PTEN M-CBR3, a Versatile and Selective Regulator of Inositol 1,3,4,5,6-Pentakisphosphate (Ins(1,3,4,5,6)P$_5$)  

EVIDENCE FOR Ins(1,3,4,5,6)P$_5$ AS A PROLIFERATIVE SIGNAL*  

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Elaine A. Orchiston‡, Deborah Bennett‡, Nick R. Leslie‡, Rosemary G. Clarke§¶, Lucinda Winward‡, C. Peter Downes‡, and Stephen T. Safrany‡‡  

From the Divisions of §Cell Signalling and of ¶Cell Biology and Immunology, Faculty of Life Sciences, MS1/WTB Complex, Dow St., The University of Dundee, Dundee DD1 5EH, United Kingdom  

The PTEN (phosphatase and tensin homologue deleted on chromosome 10) tumor suppressor is a phosphatidylinositol 3,4-trisphosphate (PtdIns(3,4)P$_2$) 3-phosphatase that plays a crucial role in regulating many cellular processes by antagonizing the phosphoinositide 3-kinase signaling pathway. Although able to metabolize soluble inositol phosphates in vitro, the question of their significance as physiological substrates is unresolved. We show that inositol phosphates are not regulated by wild type PTEN, but that a synthetic mutant, PTEN M-CBR3, previously thought to be inactive toward inositides, can selectively regulate inositol 1,3,4,5,6-pentakisphosphate (Ins(1,3,4,5,6)P$_5$). Transfection of U87-MG cells with PTEN M-CBR3 lowered Ins(1,3,4,5,6)P$_5$ levels by 60% without detectable effect on PtdInsP$_3$. Although PTEN M-CBR3 is a 3-phosphatase, levels of myo-inositol 1,4,5,6-tetraakisphosphate were not increased, whereas myo-inositol 1,3,4,5,6-tetraakisphosphate levels increased by 80%. We have used PTEN M-CBR3 to study the physiological function of Ins(1,3,4,5,6)P$_5$ and have found that Ins(1,3,4,5,6)P$_5$ does not modulate PKB phosphorylation, nor does it regulate clathrin-mediated endosomal growth factor receptor internalization. By contrast, PTEN M-CBR3 expression, and the subsequent lowering of Ins(1,3,4,5,6)P$_5$, are associated with reduced anchorage-independent colony formation and anchorage-dependent proliferation in U87-MG cells. Our results, together with previously published data, suggest that Ins(1,3,4,5,6)P$_5$ has a role in proliferation.  

PTEN (phosphatase and tensin homologue deleted on chromosome 10) is a dual specificity phosphatase that is mutated in a wide range of human sporadic tumor types (1). The PTEN gene encodes a 403-amino acid protein, which is a member of the protein-tyrosine phosphatase family. However, there have been no good phosphoprotein substrates identified to date. The tumor suppressor function of PTEN relies on its ability to metabolize acidic nonprotein substrates (2, 3). Indeed, PTEN dephosphorylates the signaling molecules, phosphatidylinositol 3-phosphate (PtdIns(3)P), phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P$_2$), phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)P$_2$), and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P$_3$) in vitro by removal of the phosphate at the 3-position of these substrates. The preferred substrate was found to be PtdIns(3,4)P$_2$ by a factor of some 200-fold (4). In this respect, PTEN acts as a functional antagonist of phosphoinositide 3-kinase signaling pathways, promoting apoptosis and inhibiting cell-cycle progression (5–7). Evidence for this includes a naturally occurring mutation (PTEN G129E), identified in sufferers of Cowden disease, in which patients encounter multiple hamartomatous lesions, especially of the skin, mucous membranes, breast, and thyroid. The PTEN G129E mutant has comparable activity to PTEN against the synthetic phosphoprotein substrate, polyGluTyr$_3$, but is unable to dephosphorylate inositol lipids, indicating that the lipid phosphatase activity and not protein phosphatase activity is required for tumor suppressor function (3).  

