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**The control of phosphatidylinositol 3,4-bisphosphate concentrations by activation of the Src homology 2 domain containing inositol polyphosphate 5-phosphatase 2, SHIP2**

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Short Title: Phosphorylation increases the specific activity of SHIP2.

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## Synopsis

Activation of class Ia phosphoinositide 3-kinase (PI3K) produces PtdInsP<sub>3</sub>, a vital intracellular mediator whose degradation generates additional lipid signals. Vanadate analogues that inhibit protein tyrosine phosphatases (PTPs) were used to probe the mechanisms which regulate the concentrations of these molecules allowing their independent or integrated function. In 1321N1 cells which lack PtdInsP<sub>3</sub> 3-phosphatase activity, sodium vanadate or a cell permeable derivative, potassium bisperoxo(1,10-phenanthroline)oxovanadate (V) (bpV(phen)), increased the recruitment into antiphosphotyrosine immunoprecipitates of PI3K activity and of the p85 and p110 $\alpha$  subunits of class Ia PI3K and enhanced the recruitment of PI3K activity stimulated by PDGF (platelet derived growth factor). However, neither inhibitor much increased cellular PtdInsP<sub>3</sub> concentrations but both diminished dramatically the accumulation of PtdInsP<sub>3</sub> stimulated by PDGF or insulin and increased markedly the control and stimulated concentrations of PtdIns(3,4)P<sub>2</sub>. These actions were accounted for by the ability of PTP inhibitors to stimulate the activity of endogenous PtdInsP<sub>3</sub> 5-phosphatase(s), particularly SHIP2 (Src homology 2 domain containing inositol polyphosphate 5-phosphatase 2) and to inhibit types I and II PtdIns(3,4)P<sub>2</sub> 4-phosphatases. Thus, bpV(phen) promoted the translocation of SHIP2 from the cytosol to a Triton X-100 - insoluble fraction and induced a marked (5-10 fold) increase in SHIP2 specific activity mediated by enhanced tyrosine phosphorylation. The net effect of these inhibitors was, therefore, to switch the signal output of class I PI3K from PtdInsP<sub>3</sub> to PtdIns(3,4)P<sub>2</sub>. A key component controlling this shift in the balance of lipid signals is the activation of SHIP2 by increased tyrosine phosphorylation, an effect observed in HeLa cells in response to both PTP inhibitors and epidermal growth factor.

## Introduction

Phosphoinositides (PIs) play a central role in intracellular signalling, acting as substrates for phospholipases C or PI 3-kinases (PI3K) but also by recruiting or by regulating the activity of modular proteins with appropriate, PI-specific binding domains [1-4]. The receptor-regulated (class I) PI3Ks use PtdIns(4,5)P<sub>2</sub> as their substrate to synthesize PtdInsP<sub>3</sub> [2], a vital lipid signal which regulates diverse cellular processes [3, 4]. PtdInsP<sub>3</sub> is degraded by the 3-phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome 10) [5] and by several 5-phosphatases including SHIP and SHIP2 (Src homology 2 [SH2] domain containing inositol polyphosphate 5-phosphatases 1 and 2) [6, 7] but the two routes are not redundant, perhaps reflecting their distinct lipid products [8]. The removal of PtdInsP<sub>3</sub> by 5-phosphatases produces PtdIns(3,4)P<sub>2</sub> which is then degraded, most probably to PtdIns3P [9]. However, PtdIns(3,4)P<sub>2</sub> and PtdIns3P also can be synthesized directly from PtdIns4P and PtdIns by the non-receptor-regulated class II and III PI3Ks, respectively [2]. The role of the PtdIns3P produced by class III PI3K in the endocytic pathway is well documented [10] but whether or not the formation of this lipid by PtdInsP<sub>3</sub> metabolism gives rise to a functionally and/or spatially discrete pool of PtdIns3P is uncertain. Similarly, the distinct functions of separate PtdIns(3,4)P<sub>2</sub> pools remains unclear.

Nevertheless, several lines of evidence indicate that PtdIns(3,4)P<sub>2</sub> is an important, independent lipid signal. Firstly, mechanisms exist for its synthesis both independently of and co-ordinately with that of other 3-phosphorylated phosphoinositides (3-PIs). Secondly, two enzymes, the types I and II inositol polyphosphate 4-phosphatases, exhibit a strong preference for PtdIns(3,4)P<sub>2</sub> over other possible PI or inositol phosphate substrates, at least *in vitro* [11-13]. Loss of the type I enzyme accounts for the genetic defect which underlies the neuronal loss characteristic of *Weeble* mice [14] and mouse embryo fibroblasts derived from these animals show elevated concentrations of PtdIns(3,4)P<sub>2</sub> [15], implying an important role for this enzyme in PtdIns(3,4)P<sub>2</sub> metabolism and a crucial function(s) for its substrate and/or product lipids. A report showing that the type I 4-phosphatase regulates cell growth downstream of the GATA transcription factor [16] supports a similar conclusion. Finally, several proteins bind with high selectivity to PtdIns(3,4)P<sub>2</sub> [17, 18] while others show a similar preference for PtdIns(3,4)P<sub>2</sub> and PtdInsP<sub>3</sub> over other PIs [19], suggesting that this lipid has precise molecular targets through which its function(s) may be mediated.

The sequential production of PtdInsP<sub>3</sub>, PtdIns(3,4)P<sub>2</sub> and PtdIns3P following recruitment of class I PI3Ks by activated growth factor receptor tyrosine kinases and other cell-surface receptors suggests these lipids occur at a common sub-cellular location and implies that their

distinct functions may be integrated. To allow flexible signalling however, mechanisms must exist beyond the activation of PI3K to allow the concentrations of each lipid to be modulated independently. The regulation of type I 4-phosphatase activity by Rab5 may reflect one such mechanism [15]. More pertinently however, the regulation of appropriate enzyme activities by reactive oxygen species (ROS) may be crucial in this regard. ROS are generated endogenously in response to both physiological and pathological stimuli, including growth factors that activate class I PI3Ks and may play important roles in mediating the actions of these agents [20, 21]. Key intracellular targets of ROS include members of the protein tyrosine phosphatase (PTP) family whose catalytic mechanism relies critically upon a redox-sensitive cysteine residue [21, 22]. Enzymes in this category include the PTPs which reverse the tyrosine phosphorylation of growth factor receptors stimulated by ligand binding but also may include the PtdInsP<sub>3</sub> 3-phosphatase, PTEN [23, 24] and the 4-phosphatases that probably limit PtdIns(3,4)P<sub>2</sub> concentrations [13]. Significantly, exogenous oxidative stress not only stimulates a PtdInsP<sub>3</sub>-dependent increase in the activity of Akt mediated by inhibition of PTEN [23] but also evokes selective accumulation of PtdIns(3,4)P<sub>2</sub> [25]. Thus, ROS may play an important physiological role as modulators of PI3K signalling [22, 26].

In the current study we used vanadate derivatives which are established inhibitors of PTP activity [27] to modify the balance of the 3-PIs produced following the stimulation of PI3K and to identify the vulnerable targets. These are likely to include endogenous tyrosine kinase/phosphatase cycles as well as other enzymes that are also common, physiological targets of ROS. Our results in PTEN null, 1321N1 astrocytoma cells reveal endogenous mechanisms that allow independent control of the concentrations of the PtdInsP<sub>3</sub> and PtdIns(3,4)P<sub>2</sub> synthesized sequentially following stimulation of class I PI3K. This control, exerted unexpectedly by the concerted activation of PtdInsP<sub>3</sub> 5-phosphatase(s) and inhibition of cysteine-dependent PtdIns(3,4)P<sub>2</sub> 4-phosphatase(s), switches the output of class I PI3K from PtdInsP<sub>3</sub> to PtdIns(3,4)P<sub>2</sub> and has important implications for the physiological regulation of PI3K signalling by growth factor receptors.

## **Experimental**

### **Materials:**

Cells were from the European Tissue Culture Collection. Antibodies against the following were obtained from the sources indicated: p85 and anti-phosphotyrosine (clone 4G10), Upstate Biotechnology; p110 $\alpha$ , Cell Signalling Technology; type I inositol

polyphosphate 4-phosphatase, Santa Cruz; anti-SHIP2 antibodies were raised in sheep against a peptide (DPAHKRLLLDLTLQLSK) in the C-terminus of the human enzyme and purified by affinity chromatography on Sepharose columns coupled to the immunizing peptide by the Division of Signal Transduction Therapy, University of Dundee. SHP1 (Src homology 2 domain tyrosine phosphatase 1) was generously provided by Dr. N. Leslie, Division of Molecular Physiology, University of Dundee. Potassium bisperoxo(1,10-phenanthroline)oxovanadate (V) (bpV(phen)) and thin layer chromatography (TLC) plates were from Merck. Sodium vanadate was treated prior to use as described previously [28]. EDTA free, protease inhibitor cocktail tablets were from Roche. Radiolabels were from Perkin-Elmer. Synthetic inositol phosphates and phosphoinositides (dipalmitoyl form) were from Cell Signaling. Other reagents were from sources defined previously [29-31].

