text{P}_3$ compared with normal PTEN-expressing cells, that is dramatically reduced upon expression of the wild-type enzyme. These experiments show that while the expression of M-CBR3 PTEN caused no detectable reduction in the levels of $\text{PtdIns}(3,4,5)\text{P}_3$, the expression of 399Stop PTEN had a similar effect on the level of this lipid compared with the wild-type enzyme (Figure 3).

The activation of PKB (also known as Akt) has been shown to require $\text{PtdIns}(3,4,5)\text{P}_3$ and/or $\text{PtdIns}(3,4)\text{P}_2$ [37,38], and thus has been used in several studies as a cellular read-out of PTEN function [8,10]. The role of PDZ-targeting in the regulation of PKB by PTEN is somewhat controversial, and data have been largely qualitative and often used overexpressed transfected PKB protein. While it has been shown that PDZ-dependent targeting is apparently not required for PTEN to greatly inhibit PKB activation [18,19], a small enhancement of this effect was reported

Figure 4 Effects of PTEN on PKB activation

(A) U87MG cells were infected with viruses encoding GFP or GFP-PTEN fusion proteins for 24 h before starvation in 1% serum for 2 h. Cells were then incubated with or without 50 ng/ml PDGF for 10 min. Cells were then lysed and the phosphorylation state of endogenous PKB analysed by Western blotting with phosphoserine-473 PKB specific antibodies. This blot was then stripped and re-probed with antibodies raised against total PKB. Expression of PTEN proteins was also verified by Western blotting. (B) U87MG cells were infected with viruses encoding wild-type (WT) or 399Stop GFP-PTEN proteins for 24 h, starved for 2 h in 1% serum, and stimulated with 0, 0.1, 1 or 10 ng/ml PDGF for 10 min before lysis. Endogenous PKB was then immunoprecipitated (IP) and assayed *in vitro*. Data points are shown as the means of duplicate independent infections. Expression of PTEN proteins in the first lysate of each experimental set was verified by blotting with PTEN antibodies. This experiment was performed three times with similar results.

when PTEN was co-expressed with the PDZ-domain containing MAGI proteins [22,23]. Therefore the activity state of endogenous PKB was analysed in U87MG cells transiently expressing PTEN and PTEN mutants, using both phosphospecific antibodies (Figure 4A) and kinase assays of immunoprecipitated PKB (Figure 4B). U87MG cells display a very high basal PKB activity, that can be increased slightly by growth factor stimulation. Expression of wild-type or 399Stop PTEN each dramatically reduced PKB phosphorylation in both resting and

PDGF-stimulated cells, whereas phosphatase dead PTEN did not. Expression of the C2 domain mutant PTEN M-CBR3 had a reproducibly small inhibitory effect on PKB phosphorylation and activity (Figure 4A and results not shown). In order to make a more detailed comparison of the ability of wild-type and 399Stop PTEN to inhibit PKB activation, PKB activity was assayed in cells grown to confluence in culture and those growing at low cell density from unstimulated and PDGF-stimulated cells. In all of these experiments there was no significant difference observed between the inhibition of PKB activity by wild-type and 399Stop PTEN (Figure 4B and results not shown).

Our data indicate that PDZ domain-mediated interactions are not required for the regulation by PTEN of cellular PKB activity in U87MG cells, and contrast with data which show that overexpression of MAGI proteins enhanced the inhibition of PKB activity by PTEN using overexpressed proteins in HEK-293 cells [22,23]. It is possible that such a mechanism has physiological relevance, but that the correct targeting components are not expressed in U87MG cells. However, since U87MG cells are glioblastoma-derived, our data support the hypothesis that processes other than the regulation of PKB may be important for the selection of C-terminal PTEN mutations in glioblastomas.

Extending this analysis of PKB activation, the phosphorylation of a number of other proteins was investigated, the regulation of which has been reported to be dependent on PI 3-kinase signalling. GSK3 α and the forkhead transcription factor FKHRL1 have been proposed to be substrates of PKB [37], and p70S6K activation has been shown to rely, at least in part, on PI 3-kinase-dependent phosphorylation, that is probably independent of PKB [39]. The phosphorylation of these proteins in unstimulated U87MG cells transiently expressing wild-type and mutant PTEN proteins was investigated by the use of phospho-specific antibodies. In each case, the phosphorylation of these proteins was greatly reduced by the expression of wild-type or 399Stop PTEN, but not PTEN enzymes lacking lipid phosphatase activity or M-CBR3 PTEN (Figure 5). These data also suggest that expression for several hours of active PTEN reduces not only FKHRL1 phosphorylation, but also cellular abundance (in contrast to a 30 min wortmannin incubation). Similar data have recently been presented by Nakamura et al. [40].