PTEN has several structural features, including an N-terminal phosphatase domain requiring a reduced cysteine (Cys$^{124}$), a calcium-independent C2 domain, that has been shown to bind lipid vesicles in vitro, and a sequence shown to bind PDZ domains (see Ref. 1). In cells, PTEN exists as a phosphoprotein, with phosphorylation occurring at a region of the C terminus of the C2 domain (8, 9). It has recently been shown that the C2 domain, and not the PDZ-binding sequence, plays a crucial role in membrane targeting and substrate specificity (10–12). Functional interference with this C2 domain, exemplified by the artificially modified PTEN M-CBR3 protein first described by Lee et al. (10), causes a reduction in the ability to interact with lipid membranes but marginally increases phosphatase activity toward inositol phosphate substrates (4, 10). Expression of GFP-tagged PTEN suggests that it is predominantly cytoplasmic, in agreement with most studies utilizing PTEN-selective antibodies (11, 13, 14). Its role as a lipid phosphatase, however, requires interaction with membranes, such that PTEN M-CBR3 is unable to regulate PtdInsP$_3$ levels, whereas myristoylated PTEN, which is anchored to the membrane, is more effective than PTEN in altering effects downstream of PtdInsP$_3$ (12, 15).
It has recently been suggested that the cellular substrates of PTEN may include inositol phosphates, particularly Ins(1,3,4,5,6)P_5 (16). Therefore, the effects of PTEN could be mediated by regulation of these inositol phosphates. In this study we have clarified the effects of PTEN expression on inositol lipid and inositol phosphate levels in cells. PTEN expression lowered PtdInsP_2 and PtdIns(3,4)P_2. We found no significant effect on PtdInsP_3, but a physiological regulation of inositol phosphates. The PTEN M-CBR3 mutant, however, selectively affected inositol phosphate levels, especially Ins(1,3,4,5,6)P_5, without altering the levels of 3-phosphoinositide lipids. With this new insight into the effects of PTEN M-CBR3 we have re-evaluated previously published data in determining the roles played by Ins(1,3,4,5,6)P_5 and suggest that Ins(1,3,4,5,6)P_5 is a proliferative signal.

EXPERIMENTAL PROCEDURES

Cell Culture—Tissue culture media and additives were provided by Invitrogen. U87-MG cells, obtained from the European Collection of Animal Cell Cultures, were maintained in minimal essential medium, plus 10% fetal bovine serum, 1% nonessential amino acids, 1 mM sodium pyruvate, and 10% fetal bovine serum. Expression vectors were introduced into the U87-MG cells using a previously described baculovirus delivery system adapted for mammalian expression (12). Assays were performed 24 h following DNA delivery for all experiments except FACS analysis, where cells were analyzed after 36 or 60 h. Levels of expressed protein were determined using fluorescence, as well as Western blotting of extracts from U87-MG cells expressing GFP-tagged protein. Transfection of U87-MG cells for proliferation assays was performed as described below. Vectors were prepared as previously described, except PTEN M-CBR3/G129E, which was prepared by cleaving PTEN G129E and PTEN M-CBR3 with PpuMI and BamHI in FastBacMam-EGFP, and replacing the wild type C-terminal region of the protein (from PTEN G129E) with that containing the M-CBR3 mutation. The sequence was verified, and virus was prepared as above.

Analysis of Inositol Phosphate and Lipid Levels—Cells were labeled with 50 μCi/ml myo-[3H]inositol (Amerham Biosciences) in inositol-free medium (PerkinElmer Life Sciences) for 96 h, prior to lysis in 0.6 M HClO₄. The supernatant was neutralized with 1 M K₂CO₃ containing 5 mM EDTA, and the inositol phosphates were resolved on an anion-exchange column (125-mm Partisphere SAX, 5 m; Whatman) using a gradient generated by mixing buffer A (1 mM Na₂EDTA) and buffer B (1.3 M (NH₄)₂HPO₄, pH 4.8, with H₃PO₄) as follows: 0–10 min, 0% B; 10–20 min, 0–10% B; 20–75 min, 10–35% B; 75–111 min, 35–100% B; 111–115 min, 100% B; 116–126 min, 0% B. Fractions were collected at 1-min intervals. Inositol lipids were extracted from the HClO₄ pellet, prepared, and analyzed as previously described (17). Peaks were ascribed by co-elution of standards in parallel runs.