#### **Cell culture:**

1321N1 astrocytoma cells and HeLa cells were cultured in 6 well dishes as described previously [29, 30].

#### **Preparation of cell lysates:**

Cells were incubated with and without stimuli or inhibitors and detergent lysates prepared as described previously [31] or with the following modifications. Extracts for SHIP2 analysis were prepared using an alternative lysis buffer (lysis buffer B : 50mM Tris, pH 7.5 with HCl, 1mM EDTA, 1mM EGTA, 50mM NaF, 5mM sodium pyrophosphate, 10mM sodium  $\beta$ -glycerophosphate, 1mM sodium vanadate, 0.1% (v/v)  $\beta$ -mercaptoethanol, 0.5% (w/v) Triton X-100, 0.1mM benzamidine and 0.1mM PMSF). Cell lysates for analysis of inositol polyphosphate 4-phosphatase were prepared as follows. A 75cm<sup>2</sup> flask of cells (typically ~5-10mg protein) was rinsed twice with 10ml of buffer containing 154mM NaCl and 10mM Hepes/NaOH at pH 7.4, then lysed into 2.5ml of ice-cold buffer (lysis buffer C: 100mM KCl, 20mM NaCl, 25mM Hepes/KOH pH 7.4 and 0.25% (w/v) Triton X-100, supplemented freshly with 25mM dithiothreitol, 0.25mM PMSF, 0.25mM benzamidine and 0.05mM leupeptin). The cell debris was collected and centrifuged at 20,000g and 4°C for 10 min. The supernatant was then mixed with 0.25ml of packed Chelex 100 beads (100-200 mesh, sodium form, pre-washed with lysis buffer) on ice for 15 min to remove Mg<sup>2+</sup> ions and thereby minimize in subsequent assays the activity of inositol 1,3,4-trisphosphate (Ins(1,3,4)P<sub>3</sub>) 1-phosphatase. The latter activity was eliminated more conveniently and routinely by using a final assay buffer containing an excess of EDTA over Mg<sup>2+</sup> ions (5mM and 2mM

respectively) but the ability of EDTA to chelate vanadate [32] precluded this approach in some experiments. Cell extracts were used freshly or stored at -80 °C.

### **Cell fractionation:**

The incubation buffer was aspirated and replaced with 1ml of ice-cold hypotonic lysis buffer (20mM Hepes, pH 7.5 with NaOH, 10mM KCl, 1.5mM MgCl<sub>2</sub>, 5mM NaF, 5mM sodium pyrophosphate, 5mM sodium β-glycerophosphate and EDTA-free protease inhibitor cocktail) and the cells allowed to swell for 15 min on ice. The cells were then scraped and the debris passed 5 times through a 25mm gauge needle before centrifugation at 20,000g and 4°C for 10 min. The supernatants (designated as the cytosolic fraction) were adjusted to final concentrations of 1mM EDTA, 1mM EGTA and 0.5% (w/v) Triton X-100 and aliquots (30μl) analyzed for SHIP2 by SDS/PAGE and immunoblotting. The pellets were washed once with 1ml of ice-cold hypotonic lysis buffer then extracted with 0.5ml of ice-cold lysis buffer B for 15 min on ice. After centrifugation for 10 min at 20,000g and 4°C, the supernatants (reflecting the Triton-soluble membrane fraction) were collected and aliquots (30μl) analyzed for SHIP2 as described above. The residual pellets (reflecting the Triton-insoluble membrane fraction) were washed once more with 1ml ice-cold lysis buffer B then solubilized in SDS/PAGE sample buffer (100μl) and aliquots (30μl) also analyzed for SHIP2.

### **Cellular [<sup>3</sup>H]phosphoinositides:**

These were analyzed as described previously [29, 30] except that for some experiments the final hplc analysis used a modified gradient elution which afforded better resolution of the [<sup>3</sup>H]GroPInsPs derived from PtdIns3P, PtdIns4P and PtdIns5P. This was achieved by elution with 1.0M NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> adjusted to pH 3.0 with H<sub>3</sub>PO<sub>4</sub> (B) at 1ml/min and the following gradient: 0min, 0%B; 5min, 0%B; 25min, 7.5%B; 55min, 7.5%B; 130min, 82.5%B; 131min 0%B; 150min 0%B.

### **Preparation of radiolabelled substrates:**

The preparation of [3-<sup>33</sup>P]PtdInsP<sub>3</sub> and [3-<sup>32</sup>P or 3-<sup>33</sup>P]Ins(1,3,4,5)P<sub>4</sub> was as described previously [33, 34] except that the latter product was purified by chromatography on Dowex anion exchange resin. This was achieved on a column (2.0 x 0.8cm) of AG1X8 resin (200-400 mesh, formate form) eluted sequentially with water (10ml), 0.35M ammonium formate

pH 3.0 with formic acid (40ml, to remove residual ATP), 0.75M ammonium formate/0.1M formic acid (10ml, to remove residual InsP<sub>3</sub>) and 1.0M ammonium formate/0.1M formic acid (10ml, to recover the InsP<sub>4</sub> product). The final fraction was spiked with 5µmoles of NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> and dried under vacuum until all the ammonium formate had sublimed. The [<sup>32</sup>P or 3-<sup>33</sup>P]Ins(1,3,4,5)P<sub>4</sub> recovered was dissolved in water and stored at -20 °C.

The degradation of [<sup>32</sup>P or 3-<sup>33</sup>P]Ins(1,3,4,5)P<sub>4</sub> to [<sup>32</sup>P or 3-<sup>33</sup>P]Ins(1,3,4)P<sub>3</sub> was achieved using a recombinant, catalytically-active fragment of SHIP2 corresponding to amino acids 418-884 of the human protein. Radiolabelled InsP<sub>4</sub> was incubated at 37°C for 3h in 0.5ml of buffer (100mM KCl, 20mM NaCl, 2mM MgCl<sub>2</sub>, 50mM Hepes/KOH at pH 7.4 and 1mg/ml bovine serum albumin) containing SHIP2 (25µg/ml). The radiolabelled InsP<sub>3</sub> produced was then extracted and purified as described previously [29], spiked with NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> and desalted as described above then dissolved in water (0.25-0.5ml) and stored at -20°C. The radiochemical purity of the [<sup>32</sup>P or 3-<sup>33</sup>P]Ins(1,3,4,5)P<sub>4</sub> and of [<sup>32</sup>P or 3-<sup>33</sup>P]Ins(1,3,4)P<sub>3</sub> obtained was verified routinely by polyethyleneimine (PEI) thin layer chromatography (TLC) on plates developed in 2.5M ammonium formate/ 1.0M formic acid.

#### **Immunoprecipitation and *in vitro* assays:**

PI 3-kinase was immunoprecipitated from cell lysates using 1µg of anti-phosphotyrosine antibody and assayed using PtdIns as substrate as described previously [31] except that after separation of the reaction products by TLC, the spots corresponding to [<sup>32</sup>P]PtdIns3P were detected by phosphorimaging and quantified in arbitrary units using Advanced Image Data Analysis (AIDA) software. The identity of the [<sup>32</sup>P]PtdIns3P formed was confirmed by analysis of its [<sup>32</sup>P]GroPIns3P deacylation product in the presence of appropriate internal standards by hplc performed as described previously [29, 30] except that the separation of the GroPInsP isomers was achieved by isocratic elution at a flow rate of 1ml/min with 50mM NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> adjusted to pH 3.0 with H<sub>3</sub>PO<sub>4</sub>.