Several complex cellular processes have been identified that rely to some degree on PI 3-kinase signalling and can be inhibited by PTEN, including colony formation in soft agar and cell spreading [14,15,17]. Therefore the requirements for targeting of PTEN in the inhibition of these processes were investigated using the M-CBR3 and 399Stop mutants. As described previously, we found that deletion of the extreme C-terminus of PTEN had no significant effect on the efficiency of growth inhibition by PTEN in a colony formation assay, whereas the CBR3 mutation dramatically reduced the effects of PTEN expression in this assay (results not shown). We have previously shown that overexpression of PTEN can inhibit PDGF-induced membrane ruffling in Swiss 3T3 fibroblasts, and that in contrast with the effect on PKB activity, this inhibition requires the C-terminal PDZ-binding sequence [18]. In the present paper we extend these studies to include an analysis of the effects of PTEN on cell spreading in U87MG cells. The inhibition of cell spreading was one of the first identified cellular effects of PTEN, where it was shown that PTEN is able to delay but not prevent cell spreading [17]. However, the requirement for lipid phosphatase activity of PTEN in the inhibition of this process remains somewhat controversial, and while PI 3-kinase signalling appears to be necessary for lamellipodium formation/membrane ruffling in fibroblasts [41,42] and cell spreading and pseudopod extension

Figure 5 Effects of PTEN on downstream signalling molecules

U87MG cells were infected with viruses encoding GFP or GFP-PTEN proteins for 24 h before lysis. One sample of GFP-transfected cells was treated with the PI 3-kinase inhibitor, wortmannin (100 nM), for 30 min. The phosphorylation of serine-21 of GSK3 α , threonine-32 of FKHL1, threonine-389 of p70S6K were investigated by the use of phospho-specific antibodies. Total levels of these proteins and the expression of PTEN in these cells was also verified by Western blotting. wt, wild-type.

Figure 6 Inhibition of cell spreading by PTEN proteins

U87MG cells were infected with viruses encoding GFP and GFP-PTEN proteins for 24 h. Cells were then trypsinized thoroughly and plated on fibronectin (A) or collagen (B) for 20 min before fixation. Data are presented as the fraction of cells spread across the substrate as a mean $\pm$ S.E.M. of eight measurements from two independent infections. This experiment was performed five times on fibronectin and twice on collagen with similar results. WT, wild-type.

in macrophages [43], precise roles for PI 3-kinase signalling in components of this complex process remain to be identified. Significantly, evidence suggests that these processes are downstream of PI 3-kinase, but independent of PKB [44,45]. We investigated the ability of U87MG cells expressing wild-type and mutant PTEN proteins to adhere to and spread across different substrates immediately after resuspension by trypsinization (Figure 6). As described previously, wild-type PTEN appeared not to affect the adhesion of U87MG cells, but caused a significant

Figure 7 Sensitivity of PKB and cell spreading to inhibition by LY294002

U87MG cells were pre-incubated at the indicated concentration of LY294002 (LY) for 30 min before assaying of unstimulated PKB activity (○) and cell spreading (□). Inhibitor was included in all solutions and media during assaying of cell spreading. Data are presented as a percentage of uninhibited activity as the means $\pm$ S.E.M. from five replicates for spreading and as the means $\pm$ the range of duplicates for PKB activity.

delay in cell spreading on fibronectin and collagen. This effect was absent from phosphatase dead PTEN C124S, the M-CBR3 C2 domain impaired PTEN mutant, and in contrast to a previous report [17], from the specifically lipid phosphatase impaired G129E. However, in contrast to most other cellular processes examined, the PDZ-impaired PTEN protein 399Stop was significantly less efficient than wild-type PTEN in the inhibition of cell spreading, implicating a role for PDZ targeting in the regulation of this process by PTEN.