Immunoprecipitation and Western Blotting—Cells were lysed in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P40, 1% glycerol, 1 mM sodium orthovanadate, 5 mM EDTA, 5 mM β-glycerophosphate, 10 mM sodium fluoride, 10 μg/ml leupeptin, 100 μM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 1 mM dithiothreitol, and 1 mM benzamidine. Lysates were pre-cleared by centrifugation at 15,000 g for 5 min. PAGE was performed using gels and reagents purchased for 5 min. PAGE was performed using gels and reagents purchased for 5 min. PAGE was performed using gels and reagents purchased for 5 min. PAGE was performed using gels and reagents purchased for 5 min. PAGE was performed using gels and reagents purchased for 5 min. PAGE was performed using gels and reagents purchased for 5 min. PAGE was performed using gels and reagents purchased for 5 min. PAGE was performed using gels and reagents purchased for 5 min.

Internalization of 125I-EGF—We performed 125I-human EGF (Amerham Biosciences) internalization assays as described by Sorkina et al. (19). Cells were grown in 24-well dishes. Unless otherwise stated, 125I-EGF was added to cells in minimum essential medium with Earle’s salts containing 0.1% bovine serum albumin at 37 °C for up to 10 min. At the end of the incubation, the medium was aspirated and the monolayers were washed three times with ice-cold minimum essential medium with Earle’s salts to remove unbound ligand. The cells were then incubated for 5 min with 0.2 μg acetic acid (pH 2.8) containing 0.5 M NaCl at 4 °C. The acid wash was combined with another short rinse in the same buffer and used to determine the amount of surface-bound 125I-EGF. The cells were then lysed in 1 μl NaOH to determine the intracellular (internalized) radioactivity. The ratio of internalized to surface radioactivity was plotted against time. Nonspecific binding was determined in the presence of 200 ng/ml unlabelled EGF.

Flow Cytometric Analysis of Cell Cycle Distribution—Adherent cells were harvested by trypsinization, washed once in PBS, and re-suspended in ice-cold 70% (v/v) ethanol in water. Cells were washed twice in PBS plus 1% (w/v) bovine serum albumin and stained for 20 min in PBS plus 0.1% (v/v) Triton X-100 containing 50 μg/ml propidium iodide and 50 μg/ml RNase A. The DNA content of cells was determined using a FACSCalibur flow cytometer (BD Biosciences) and CellQuest software. Red fluorescence (585 ± 42 nm) was acquired on a linear scale, and pulse width analysis was used to exclude doublets. Cell cycle distribution was determined using FlowJo software (Tree Star Inc.).

Proliferation Assays—Anchorage-independent colony assays were adapted from those described previously (20). Briefly, U87-MG cells were transiently transfected (FuGENE 6, Roche Applied Science) with pCDNA3.1+ alone, or PTEN expression constructs. 24 h after transfection, cells were suspended in 15% serum-containing media with 0.5 mg/ml G418 and 0.3% agar and layered in triplicate onto 0.6% agar medium in 6-well plates. Plates were then incubated for 3 weeks, with the addition of 0.5 ml of fresh medium after 10 days. To test anchorage-dependent growth, a similar method was employed to that used by Furnari et al. (21). U87-MG cells were transfected and changed to fresh medium with non-transfected G418 24 h post-transfection. Five days after transfection, nontransfected controls had very little viability. Cell numbers were determined at days 5, 7, and 9 using CellTitre96 reagent (Promega) according to the manufacturer’s instructions.