SHIP2 was immunoprecipitated with 2µg of anti-SHIP2 antibody from cell lysates pre-cleared by prior incubation with 5µl of packed protein-G-coupled Sepharose beads for 45min at 4°C. The immunoprecipitated material was washed at 4°C, once with 1ml of lysis buffer B and twice with 1ml of assay buffer lacking MgCl<sub>2</sub>. After aspirating the final wash, immunoprecipitates were mixed with assay buffer (50µl) (50mM Tris, pH 7.5 with HCl, 0.1mM EGTA, 0.03% (v/v) Brij-35, 0.1% (v/v) β-mercaptoethanol and 2.5mM MgCl<sub>2</sub>) and [<sup>32</sup>P or 3-<sup>33</sup>P]Ins(1,3,4,5)P<sub>4</sub> substrate (~0.002 MBq/assay) and the samples incubated with



continuous shaking at 30°C. At the times indicated aliquots (0.5µl) of reaction mix were spotted onto PEI-TLC plates and these developed as above to separate the [3-<sup>33</sup>P]Ins(1,3,4)P<sub>3</sub> released. The PEI-TLC plates were dried, phosphorimaged and radioactive spots quantified as described above. Alternatively, immunoprecipitated SHIP2 activity was measured using [3-<sup>33</sup>P]PtdInsP<sub>3</sub> (1µM, 0.002MBq/assay) as substrate under identical conditions but with 1% (w/v) Triton X-100 in place of Brij 35. The reactions were stopped by the addition of 0.6ml methanol: chloroform: 12M HCl (80:40:1, by vol.), 0.2ml chloroform and 0.31ml 0.1M HCl, the lower phases dried under vacuum and the [3-<sup>33</sup>P]lipid products separated by TLC on silica gel 60 plates pre-dipped in 50mM potassium oxalate/ 5mM EDTA at pH 8.5 in 50% (v/v) methanol and developed in chloroform: methanol: acetone: acetic acid: water (7:5:2:2:2, by vol.). The radioactive spots were quantified as described above.

Inositol polyphosphate 4-phosphatase activity was measured in cell lysates. When required, these were immunodepleted of 4-phosphatase by two sequential treatments each with 5µg of antibody coupled to protein-G-coupled Sepharose beads. The 4-phosphatase activity was determined by measuring the dephosphorylation of [3-<sup>32</sup>P or 3-<sup>33</sup>P]Ins(1,3,4)P<sub>3</sub> to [3-<sup>32</sup>P or 3-<sup>33</sup>P]Ins(1,3)P<sub>2</sub>. Assays were performed at 37°C in 25µl of reaction buffer (100mM KCl, 20mM NaCl, 25mM Hepes/KOH pH 7.4, 10mM dithiothreitol, 0.1mM PMSF, 0.1mM benzamidine, 0.02mM leupeptin, 0.1% (w/v) Triton X-100 and, where appropriate, inhibitors as indicated or 5mM EDTA and 2mM MgCl<sub>2</sub>) containing 0.003-0.03MBq of [3-<sup>32</sup>P or 3-<sup>33</sup>P]Ins(1,3,4)P<sub>3</sub> at the concentrations indicated. Assays were started by the addition either of substrate (5µl) or cell extract (~20-40µg protein) and aliquots (0.5µl) of the reaction mix collected immediately and at further intervals as indicated. The samples were spotted immediately onto 10cm x 10cm PEI-TLC plates and these developed in 1.25M ammonium formate/ 0.5M formic acid and the reaction products quantified as described above. The 4-phosphatase activity was calculated as the sum gain in radiolabelled Ins(1,3)P<sub>2</sub> plus P<sub>i</sub> above the same at zero time.

#### **SDS/PAGE and immunoblotting:**

This was performed as described previously [31]. Primary antibodies were applied for either 2h at room temperature using 0.1µg/ml (SHIP2 and anti-phosphotyrosine) or overnight at 4°C using 2µg/ml (inositol polyphosphate 4-phosphatase). Proteins were visualized using appropriate horse radish peroxidase-coupled secondary antibodies and Enhanced Chemiluminescence reagent (Amersham).

### **SHIP2 dephosphorylation:**

SHIP2 was immunoprecipitated as described above. After washing twice with 1ml SHP1 phosphatase buffer (50mM Tris, pH 7.5 with HCl, 0.1mM EGTA, 0.03% (v/v) Brij and 10mM dithiothreitol) samples were shaken for 30 min at 30°C with recombinant, full length GST-SHP1 (0.4mg/ml) or with their buffer controls. After transfer on to ice, washing twice with ice-cold lysis buffer B and twice with SHIP2 assay buffer, samples were analyzed by immunoblotting or for activity as described above.

### **Fluorescence microscopy of 1321N1 cells transfected with GFP-SHIP2:**

1321N1 astrocytoma cells, grown to 70% confluence on glass coverslips, were transfected with full length SHIP2 cloned into an EGFP C1 vector (from Clontech), using Eugene6 according to the manufacturer's recommendation. After 24 hours, the medium was replaced with incubation buffer and cells treated as indicated. After aspiration of the buffer, cells were fixed with 3% (w/v) paraformaldehyde in phosphate buffered saline (PBS) for 30 min at room temperature, washed 3 times with PBS and mounted in DABCO/Mowiol (13% (w/v) Mowiol 4-88, 33% (v/v) glycerol, 2.5% (w/v) DABCO, 13mM Tris, pH 8.5 with HCl). Images were recorded using an Improvion microscope with GFP filter set.

## **Results**

### **The inhibition of PTPs reveals a mechanism which switches the output of receptor-stimulated PI3K from PtdInsP<sub>3</sub> to PtdIns(3,4)P<sub>2</sub>:**

To study the effects of PTP inhibitors on the production of 3-PIs in response to growth factor stimulation of PI3K we used a 1321N1 astrocytoma cell line which lacks PTEN so that PtdIns(3,4,5)P<sub>3</sub> catabolism is channelled exclusively via 5-phosphatase(s). Table 1 compares the effects on PI concentrations in these cells of two potent PTP inhibitors, sodium vanadate and bpV(phen), a cell permeable vanadate analogue [35]. The data show that these inhibitors evoked changes in the cellular concentrations of several PIs, inducing similar, though not identical effects, presumably reflecting some differences in both their cellular targets and accessibility. Most strikingly however, Table 1 reveals that sodium vanadate and bpV(phen) induced dramatic increases of >10-1000 fold in the concentration of PtdIns(3,4)P<sub>2</sub>, implying that inhibitor-sensitive targets are essential for the synthesis and/or degradation of this lipid in particular. The profound increase in the accumulation of PtdIns(3,4)P<sub>2</sub> in response to bpV(phen), though not the similar, smaller effect of sodium vanadate, was accompanied by a

marked reduction in the concentration of PtdIns(4,5)P<sub>2</sub>, suggesting the conversion of this lipid to PtdIns(3,4)P<sub>2</sub> via class I PI3K. Significantly however, neither PTP inhibitor altered much the concentration of PtdInsP<sub>3</sub>. Thus, we next examined the effects of both inhibitors on the accumulation of PtdInsP<sub>3</sub> and PtdIns(3,4)P<sub>2</sub> stimulated by PDGF in these cells.

Figures 1A and B show that PDGF stimulated a ~10-20 fold and a ~5-10 fold increase in the cellular concentrations of PtdInsP<sub>3</sub> and of PtdIns(3,4)P<sub>2</sub> respectively, consistent with the established recruitment of class I PI3K by the PDGF receptor [36]. The increased concentrations of both lipids were persistent over at least 15min and, importantly, the stimulated, steady-state concentration of PtdInsP<sub>3</sub> was ~10 fold greater than that of PtdIns(3,4)P<sub>2</sub>, as is also the case in response to activation of the IGF-1 receptor in 1321N1 cells [30]. By comparison, sodium vanadate alone increased the concentration of PtdIns(3,4)P<sub>2</sub> but not that of PtdInsP<sub>3</sub>. Unexpectedly however, sodium vanadate altered markedly the response to receptor stimulation, potentiating PDGF-stimulated accumulation of PtdIns(3,4)P<sub>2</sub> but suppressing that of PtdInsP<sub>3</sub>. Figure 2 reveals similar effects of bpV(phen) but with some important distinctions. Figures 2A-C show that bpV(phen) alone had little effect on the cellular concentration of PtdInsP<sub>3</sub> but caused an impressive increase in the concentration of PtdIns(3,4)P<sub>2</sub> which was manifest only after a delay of several minutes and was mirrored by an almost quantitatively identical reduction in the concentration of PtdIns(4,5)P<sub>2</sub>. When added together with PDGF however, bpV(phen) attenuated rapidly and powerfully the accumulation of PtdInsP<sub>3</sub> stimulated by receptor activation of PI3K but also induced a faster and more pronounced accumulation of PtdIns(3,4)P<sub>2</sub> and loss of PtdIns(4,5)P<sub>2</sub> than when present alone. Figure 2D however, also shows that, under the same conditions, bpV(phen) increased by ~2 fold the recruitment of PI3K stimulated by PDGF. Sodium vanadate also increased the PDGF-stimulated PI3K activity measured in antiphosphotyrosine or PDGF receptor immunoprecipitates (data not shown) and the IGF-1 receptor-mediated association of PI3K with insulin receptor substrate-1 [31]. Thus, these results show that the net effect of these PTP inhibitors is to alter the balance of PtdInsP<sub>3</sub> and PtdIns(3,4)P<sub>2</sub> accumulated in response to the activation of class I PI3K by growth factor receptors and suggest that this is achieved by their concerted intervention at three key steps.