It is possible that different sensitivities of the different cellular processes analysed to inhibition by PTEN proteins could, in part, reflect different sensitivities of these processes to changes in cellular PtdIns(3,4,5) P_3 levels. Therefore we analysed the sensitivity of PKB activation and of cell spreading, under the assay conditions we had previously used, to inhibition of PI 3-kinase by the compound LY294002. These experiments do indeed show a difference in the sensitivity of these processes to inhibition, the IC_{50} being approx. 2 μ M for the inhibition of PKB, but approx. 10 μ M for the inhibition of cell spreading (Figure 7), supporting the above proposal. The sensitivity of unstimulated p70S6K activity to inhibition by LY294002 was also analysed and found to be very similar to that of PKB (results not shown).

It has been shown that truncation of PTEN C-terminal to the end of the C2 domain (at amino acid 351) has little or no effect on the phosphatase activity of the enzyme or its inhibitory activity in a colony formation assay in culture (Figure 2, results not shown and [19,21]). However, mutations in this C-terminal region of PTEN are found in some human tumours, most having been identified in glioblastomas. Approx. 5% of mutations in PTEN in glioblastomas are found C-terminal to the end of the C2 domain [1,2]. Our data analysing the cellular effects of targeting mutations on PTEN function in cultured cells show that in most of the cellular assays used, the PDZ-impaired protein, 399Stop, functions as efficiently as the wild-type enzyme. This suggests that, whereas tumour cells carrying mutations that abolish the phosphatase activity of PTEN are likely to have highly elevated levels of PtdIns(3,4,5) P_3 and PKB activity [10,11,13], tumour cells carrying mutations in the extreme C-terminus of PTEN, similar to the 399Stop mutation, would not. In order to test this hypothesis, we performed an analysis of the

Figure 8 Analysis of a tumour carrying a mutation in the stop codon of PTEN

(A) Purified GST-tagged PTEN wild-type (wt) and X404L were assayed *in vitro* against the soluble substrate $\text{Ins}(1,3,4,5)\text{P}_4$. A quantity of 25 ng of enzyme was used, and activity is presented as c.p.m. labelled phosphate released and as percentage conversion of the substrate. (B) GFP and GFP-PTEN proteins (wild-type, 399Stop, X404L and M-CBR3) were expressed in U87MG cells for 24 h before lysis. Purified GST-hDIg was then incubated with U87MG cell

lysates and affinity-purified with GSH-Sepharose. Proteins in this pull-down were analysed by Western blotting with PTEN antibodies and then by general protein stain. PTEN expression in the original lysate was also determined by immunoblotting with antibodies raised against GFP. (C) Glioblastoma fragments previously identified as carrying mutations in PTEN (X404L and a frameshift at amino acid 247, as described in the Results section and in [31]) were disrupted and lysed. Cellular PKB was then immunoprecipitated (IP) and analysed by Western blotting (WB) with antibodies against total PKB and against phosphoserine-473 PKB.

DISCUSSION

PTEN is a tumour suppressor protein that is mutated in many types of human tumour, but at particularly high frequencies in glioblastomas and endometrial carcinomas. Evidence indicates that deletion of the phosphatase activity of PTEN in tumours leads to a dramatic increase in the activity of the PI 3-kinase/PKB signalling pathway and, in turn, cell-cycle progression and resistance to apoptosis. In the present study we have analysed the requirements for cellular targeting of PTEN and suggest that not all tumour-derived mutations would be expected to lead to significantly elevated cellular $\text{PtdIns}(3,4,5)\text{P}_3$ levels and PKB activity. Our analysis of PKB phosphorylation in glioblastoma tumour samples supports this conclusion. This suggests that, in a subset of glioblastomas, mutation of PTEN does not lead to the deregulation of processes involving PKB activation, such as cell-cycle progression and anchorage-independent growth, but of other cellular processes that may include rearrangement of the cytoskeleton, as seen in cell-spreading and membrane-ruffling assays. Since PI 3-kinase is a known mediator of tumour cell invasion [46], a process that can be inhibited by PTEN [47,48], it will be interesting to investigate the requirement for cellular targeting in the inhibition of this pathologically relevant event.

Approx. 5% of mutations found in PTEN in glioblastomas are C-terminal to the end of the C2 domain (recent reviews describe six out of 159 [1] and seven out of 116 [2]). This is an interesting contrast to endometrial tumours and the PTEN-associated inherited conditions, in which mutations have never been identified C-terminal to the C2 domain (from over a

hundred mutations identified in each). One possibility is that this might reflect the finding that PTEN mutation appears to be an early event in endometrial tumour progression, but is associated with advanced malignant brain tumours.