RESULTS

As previously described, expression of PTEN in U87-MG cells caused a decrease in PtdInsP_2 levels. Greater expression of PTEN (far above the levels of endogenous expression in PTEN-positive cells) caused a significant decrease in PtdIns(3,4)P_2, but not of PtdIns(3,5)P_2 (Fig. 1). We have attempted to express lower levels of wild type and mutant PTEN to study the cellular consequences of such protein expression. A comparison with extracts obtained from cells with normal PTEN status showed that levels of expression of GFP-PTEN were comparable with the range of PTEN expression found in a variety of cell types (Fig. 2). We were surprised to see that 1321N1 astrocytoma cells were either PTEN-null or expressed levels of protein below the detection limit of these assays, because this had not previously been reported and these cells have normally low basal levels of PtdInsP_2 and PKB phosphorylation. Under the conditions used, levels of PtdIns(3)P, PtdIns(3,4)P_2, and PtdIns(3,5)P_2 were not significantly decreased (Table I). Expression of similar levels of the catalytically dead mutant, PTEN C124S, and the lipid phosphatase-dead mutant, PTEN G129E (which retains phosphoprotein phosphatase activity), slightly raised PtdInsP_3 levels. It has been suggested that this is due to “substrate trapping,” resulting in a stable lipid-enzyme complex, protecting the PtdInsP_3 from metabolism by other phosphatases (3). PTEN M-CBR3, PTEN G129E/M-CBR3, and EGF did not alter inositol lipid levels (Table I). Inositol phosphate levels were also determined. Under the conditions used, PTEN lowered Ins(1,3,4,5,6)P_5 levels 30% below ExCell and EGF controls. However, because PTEN C124S, PTEN G129E, and PTEN G129E/M-CBR3 mimicked the effects of PTEN expression, this appears, surprisingly, to be unrelated to the catalytic activity of PTEN. In contrast PTEN M-CBR3 significantly lowered Ins(1,3,4,5,6)P_5 levels by 60% below ExCell control levels, and raised the levels of an inositol tetraakisphosphate by 80%. To our surprise the latter was not the direct product of the 3-phosphatase activity of PTEN M-CBR3, Ins(1,4,5,6)P_4, but was inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,6)P_4), the metabolic precursor to Ins(1,4,5,6)P_4 (Table II and Fig. 3). De磷酸orylation of...
Ins(1,3,4,5,6)P₅ by PTEN M-CBR3 in vitro yielded solely Ins(1,4,5,6)P₄, which was stable to further metabolism, indicating that, like PTEN, PTEN M-CBR3 was acting as a 3-phosphatase (data not shown). The lack of effect of PTEN G129E/M-CBR3 implies that these effects are related to the "lipid phosphatase" activity of PTEN and not mediated by any phosphoprotein phosphatase activity of PTEN M-CBR3. Because PTEN M-CBR3 specifically reduces Ins(1,3,4,5,6)P₅ levels without any effect on inositol lipids, we have utilized this mutant to assess possible biological roles of endogenous Ins(1,3,4,5,6)P₅. Specifically, we tested whether Ins(1,3,4,5,6)P₅ can regulate PKB phosphorylation, anchorage-independent colony formation, proliferation, cell cycle progression, or clathrin-mediated EGFR internalization.

Expression of PTEN M-CBR3 in U87-MG cells was unable to alter the phosphorylation state of PKB. Expression of similar levels of wild type PTEN, which lowers PtdInsP₃ levels, caused a marked decrease in phospho-P Thr³⁰⁸ and S⁴⁷³-PKB, whereas PDGF receptor activation increased phosphorylation at both sites (Fig. 4). These results suggest that Ins(1,3,4,5,6)P₅ does not affect PKB phosphorylation directly or interfere with PtdInsP₃ dependent phosphorylation of PKB. In this respect, PKB appears to have a higher selectivity toward inositol lipids than previously suggested (22, 23). These results reiterate our recent findings with the substrate specificity of PTEN, in that short-chain inositol lipid analogues, or deacylated lipids can yield misleading data with respect to ligand affinities, because such studies fail to replicate binding in the context of a complete biological membrane surface.

Inositol phosphates, including Ins(1,3,4,5,6)P₅, have been implicated in inhibition of clathrin-mediated internalization, by means of preventing triskelion formation (24, 25). EGFR internalization was monitored by means of incubating ¹²⁵I-EGF with U87-MG cells. Rates of internalization were identical whether PTEN M-CBR3 was expressed or not. Incubating U87-MG cells with ¹²⁵I-EGF at 4 °C prevented any internalization (Fig. 5). Similarly, PD158780, an EGFR kinase inhibitor previously shown to block receptor phosphorylation, and hence internalization, was found to behave as expected, significantly inhibiting internalization (data not shown).