**PTP inhibitors promote selective PtdIns(3,4)P<sub>2</sub> accumulation by activation of class I PI3K, inhibition of 4-phosphatases and stimulation of PtdInsP<sub>3</sub> 5-phosphatase(s):**

Figure 3A shows that bpV(phen) stimulated the recruitment of the p85 and p110 $\alpha$  regulatory and catalytic subunits, respectively, of class Ia PI3K into complexes

immunoprecipitated by antiphosphotyrosine antibodies. This occurred in a time- and concentration-dependent manner which correlated closely with the recruitment of PI3K activity into similar immunoprecipitates as shown in Figures 3B and C. Further, the product of this activity when determined using PtdIns as substrate was confirmed as PtdIns3P (data not shown), excluding the possibility that this reflects activity other than that of 3-kinases and consistent with the *in vitro* capability of class I PI3K(s) to 3-phosphorylate each of the three major PIs [2]. Most importantly however, Figure 3D demonstrates that the cellular accumulation of PtdIns(3,4)P<sub>2</sub> stimulated by bpV(phen) also occurs with a very similar time- and concentration-dependence suggesting that the synthesis of this lipid in response to bpV(phen) is mediated predominantly by class Ia PI3K. In support of this, Figure 3D shows additionally that the bpV(phen)-stimulated accumulation of PtdIns(3,4)P<sub>2</sub> was prevented almost completely by pre-treatment of cells with wortmannin, a broad spectrum inhibitor of PI3K(s). However, the dramatic, linear accumulation of this lipid shown in Figure 3D cannot result from activation of PI3K alone and must also reflect the ability of bpV(phen) to inhibit PtdIns(3,4)P<sub>2</sub> hydrolysis.

To address directly the susceptibility of PtdIns(3,4)P<sub>2</sub> degradation to PTP inhibitors, we developed an assay for the inositol polyphosphate 4-phosphatases thought to regulate the cellular concentrations of this lipid. This assay exploits the ability of these 4-phosphatases to hydrolyze Ins(1,3,4)P<sub>3</sub> *in vitro* [11-13] providing a specific index of 4-phosphatase activity in crude cell extracts. Immunodepletion experiments using antibodies specific for the type I 4-phosphatase revealed that the type I enzyme accounted for about 60% of the activity in cell lysates with the remainder presumably reflecting the presence of the type II enzyme. Our data (not shown) suggest that both enzymes are potently and non-competitively inhibited by sodium vanadate and bpV(phen) with indistinguishable IC<sub>50</sub> values of ~0.1 μM. These results establish the vulnerability of both 4-phosphatases to PTP inhibitors and support the view that blockade of these enzymes is likely to contribute to the cellular accumulation of PtdIns(3,4)P<sub>2</sub> induced by these agents.

Our results showing the ability of PTP inhibitors to attenuate agonist-stimulated PtdInsP<sub>3</sub> accumulation suggest that they also activate PtdInsP<sub>3</sub> 5-phosphatases. As 1321N1 cells lack PTEN, this issue could be addressed directly by determining the rate of PtdInsP<sub>3</sub> hydrolysis in these cells as illustrated in Figure 4. This shows the results of experiments in which cells pre-treated in the absence or presence of sodium vanadate were then stimulated briefly with PDGF to promote accumulation of PtdInsP<sub>3</sub> before the addition of a high concentration of

wortmannin to prevent rapidly further PI3K activity and allow measurement of the rate at which existing PtdInsP<sub>3</sub> was then removed by 5-phosphatases. Figure 4 shows that the PtdInsP<sub>3</sub> accumulated in response to PDGF alone was metabolized with a half-time of ~3 minutes. By contrast, the PtdInsP<sub>3</sub> accumulated in the co-presence of PDGF and sodium vanadate was removed with a half-time of ~30-60 seconds demonstrating a several fold increase in the rate of 5-phosphatase activity that is consistent with the lower accumulation of PtdInsP<sub>3</sub> under these conditions (see Figure 1A). These results show clearly that PTP inhibitors can increase PtdInsP<sub>3</sub> 5-phosphatase activity but it should be emphasized that they likely underestimate the maximum extent to which this can occur. Thus, while we elected for practical purposes to use sodium vanadate for these experiments, it is apparent that bpV(phen) must act more powerfully, since despite increasing PDGF-stimulated PI3K activity ~2 fold, it abolished concomitantly the 10-20 fold PDGF-stimulated rise in PtdInsP<sub>3</sub> concentrations (cf Figures 2A and D), suggesting an increase in PtdInsP<sub>3</sub> 5-phosphatase activity of 1-2 orders of magnitude.

**PTP inhibitors stimulate the translocation of SHIP2 and a tyrosine phosphorylation-mediated increase in SHIP2 specific activity:**

SHIP2 is a widely expressed PtdInsP<sub>3</sub> 5-phosphatase [6, 7] which undergoes reversible tyrosine phosphorylation [37, 38] and can be localized through its interaction with a variety of tyrosine phosphorylated binding partners [6, 7]. Thus, we speculated that this enzyme might be responsible for the increased 5-phosphatase activity induced by PTP inhibitors. Figure 5A shows that SHIP2 is expressed in 1321N1 cells and that its tyrosine phosphorylation is increased markedly above negligible resting levels in response to bpV(phen). Similar results were also obtained using sodium vanadate (data not shown). Both the time- and concentration-dependent tyrosine phosphorylation of SHIP2 evoked by bpV(phen) correlated with that for the recruitment of class I PI3K and the accumulation of PtdIns(3,4)P<sub>2</sub> induced by this agent, consistent with a role for SHIP2 in mediating the increase in 5-phosphatase activity which minimizes the contemporary accumulation of PtdInsP<sub>3</sub>. The data presented in Table 2 however, suggest that only limited phosphorylation of SHIP2 may be required, showing that as little as 3 μM bpV(phen) reduced dramatically the cellular accumulation of PtdInsP<sub>3</sub> stimulated by PDGF or insulin. Figures 5B and C demonstrate further that bpV(phen) treatment also induced a profound re-localization of over-expressed and endogenous SHIP2, respectively in 1321N1 cells. Thus, in resting cells

GFP-tagged SHIP2 was predominantly cytosolic but translocated to the cell periphery in response to bpV(phen). Interestingly however, this re-localization appears to be independent of PI3K activity as it was not blocked by concentrations of wortmannin that prevented by  $\geq 95\%$  the bpV(phen)-stimulated increase in cellular PtdIns(3,4)P<sub>2</sub> concentrations and which altered markedly the resting cell morphology. Figure 5C shows that a significant proportion ( $\geq 50\%$ ) of endogenous SHIP2 also translocated from the cytosol of 1321N1 cells following bpV(phen) treatment and was recovered predominantly in the Triton X-100-insoluble fraction, consistent with the known ability of SHIP2 to interact with cytoskeletal proteins [6, 7], a feature which may be associated intimately with its tyrosine phosphorylation.

Earlier studies however, have not shown that increased tyrosine phosphorylation of SHIP2 causes an enhanced capacity of this enzyme to degrade PtdInsP<sub>3</sub> [39-41]. In contrast, our results imply that this is likely and the data presented in Figure 6 confirm this view. Figures 6A and B compare directly SHIP2 tyrosine phosphorylation and activity measured in immunoprecipitates of this protein from Triton X-100 lysates of control and bpV(phen) treated 1321N1 cells. Figure 6A indicates the relative amount of SHIP2 immunoprecipitated from control and stimulated cells (upper panel) and its tyrosine phosphorylation (lower panel) and confirms that bpV(phen) stimulates both a reduction of  $\geq 2$  fold in the amount of SHIP2 recovered in the Triton X-100 soluble fraction from these cells and a marked increase in its phosphorylation. Figure 6B shows the SHIP2 activity, measured using Ins(1,3,4,5)P<sub>4</sub>, the water-soluble headgroup of PtdInsP<sub>3</sub> against which SHIP2 is also active [42], as a substrate. These results show that although a reduced amount of SHIP2 protein is present in immunoprecipitates from stimulated cells, this nevertheless hydrolyzed Ins(1,3,4,5)P<sub>4</sub>  $\sim 3$ -4 fold faster than that from control cells, revealing that bpV(phen) stimulates an increased tyrosine phosphorylation of SHIP2 and a coincident increase of  $\sim 5$ -10 fold in its specific activity. A very similar increase in the SHIP2 activity stimulated by bpV(phen) was also measured with PtdInsP<sub>3</sub> as substrate (data not shown). By contrast however, bpV(phen) did not stimulate SHIP2 activity from control or stimulated cells when added directly to *in vitro* assays but rather inhibited activity modestly ( $\sim 25\%$ ) at high concentrations (100 $\mu$ M), consistent with an indirect cellular action of this reagent.