Our data show differential requirements for targeting of PTEN in different cellular functions. We have shown that deletion of the C-terminal PDZ-binding sequence from PTEN has no detectable effect on the inhibition of PKB by PTEN, but dramatically reduces the ability of the enzyme to inhibit cell spreading in comparison with the wild-type enzyme in the same experimental system. There are several possible explanations for this difference. First, it is possible that the lack of PDZ-targeting of the 399Stop PTEN mutant causes a small quantitative impairment in its ability to regulate a single functional pool of cellular PtdIns(3,4,5) P_3 , too small to be detected by our direct measurements of the levels of this lipid (Figure 3). To address this question we compared the sensitivities of cell spreading and PKB activity to a pharmacological inhibitor of PI 3-kinase signalling. The greater sensitivity of PKB (and p70S6K) activity to LY294002 is thus compatible with an apparently greater sensitivity to PTEN expression revealed by the ability of a PDZ-targeting-impaired PTEN mutant to inhibit PKB (and p70S6K), but not cell spreading. A limitation of this approach is that cell spreading and PKB activity could be controlled by distinct PI 3-kinase isoforms with differing sensitivities to LY294002. Another possible explanation for our data is that cell spreading and PKB are activated by different pools of cellular PtdIns(3,4,5) P_3 , and that the PDZ targeting of PTEN is required to localize enough PTEN to the 'cell spreading pool', but not to the 'PKB-dedicated pool'. Evidence demonstrating that both the production of PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 , and the activation of the GTPase rac, can be tightly localized to regions of the cell supports this possibility [26,27,49]. It is also possible that the requirements for targeting of PTEN in the regulation of many different processes could vary between cell types depending upon the expression of particular PDZ domain-containing proteins or other downstream components.

There are recent interesting reports [19,20,50] suggesting that the C-terminus of PTEN can also regulate the stability of PTEN, potentially through several phosphorylation sites identified in this region between the C2 domain and the PDZ-binding sequence. However, our findings would appear to be unrelated, as our experiments with the 399Stop C-terminal deletion mutant (which retains these identified phosphorylation sites) are controlled for protein expression, and similar constructs have been shown to retain approximately wild-type levels of phosphorylation and to have similar stability to the wild-type protein.

Using fluorescence microscopy and cellular fractionation, we did not observe any significant effects of either the 399Stop mutation or the M-CBR3 mutation on the cellular localization of PTEN. Only a small fraction of PTEN appears to be membrane-associated, and the functional significance of this is unclear. It seems likely that only a small pool of the cellular protein would be bound to PDZ domain-containing proteins at one time. Also, the lack of an effect of the M-CBR3 mutation on the localization suggests that the major effects of this mutation on the functioning of PTEN may be through an impairment of the ability of the enzyme to metabolize lipid substrates at the membrane, rather than through impairment of the ability of the protein to localize to the membrane [51].

It is worthy of comment that in these and previous studies we have analysed the activity of the PTEN mutant G129E in all of the cellular processes described. This mutant shows remarkable selectivity *in vitro*, displaying almost no detectable phosphatase activity against phosphoinositide substrates, but retaining

approximately full activity against a phosphotyrosine peptide substrate [8,35]. Therefore with the assumption that this *in vitro* selectivity reflects that *in vivo*, this tumour-associated mutant has been used to implicate the lipid phosphatase activity of PTEN in the tumour suppressor function of the enzyme. In our studies we have failed to identify any activity of this mutant in any of the cellular assays reported. This is also in agreement with other recent studies [14,16].

Our data indicate that, while PDZ domain-dependent targeting of PTEN is not required to reduce total cellular PtdIns(3,4,5) P_3 levels, it seems to play a part in the regulation of some PTEN-dependent functions but not others. They also suggest that these functions could be significant in the development of some glioblastomas, and that upregulation of PKB activity is not a universal feature of tumours carrying mutations in PTEN. A better understanding of the role of PDZ targeting in PTEN function will come with the more complete characterization of signalling complexes into which PTEN is recruited by particular PDZ domain-containing proteins and the identification of the processes they can regulate.

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