Several complex cellular processes have been identified that rely to some degree on phosphoinositide 3-kinase signaling and can be inhibited by PTEN, including colony formation in soft agar (10, 26) and cell spreading (12). The ability of PTEN M-CBR3 to inhibit anchorage-independent colony formation was compared with that of wild type PTEN. U87-MG cells were transfected with expression vectors for untagged PTEN proteins carrying a Neomycin/G418 resistance gene and after 24 h seeded into soft agar with G418 selection. Cells transfected with vector alone or phosphatase-dead PTEN formed large numbers of colonies within 3 weeks (Fig. 6a). PTEN greatly inhibited colony formation, but this was not mimicked by the PTEN C124S, PTEN G129E, or PTEN G129E/M-CBR3 mutants. PTEN M-CBR3, again, had a small, but significant effect in reducing colony number (Fig. 6a), confirming its effects on anchorage-independent growth.

Similarly, expression of PTEN lowered cellular proliferation under adherent conditions, as determined using an MTS assay (Fig. 6b). These effects were not mimicked by the PTEN C124S, PTEN G129E, or PTEN G129E/M-CBR3 mutants. PTEN M-CBR3, again, had a small, but significant effect in reducing proliferation (Fig. 6b), showing that Ins(1,3,4,5,6)P₅ plays a role in proliferation.

The effects of PTEN on anchorage-independent growth and cellular proliferation appear to be mediated by its ability to cause G₁ arrest. The effects of PTEN M-CBR3 on cell-cycle distribution were also monitored. Cells treated with hauconival expression vectors for 36 or 60 h were analyzed by FACS. As
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### Table I

Effects of protein expression on inositol lipid levels in U87-MG cells

| ExCell | EGFP | PTEN | C124S | G129E | M-CBR3 | G129E/M-CBR3 |
|--------|------|------|-------|-------|--------|--------------|
| PtdIns(3)P₂ | 22 ± 2* | 20 ± 3 | 17 ± 3 | 21 ± 2 | 20 ± 1 | 21 ± 2 | 18 |
| PtdIns(3,4)P₂ | 1.5 ± 0.2 | 1.0 ± 0.3 | 0.6 ± 0.2 | 0.8 ± 0.2 | 1.2 ± 0.2 | 0.7 ± 0.2 | 1.2 |
| PtdIns(3,5)P₂ | 0.5 ± 0.1 | 0.7 ± 0.2 | 0.6 ± 0.2 | 0.8 ± 0.1 | 1.2 ± 0.2 | 0.7 ± 0.2 | 1.2 |
| PtdInsP₃ | 0.5 ± 0.1 | 0.7 ± 0.2 | 0.6 ± 0.2 | 0.8 ± 0.1 | 1.2 ± 0.2 | 0.7 ± 0.2 | 1.2 |

* Values are presented as 100 × % of label in the PtdIns peak, mean ± S.E. from six independent experiments, except for PTEN G129E/M-CBR3, where n = 2.

### Table II

Effects of protein expression on inositol phosphate levels in U87-MG cells

| ExCell | EGFP | PTEN | C124S | G129E | M-CBR3 | G129E/M-CBR3 |
|--------|------|------|-------|-------|--------|--------------|
| Ins(1,4,5,6)P₄ | 22 ± 2* | 18 ± 1 | 20 ± 2 | 18 ± 2 | 25 ± 4 | 18 ± 2 | 20 ± 3 |
| Ins(1,3,4,5,6)P₅ | 6.7 ± 0.5 | 4.8 ± 0.4 | 4.0 ± 0.8 | 4.0 ± 0.7 | 5 ± 1 | 5.4 ± 0.9 | 7 ± 1 |
| Ins(1,3,4,6)P₄ | 36 ± 2* | 37 ± 3 | 24 ± 2* | 28 ± 2* | 29 ± 3* | 15 ± 2* | 26 ± 2* |

* Values for inositol tetakisphosphates are presented as 10 × % of label in the InsP₄ peak, mean ± S.E. from seven independent experiments.