To confirm the causal relationship between the tyrosine phosphorylation and the increased specific activity of SHIP2, we prepared immunoprecipitates from control and bpV(phen)-treated cells and incubated these in the absence or presence of the phosphotyrosine specific phosphatase SHP1 prior to measurement of SHIP2 activity and

tyrosine phosphorylation as shown in Figures 6C and D respectively. The results show that SHP1 had no effect on the activity of SHIP2 from control cells, consistent with the minimal tyrosine phosphorylation of this protein under resting conditions, and that mock incubations did not alter the activity or tyrosine phosphorylation of SHIP2 from either control or stimulated cells. By contrast however, SHP1 reversed completely both the increase in SHIP2 activity and tyrosine phosphorylation stimulated by bpV(phen). This demonstrates unequivocally that enhanced tyrosine phosphorylation of SHIP2 can increase directly the specific activity of this PtdInsP<sub>3</sub> 5-phosphatase and provides strong support for the role of SHIP2 in the mechanism by which PTP inhibitors switch the output of PI3K from PtdInsP<sub>3</sub> to PtdIns(3,4)P<sub>2</sub>. To date our attempts to confirm this using SHIP2 directed siRNAs have been confounded by insufficiently reduced protein expression, making adequate assessment of the effects on cellular PtdInsP<sub>3</sub> concentrations impractical. However, the limited reduction in SHIP2 expression achieved with siRNAs was sufficient to show a corresponding reduction in the phosphatase activity and tyrosine phosphorylation measured in SHIP2 immunoprecipitates (data not shown), suggesting that both reflect properties of SHIP2 itself rather than of other, contaminating proteins.

#### **Epidermal growth factor receptor-stimulated tyrosine phosphorylation of SHIP2 correlates with increased SHIP2 5-phosphatase activity:**

To determine how phosphorylation modulates the activity of SHIP2, we have begun recently to map the SHIP2 residues phosphorylated in response to bpV(phen) stimulation of 1321N1 and HeLa cells. The preliminary results of these studies indicate a number of sites at which phosphorylation is induced by this reagent, including the NPXY motif C-terminal to the active site and tyrosine residue 1162 which is also located toward the C-terminus. As the phosphorylation of these residues in response to EGF has been reported previously [43, 44], we compared the ability of EGF and bpV(phen) to stimulate SHIP2 activity and tyrosine phosphorylation. In 1321N1 cells EGF had little effect on either parameter (data not shown) but in HeLa cells the growth factor induced a transient increase in both SHIP2 activity and tyrosine phosphorylation as shown respectively in Figures 6E and F. Thus, within 2 min EGF increased the specific activity of SHIP2 ~2 fold and induced a marked increase in tyrosine phosphorylation while by 20 min after EGF addition both the activity and phosphorylation returned close to control values. By comparison, stimulation with bpV(phen) resulted in a more profound increase in SHIP2 tyrosine phosphorylation and increased 5-phosphatase specific activity ~3 fold. In contrast to earlier studies [39-41], these results show that

receptor-stimulated tyrosine phosphorylation of SHIP2 can modify the specific activity of this 5-phosphatase. Importantly, however, our data also imply that PTP inhibitors and receptor stimuli that activate PI 3-kinase signalling can induce tyrosine phosphorylation of SHIP2 at co-incident sites, including at least some of those through which increases in SHIP2 phosphatase activity is mediated. Thus, the phosphorylation-mediated regulation of SHIP2 activity that we have demonstrated in response to PTP inhibitors may also serve to regulate the balance of lipid signals produced in response to physiological stimulation of PI3K.

## Discussion

We have examined the effects of vanadate analogues which are powerful inhibitors of protein tyrosine phosphatases to determine how the concentrations of the separate 3-PI signals generated by the activation of class I PI3K can be regulated independently. Our results show how the action of these agents at key points can switch the balance of lipid signals generated in response to the activation of class I PI3K from PtdInsP<sub>3</sub> to PtdIns(3,4)P<sub>2</sub>. Thus, although PTP inhibitors increase the productivity of PI3K they also stimulate the activity of PtdInsP<sub>3</sub> 5-phosphatase(s) and inhibit the action of PtdIns(3,4)P<sub>2</sub> 4-phosphatases. Our results therefore exemplify means by which endogenous mediators might exert independent control over the concentrations of the separate 3-PIs produced in response to the activation of a single class of PI3K. The established sensitivity of PTEN to inhibition by ROS [23, 24] may confer further flexible control, while the ability of hydrogen peroxide to induce a marked, accumulation of PtdIns(3,4)P<sub>2</sub> but not of PtdInsP<sub>3</sub> [25] suggests that exogenously applied ROS evoke effects qualitatively similar to PTP inhibitors. The regulatory mechanisms revealed by PTP inhibitors are important not only in the context of endogenous ROS however, but also have wider physiological and therapeutic relevance. Firstly, because the stimulation of SHIP2 activity by PTP inhibitors also occurs in response to ligands for endogenous receptors that activate PI3K. Secondly, because although vanadate analogues are powerful insulin mimetics [27] and inhibit PTEN [45], our results show that their net effect on PI3K output can be to reduce rather than to augment cellular PtdInsP<sub>3</sub> concentrations.

PtdIns(3,4)P<sub>2</sub> can, in principle, arise from the degradation of PtdInsP<sub>3</sub> or can be synthesized directly by class II PI3Ks. Several lines of evidence presented support the former as the predominant route leading to PtdIns(3,4)P<sub>2</sub> accumulation in response to PTP inhibitors. Firstly, PTP inhibitors acting alone cause recruitment of both the regulatory (p85) and



catalytic (p110 $\alpha$ ) components of class Ia PI3K into antiphosphotyrosine immunoprecipitates which also display appropriate PI3K activity. Secondly, this occurs with time- and concentration-dependences that correlate closely with that for the wortmannin-sensitive accumulation of PtdIns(3,4)P<sub>2</sub> induced by PTP inhibitors. Thirdly, sodium vanadate and bpV(phen) accelerate the accumulation of PtdIns(3,4)P<sub>2</sub> produced in response to the stimulation of growth factor receptors known to recruit class Ia PI3K. Finally, the dramatic accumulation of PtdIns(3,4)P<sub>2</sub> evoked by bpV(phen) occurs at the expense of PtdIns(4,5)P<sub>2</sub>, the physiological substrate for class I PI3Ks [2]. Interestingly, this implies that PTP inhibitors stimulate the phosphorylation of PtdIns(4,5)P<sub>2</sub> by PI3K but also impede the mechanisms that normally replenish this lipid. Hence, the fraction of cellular PtdIns(4,5)P<sub>2</sub> accessible to class I PI3K can be estimated at  $\geq 70\%$ , a proportion similar to that also available to receptor-activated phospholipases C in 1321N1 cells [30, 46], suggesting that these two signalling enzymes share a substantially overlapping pool of PtdIns(4,5)P<sub>2</sub> substrate. However, comparison with our previous data [29, 30] suggests that the maximal rate of PtdIns(4,5)P<sub>2</sub> consumption following activation of endogenous PI3K in these cells is  $\sim 100$  fold slower than by phospholipase C.

Although the activation of class I PI3K is clearly essential, the near linear accumulation of PtdIns(3,4)P<sub>2</sub> in response to bpV(phen) also suggests strongly that PTP inhibitors must impair the catabolism of this lipid. The types Ia and IIa inositol polyphosphate 4-phosphatases are candidate PtdIns(3,4)P<sub>2</sub> phosphatases [11-13]. Recent studies in cells under or over-expressing [15, 47] the type I enzyme have shown significant effects on the cellular concentrations of PtdIns(3,4)P<sub>2</sub>. We have shown that both enzymes are present in 1321N1 cells and are inhibited potently by PTP inhibitors as expected of these PTP-like, cysteine-dependent phosphatases [13]. The comprehensive inhibition of these enzymes *in vitro* by sodium vanadate and bpV(phen) suggests that a similar action in cells contributes to the dramatic accumulation of PtdIns(3,4)P<sub>2</sub> which these agents induce. Conversely, the powerful cellular action of these inhibitors reflected by increases of up to 1000 fold in the concentrations of PtdIns(3,4)P<sub>2</sub> provides compelling support for the limited available evidence that either or both 4-phosphatase(s) function as essential regulators of 3-PI signals and reinforces the notion that these enzymes may be important targets of endogenously generated ROS. Indeed, as the PTPs which dephosphorylate growth factor receptors and PTEN are also ROS-sensitive, our results make apparent the extent to which even limited, similar susceptibility of the 4-phosphatases would drive the output of PI3K towards

PtdIns(3,4)P<sub>2</sub>. In addition, they suggest that PTP inhibitors may be useful pharmacological tools with which to probe the functions of these enzymes and of the lipids whose concentrations they control.

The most exciting and unexpected aspect of our study is the observation that PTP inhibitors modulate cellular PtdInsP<sub>3</sub> concentrations by activating appropriate 5-phosphatases. This is demonstrated both by the action of these agents to attenuate receptor-mediated increases in the accumulation of PtdInsP<sub>3</sub> and by their capacity to accelerate the rate of removal of PtdInsP<sub>3</sub> in PTEN-null 1321N1 cells. Our results provide strong correlative evidence that SHIP2 is an important component of this process and suggest that the novel regulation of SHIP2 activity uncovered by PTP inhibition occurs by a mechanism that is shared by physiological stimuli of PI3K.

A family of 5-phosphatases which use PIs or inositol polyphosphates as substrates has now been identified [6, 7] and recent evidence suggests that several function physiologically as PtdInsP<sub>3</sub> 5-phosphatases. These include SHIP1 and 2 [6, 7], the skeletal muscle inositol polyphosphate phosphatase (SKIP) [48] and the proline rich inositol polyphosphate phosphatase (PIPP) [49]. SHIP 1 expression is restricted primarily to cells of haematopoietic origin and was not detectable in 1321N1 cells by immunoblotting (data not shown). SHIP2 however, is expressed widely and interacts with a diverse group of intracellular proteins through its N-terminal SH2 domain and its C-terminal proline rich domain but also undergoes reversible tyrosine phosphorylation at an NPXY motif C-terminal to its active site [6, 7]. Both the tyrosine phosphorylation of SHIP2 and its translocation, mediated via its interaction with appropriate binding partners, are thought to contribute to the regulation of this enzyme but the details of the relationship between these events are not yet clear and a direct correlation between SHIP2 tyrosine phosphorylation and the specific activity of this enzyme has not been established [39-41]. Similarly, the functional consequence(s) of agonist-stimulated SHIP2 phosphorylation at other sites [43, 44] remains obscure. Our results demonstrate that PTP inhibitors induce a pronounced re-localization of SHIP2 to a Triton X-100-insoluble fraction at the cell periphery, consistent with the interaction of SHIP2 with components of the cytoskeleton [6, 7], and a marked increase in the tyrosine phosphorylation of this enzyme. The tyrosine phosphorylation of SHIP2 evoked by PTP inhibitors occurs with a time- and concentration-dependence that correlates closely with that for the stimulated accumulation of PtdIns(3,4)P<sub>2</sub> and causes a 5-10 fold increase in SHIP2 5-phosphatase activity. This is the first demonstration that phosphorylation can modulate the activity of this enzyme directly and alone can account for a significant fraction of the increase in the cellular

capacity to degrade PtdInsP<sub>3</sub> induced by PTP inhibitors. Furthermore, as the translocation of SHIP2 also is likely to influence the cellular efficiency of this enzyme, our results are consistent with SHIP2 being the predominant 5-phosphatase contributing to the cellular action of these agents. Importantly, neither the tyrosine phosphorylation nor the re-localization of SHIP2 stimulated by PTP inhibitors was sensitive to the inhibition of PI3K by wortmannin, consistent with our proposal that the ratio of the PtdInsP<sub>3</sub> : PtdIns(3,4)P<sub>2</sub> produced by PI3K can be controlled independently through the regulation of 5-phosphatase activity. Our results also illustrate the wide dynamic range displayed by cellular PtdInsP<sub>3</sub> 5-phosphatase activity from resting to stimulated conditions and emphasize the capacity of SHIP2 for activation. This suggests that unlike the 3-phosphatase PTEN, which regulates basal PtdInsP<sub>3</sub> concentrations, 5-phosphatases like SHIP2 operate at only a fraction of their maximal capacity in resting cells and may be geared specifically to control stimulated increases in PtdInsP<sub>3</sub> concentrations.

In contrast to earlier studies [39-41], our results show clearly that the specific activity of SHIP2 is increased by tyrosine phosphorylation. This may reflect methodological differences and, for the increased activity stimulated by PTP inhibitors, the intense tyrosine phosphorylation of SHIP2 induced, arguably including phosphorylation at non-physiological sites. The latter is unlikely because EGF acts similarly and because PTP inhibition would be expected primarily to amplify the steady-state phosphorylation level of proteins responsive to endogenous kinase/phosphatase cycles rather than to drive other phosphorylation events. This view is supported by our analysis of the SHIP2 sites phosphorylated in response to bpV(phen) as these show phosphorylation of tyrosine residues known to be phosphorylated in response to EGF. Further studies will be required to elucidate fully the role of tyrosine phosphorylation in the regulation of SHIP2. By revealing the existence of hitherto, unrecognised mechanisms which allow the powerful activation of cellular 5-phosphatases such as SHIP2, our results highlight novel targets for potential therapeutic intervention, the significance of which is implied by recent knockout studies [50, 51] which suggest that SHIP2 plays a particularly important role in insulin signalling.

In conclusion, we have used PTP inhibitors to uncover novel, powerful mechanisms capable of switching the predominant output of class I PI3K from PtdInsP<sub>3</sub> to PtdIns(3,4)P<sub>2</sub>. We propose that growth factor receptor stimuli which activate PI3K act, perhaps indirectly through the generation of endogenous ROS to modulate the same key targets to provide the flexible, independent control that is required for the PtdInsP<sub>3</sub>, PtdIns(3,4)P<sub>2</sub> and PtdIns3P produced following the stimulation of class I PI3K to function as separate but integrated lipid

signals. Thus, elevated ROS concentrations would act to amplify PtdInsP<sub>3</sub> formation via their inhibition of PTEN [23, 24] and receptor tyrosine phosphatase activities [20, 21] while the contemporary inhibition of 4-phosphatases by ROS and activation/translocation of SHIP2 mediated by receptor stimulation would act concertedly and adaptably to control the balance of other PtdInsP<sub>3</sub>-derived signals and ultimately, to modify the outcome of PI3K activation.

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## Abbreviations

The abbreviations used are: IGF-1, insulin-like growth factor-1; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; PI, phosphoinositide; PI3K, phosphoinositide 3-kinase; ROS, reactive oxygen species; bpV(phen), potassium bisperoxo(1,10-phenanthroline)oxovanadate (V); PTP, protein tyrosine phosphatase; PTEN, phosphatase and tensin homologue deleted on chromosome ten; SHIP and SHIP2, Src homology 2 domain containing inositol polyphosphate 5-phosphatases 1 and 2; SKIP, skeletal muscle inositol polyphosphatase; PIPP, proline rich inositol polyphosphatase; GFP, green fluorescent protein; SHP1, Src homology 2 domain tyrosine phosphatase 1; GST, glutathione S-transferase; PEI, polyethyleneimine.

## Table and Figure Legends

### Table 1. The effects of vanadate analogues on cellular phosphoinositide concentrations:

Cells pre-labelled with [<sup>3</sup>H]inositol were incubated for 30 min in the absence or presence of 1mM sodium vanadate or 0.1mM bpV(phen) before the extraction and analysis of the [<sup>3</sup>H]PIs. Data for control and sodium vanadate represent the mean  $\pm$  sem of ten experiments

performed in duplicate and for bpV(phen) of three experiments performed similarly. The lipid concentrations for control cells are expressed as percentages of the total cellular [ $^3\text{H}$ ]PI and for sodium vanadate and bpV(phen) treated cells as a proportion of the appropriate control. \*Concentrations of PtdIns4P and PtdIns5P were measured as a combined total but in a subset of four experiments in which these lipids were determined separately the concentration of PtdIns5P as a percentage of total PI was  $0.085 \pm 0.018\%$  and  $0.247 \pm 0.017\%$ \*\* respectively in control and sodium vanadate treated cells. \*\*Indicates a significant difference ( $P \leq 0.05$ ) from the appropriate control determined by Student's *t* test.