### Discussion

The biological roles of PTEN have generally been attributed to its ability to metabolize the lipid second messenger, PtdInsP₃. More recently, it has been shown that PtdIns(3,5)P₂, PtdIns(3,4,5)P₃, and PtdIns(3,5)P₂ are also substrates in vitro (28, 29). We show clearly that the primary substrate for PTEN is PtdInsP₃ and that the inositol lipid bisphosphates are not lowered following PTEN expression at close to physiological levels in U87-MG cells. Higher levels of expression can regulate the relative rates of turnover of these molecules in vivo. Alternatively, this may merely reflect the product-precursor relationship existing between PtdInsP₃ and PtdIns(3,4,5)P₃ via 5-phosphatases, such as SHIP and SHIP2 (30), whereas PtdIns(3,5)P₂ synthesis is likely to be independent of PtdInsP₃.

It has been shown that PTEN can also dephosphorylate inositol lipids in vitro, but their significance as physiological substrates has not been fully resolved. Ins(1,3,4,5,6)P₅ was shown to be a weaker substrate than PtdInsP₃ by a factor of between 10² and 10⁴ depending upon the conditions of assay (4). Ins(1,3,4,5,6)P₅ and InsP₃ are considered to be the substrates of a distinct phosphatase, MIPP. Indeed, studies involving brain and liver extracts from MIPP knockout mice were unable to detect any significant Ins(1,3,4,5,6)P₅ phosphatase activity in preparations where PTEN should have been present (31), although these assays were performed in the absence of a reducing agent, required for optimal activity of PTEN. PTEN overexpression, however, did lower cellular Ins(1,3,4,5,6)P₅ levels (16). In agreement with this study, we found that PTEN expression lowered Ins(1,3,4,5,6)P₅ levels (Table II and Fig. 3), but because these effects were also observed following expression of the catalytically inactive PTEN C124S, and the lipid-phosphatase inactive PTEN G129E mutants, we conclude that this effect is not mediated by the phosphatase activity of PTEN. They are, however, related to expression of PTEN-like proteins, because the same decline in Ins(1,3,4,5,6)P₅ is not observed when EGFP alone is expressed. We have previously found that inositol lipid metabolism by PTEN requires the C2 domain and that interfering with this domain (as with the PTEN M-CBR3 mutant) severely impedes lipid phosphatase activity but enhances the activity observed using a soluble substrate (4, 10). We now show that this artificial mutant can lower Ins(1,3,4,5,6)P₅ levels without similar effects on inositol lipids or other inositol phosphates. We also note that levels of Ins(1,3,4,5)P₄, another substrate of PTEN M-CBR3, are not affected. This observation can be explained by the relative rates of turnover of each molecule and their product-precursor relationships. The turnover of Ins(1,3,4,5)P₄ by endogenous phosphatases is far more rapid than that of Ins(1,3,4,5,6)P₅. The concomitant rise in Ins(1,3,4,6)P₄ levels is likely indicative of a compensatory increase in Ins(1,3,4,5,6)P₅ activity, because the former is considered to be the metabolic precursor of Ins(1,3,4,5,6)P₅. These cellular effects are mediated by the lipid phosphatase-like activity of PTEN M-CBR3, because PTEN G129E/M-CBR3, which should retain its protein phosphatase activity while losing activity toward inositol phosphates, was without effect. This suggests that Ins(1,3,4,5,6)P₅ is a key factor in mammalian cells and that, under normal circumstances, its level is under tight control. These effects are present without any detectable effect on any known inositol lipid. PTEN M-CBR3 thus provides a valuable tool whereby Ins(1,3,4,5,6)P₅ can be selectively regulated. This has enabled us to evaluate some of the physiological roles that have been previously ascribed to Ins(1,3,4,5,6)P₅.

The pleckstrin homology (PH) domains of many proteins have been associated with the ability of these proteins to associate with membrane surfaces. Although the ability of a small number of these proteins to bind inositol phosphates has been studied (see Refs. 22 and 32), it is only recently that these interactions have been considered to be physiologically rele-
vant. Competition between inositol phosphates and inositol lipids for PH domains is clearly observed in the case of phospholipase C\(\delta_1\) (PLC\(\delta_1\)).

We were able to address directly whether Ins(1,3,4,5,6)P\(_5\) has a role to play in PKB regulation. We showed that PDGF was able to further raise, and that PTEN expression was able to reduce, the phosphorylation status of PKB in these cells. Reduction of Ins(1,3,4,5,6)P\(_5\) levels following expression of PTEN M-CBR3 was without significant effect on PKB phosphorylation, suggesting it is not a physiological regulator and is incapable either of activating directly or competing effectively with the lipid activators of this protein kinase.