**Table 2. Receptor-mediated accumulation of PtdInsP<sub>3</sub> is suppressed by concentrations of bpV(phen) that induce limited SHIP2 tyrosine phosphorylation:** Cells pre-labelled with [ $^3\text{H}$ ]inositol were treated for 30 min in the absence or presence of  $3\mu\text{M}$  bpV(phen) then incubated for 10min in the absence or presence of PDGF (50ng/ml) or insulin ( $1\mu\text{M}$ ) before the extraction and measurement of [ $^3\text{H}$ ]PIs. PtdInsP<sub>3</sub> concentrations are expressed as a percentage of the total cellular PI and represent the mean value and range of two experiments performed in duplicate. \*\*Indicates a significant difference ( $P \leq 0.05$ ) from the appropriate control determined by Student's *t* test.

**Figure 1. Sodium vanadate attenuates receptor-mediated accumulation of PtdInsP<sub>3</sub> but potentiates that of PtdIns(3,4)P<sub>2</sub>:** Cells pre-labelled with [ $^3\text{H}$ ]inositol were treated for 30 min in the absence (circles) or presence (triangles) of 1mM sodium vanadate then further incubated as indicated in the absence (open symbols) or presence (closed symbols) of PDGF (50ng/ml) and the [ $^3\text{H}$ ]PIs then determined. The results are expressed as a percentage of the maximum accumulation of either PtdInsP<sub>3</sub> (A) or PtdIns(3,4)P<sub>2</sub> (B) and represent the mean  $\pm$  sem of three experiments performed in duplicate.

**Figure 2. bpV(phen) enhances PDGF-stimulated PI3K activity but switches its cellular product from PtdInsP<sub>3</sub> to PtdIns(3,4)P<sub>2</sub>:** (A-C) Cellular PI concentrations: Cells labelled with [ $^3\text{H}$ ]inositol were incubated as indicated with PDGF (50ng/ml) (closed circles), bpV(phen) (0.1mM) (open triangles) or both reagents (closed triangles) before the extraction and measurement of the [ $^3\text{H}$ ]PIs shown. The concentration of each lipid is expressed as a percentage of the total cellular PI and the results reflect the mean values of duplicate measurements in a single experiment representative of two that gave similar results. (D)



PI3K activity: Cells were incubated as indicated with PDGF (50ng/ml) (closed circles), bpV(phen) (0.1mM) (open triangles) or both reagents (closed triangles) and the PI3K activity present in antiphosphotyrosine immunoprecipitates then measured. The results represent the mean  $\pm$  sem of three experiments performed in duplicate.

**Figure 3. bpV(phen) stimulates a time- and concentration-dependent recruitment of class Ia PI3K that accounts for the cellular accumulation of PtdIns(3,4)P<sub>2</sub>:** (A) The recruitment of class Ia PI3K: Cells were incubated for the times and with the concentrations of bpV(phen) indicated and the recruitment of the p85 and p110 $\alpha$  subunits of class Ia PI3K into antiphosphotyrosine immunoprecipitates was then determined. The results show duplicate determinations from one experiment representative of three which gave similar data. (B) Time-course of PI3K activity: Cells were incubated with bpV(phen) at 0.01mM (circles) or 0.1mM (triangles) for the times indicated and the PI3K activity associated with antiphosphotyrosine immunoprecipitates was then measured. The results are the mean  $\pm$  sem of three experiments performed in duplicate and are expressed relative to the maximum activity achieved. (C) Concentration-dependence of PI3K activity: Cells were incubated for 10 min (triangles) or 30 min (circles) with the concentrations of bpV(phen) indicated before measurement of the PI3K activity as described above. The results are expressed as a percentage of the maximum activity achieved at the separate times and represent the mean  $\pm$  sem of five experiments performed in duplicate (triangles) or the mean and range of two experiments performed in duplicate (circles). (D) The cellular accumulation of PtdIns(3,4)P<sub>2</sub>: Cells labelled with [<sup>3</sup>H]inositol were treated for 30 min without (circles and triangles) or with (squares) wortmannin (100nM) then further incubated with 0.01mM (circles) or 0.1mM (triangles and squares) bpV(phen) for the times indicated before the measurement of PtdIns(3,4)P<sub>2</sub>. Values are the means of duplicate determinations in a single experiment representative of two which gave similar results and show PtdIns(3,4)P<sub>2</sub> concentrations as a percentage of total cellular PI.

**Figure 4. Sodium vanadate increases the rate of cellular PtdInsP<sub>3</sub> 5-phosphatase activity:** Cells labelled with [<sup>3</sup>H]inositol were treated for 30 min in the absence (circles) or presence (triangles) of 1mM sodium vanadate then incubated for 10 min in the presence of PDGF (50ng/ml) before the addition of wortmannin (10 $\mu$ M) and the [<sup>3</sup>H]PtdInsP<sub>3</sub> remaining at the intervals shown was then measured. The results are expressed as percentages of the

PtdInsP<sub>3</sub> concentrations achieved, respectively, in the absence and presence of sodium vanadate immediately prior to addition of wortmannin and represent the mean  $\pm$  sem of five experiments performed in duplicate.

**Figure 5. bpV(phen) stimulates the tyrosine phosphorylation and translocation of SHIP2:** (A) Tyrosine phosphorylation of endogenous SHIP2: Cells were incubated for the times and with the concentrations of bpV(phen) indicated, then lysed and the SHIP2 protein immunoprecipitated with anti-SHIP2 antibody and its tyrosine phosphorylation determined by SDS/PAGE and immunoblotting. The data show duplicate determinations from a single experiment representative of three that gave similar results. Lane labelled M contained markers only. (B) Translocation of GFP-SHIP2: Cells expressing GFP-SHIP2 were treated for 30 min in the absence [(i) and (ii)] or presence (iii) of wortmannin (100nM) then further incubated for 20 min without (i) or with [(ii) and (iii)] bpV(phen) (20 $\mu$ M) before the cells were fixed and examined by fluorescence microscopy. The images shown are representative of two experiments. (C) Translocation of endogenous SHIP2: Cells were incubated for 30 min with and without bpV(phen) (20 $\mu$ M) and the cellular fractions indicated then prepared and analyzed for SHIP2 by SDS/PAGE and immunoblotting. The data are from one experiment of three which gave similar results. Lane labelled M contained markers only.

**Figure 6. The specific activity of SHIP2 is increased by bpV(phen)- or EGF-stimulated tyrosine phosphorylation:** (A) and (B): 1321N1 cells were incubated for 30 min in the absence or presence of bpV(phen) (20 $\mu$ M) as indicated then lysed and SHIP2 immunoprecipitates analyzed either for (A) SHIP2 protein and SHIP2 tyrosine phosphorylation or for (B) the corresponding SHIP2 5-phosphatase activity from control (open circles) and stimulated (closed circles) cells. The data show (A) triplicate determinations and (B) the mean  $\pm$  SD of the corresponding 5-phosphatase activity from the same samples in a single experiment representative of three which gave similar results. (C) and (D): Immunoprecipitates of SHIP2 from 1321N1 cells incubated as controls or treated for 30 min with bpV(phen) (20 $\mu$ M) were analyzed either for (C) 5-phosphatase activity or (D) SHIP2 tyrosine phosphorylation either directly (untreated) or after incubation for 30 min at 30°C with GST-SHP1 (0.4mg/ml) (GST-SHP1) or with buffer alone (mock). The data in (C) show the mean and range of duplicate determinations from a single experiment and in (D) the corresponding duplicate antiphosphotyrosine immunoblots and are representative of

three experiments which gave similar results. (E) and (F): HeLa cells were incubated in the absence or presence of EGF (50ng/ml) or bpV(phen) (100 $\mu$ M) for the times indicated then lysed and SHIP2 immunoprecipitates analyzed either for (E) SHIP2 5-phosphatase activity or for (F) SHIP2 protein and SHIP2 tyrosine phosphorylation. The data show the mean  $\pm$  SD activity measured in a 30 min assay or the corresponding triplicate immunoblots for the same samples and are representative of 5 experiments which gave similar results. The vertical line dividing the upper panel in (F) indicates where images from the same immunoblot have been merged to eliminate a molecular weight marker lane and allow alignment with the lower panel.