We have also studied the effect of PTEN M-CBR3 expression on protein trafficking, because Ins(1,3,4,5,6)P\(_5\) and InsP\(_6\) have been proposed to attenuate the desensitization of substance P receptors (25). The ability of Ins(1,3,4,5,6)P\(_5\) to inhibit triskelion formation in vitro has been questioned, due to a particularly low affinity for AP-3 (24). The internalization of low levels of EGF is mediated by clathrin-coated pits (see Ref. 19). Low levels of EGF caused rapid internalization that was sensitive to the EGF receptor kinase inhibitor, PD158780, and reduced temperature. The effects were not altered by lowering Ins(1,3,4,5,6)P\(_5\), suggesting that this inositol phosphate has no role to play in trafficking of tyrosine kinase-coupled receptors.

**Fig. 3.** HPLC profile of inositol phosphates in U87-MG cells. U87-MG cells were labeled with \[^{3}H\]inositol and transfected using virus as described in the legend to Table II. Upper trace, control cells; middle trace, U87-MG cells transfected with PTEN; lower trace, U87-MG cells transfected with PTEN M-CBR3. Elution times of standards were as follows: InsP\(_1\), 17 min; InsP\(_2\), 24 min; InsP\(_3\), 33 min; Ins(1,4,5,6)P\(_4\), 51 min; Ins(1,3,4,5)P\(_4\), 54 min; Ins(1,3,4,6)P\(_4\), 57 min; Ins(1,3,4,5,6)P\(_5\), 67 min; InsP\(_6\), 91 min; and PP-InsP\(_5\), 102 min.
Our approach has been somewhat different to that of other studies, for example, in which InsP₆ is injected into oocytes or other cells and the consequences monitored (25). We have determined the consequences of PTEN M-CBR3 expression on endogenous inositol phosphate levels, whereas the injection of a particular inositol phosphate does not guarantee that it is not metabolized to generate other compounds with biological activity. It also remains possible that Ins(1,3,4,5,6)P₅-mediated trafficking is strictly limited to serpentine receptors coupled to heterotrimeric G-proteins.

The higher inositol phosphates, Ins(1,3,4,5,6)P₅ and InsP₆, have also been implicated in cell proliferation. Overexpression of cytosolic MIPP, achieved by removal of the N-terminal endoplasmic reticulum-targetting sequence and the C-terminal endoplasmic reticulum-recycling signal (Ser Asp Glu Leu), has been shown previously to lower Ins(1,3,4,5,6)P₅ by 60%, and InsP₆ levels by 40%, and to cause a decrease in the rate of cell proliferation (31). It has also been reported that the transition from proliferation to hypertrophy in chicken chondrocyte maturation is accompanied by the up-regulation of Band 17, subsequently identified as the chicken homologue of MIPP (33). These results suggest that a reduction in cell growth correlates with a reduction in the levels of Ins(1,3,4,5,6)P₅.

Expression of PTEN M-CBR3 has been shown to inhibit proliferation of U87-MG (10, 15) and LNCaP cells (15). We show that these effects correlate with a decline in Ins(1,3,4,5,6)P₅ without affecting PtdInsP₃ levels (Fig. 6, this study). These results would suggest that Ins(1,3,4,5,6)P₅ alone...
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Ins(1,3,4,5,6)P₅ without affecting PtdInsP₃. Using PTEN M-CBR3 as a selective tool, we find that Ins(1,3,4,5,6)P₅ plays no significant role in PKB phosphorylation or receptor trafficking, but plays a positive role in proliferation, albeit not as strongly as PtdInsP₃. PTEN M-CBR3 is a versatile and specific regulator of Ins(1,3,4,5,6)P₅ and can be used to determine the physiological roles played by this relatively abundant and widespread inositol phosphate.

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FIG. 7. Cell cycle effects of PTEN proteins. Flow cytometric analysis of U87-MG cells transduced using virus for 60 h. DNA was stained with propidium iodide, and cellular content was analyzed. The percentage of cells in G₁, S, or G₂/M phases were calculated using CellQuest software.