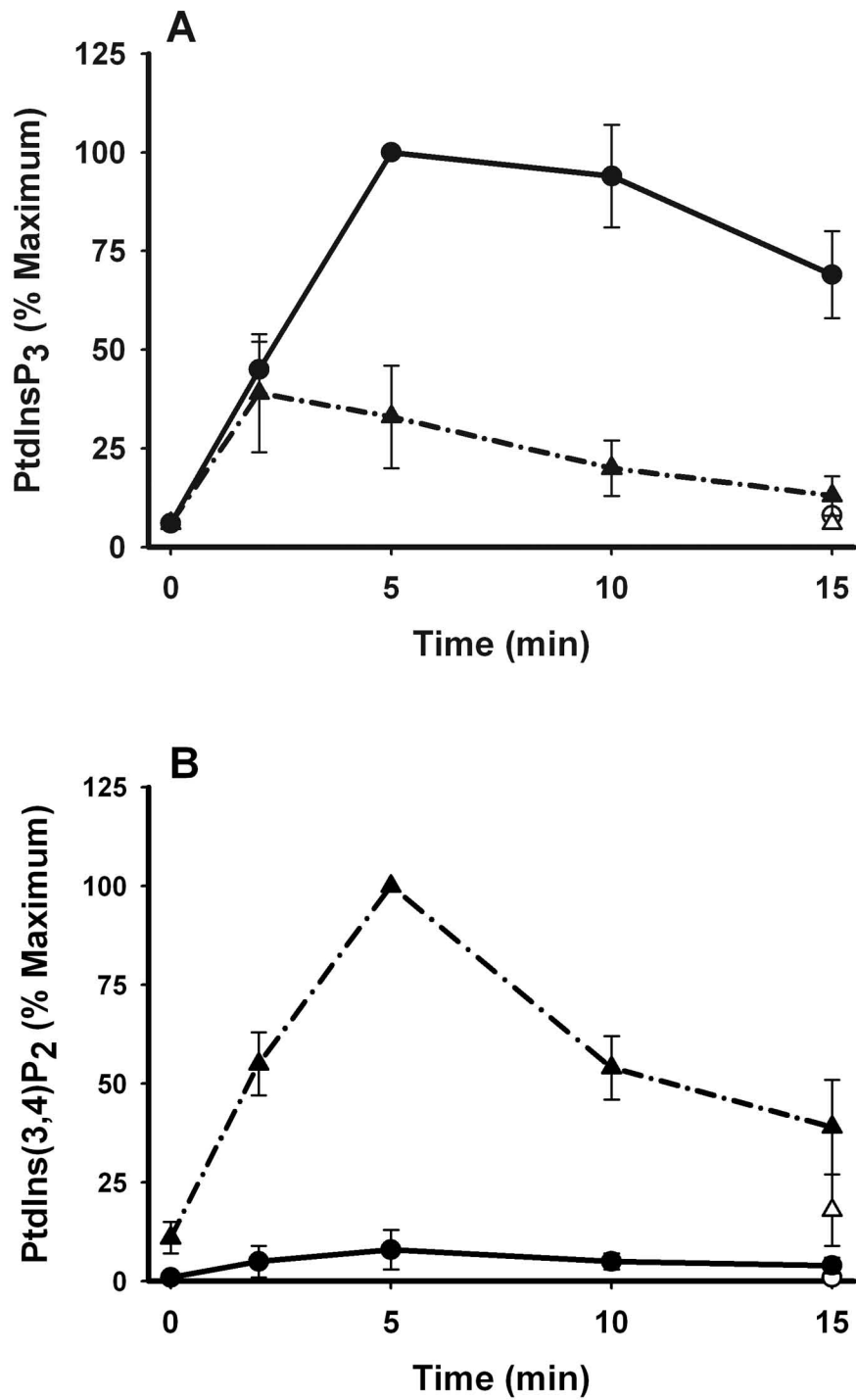
**Table 1**

<b>Lipid</b>	<b>Stimulus</b>		
	<b>Control (% Total PI)</b>	<b>Sodium Vanadate (1mM) (Fold control)</b>	<b>bpV(phen) (0.1mM) (Fold control)</b>
<b>PtdIns</b>	<b>80.615 ± 1.556</b>	<b>0.89 ± 0.01</b>	<b>0.95 ± 0.03</b>
<b>PtdIns3P</b>	<b>0.425 ± 0.039</b>	<b>1.73 ± 0.01**</b>	<b>3.95 ± 1.86</b>
<b>PtdIns4/5P*</b>	<b>7.511 ± 0.586</b>	<b>2.30 ± 0.26**</b>	<b>0.77 ± 0.06</b>
<b>PtdIns(3,5)P<sub>2</sub></b>	<b>0.022 ± 0.003</b>	<b>1.96 ± 0.22**</b>	<b>8.63 ± 4.77</b>
<b>PtdIns(3,4)P<sub>2</sub></b>	<b>0.004 ± 0.001</b>	<b>13.68 ± 4.59**</b>	<b>5164.00 ± 2243**</b>
<b>PtdIns(4,5)P<sub>2</sub></b>	<b>11.407 ± 1.026</b>	<b>1.09 ± 0.02</b>	<b>0.27 ± 0.11**</b>
<b>PtdInsP<sub>3</sub></b>	<b>0.011 ± 0.001</b>	<b>1.15 ± 0.015</b>	<b>2.17 ± 1.25</b>

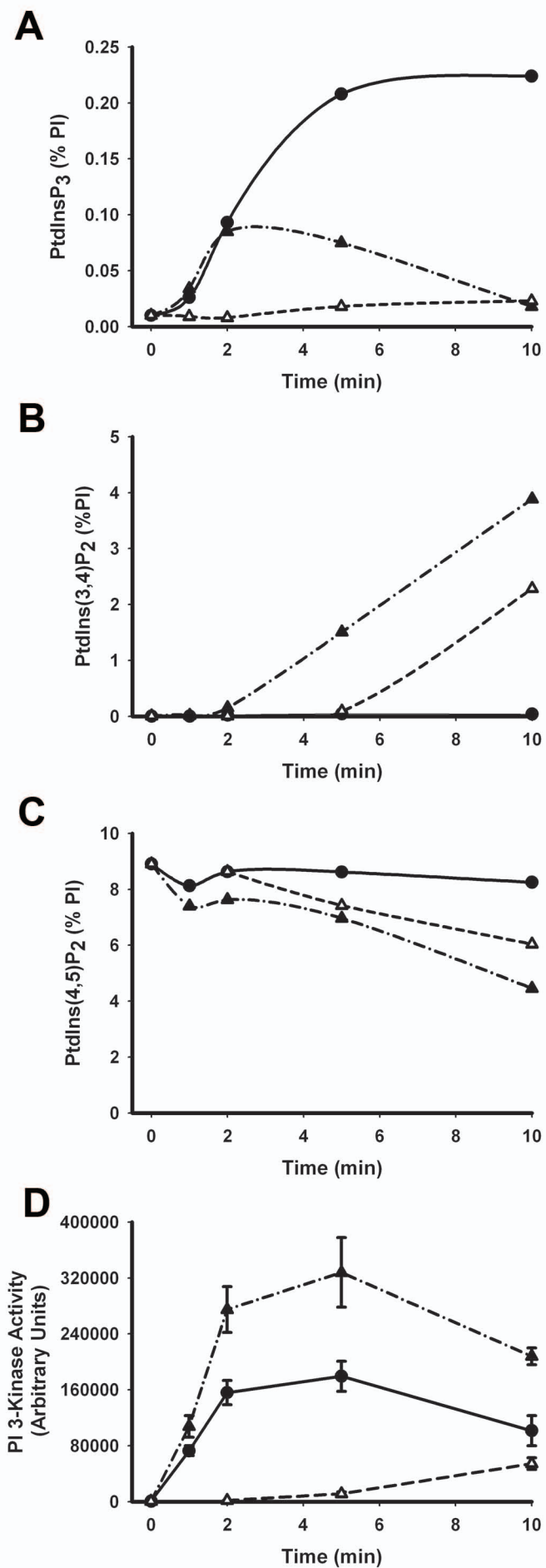
**Table 2**

<b>Stimulus</b>	<b>Cellular PtdInsP<sub>3</sub> (% total PI)</b>	
	<b>-bpV(phen)</b>	<b>+bpV(phen)</b>
<b>None</b>	<b>0.015 ± 0.003</b>	<b>0.012 ± 0.006</b>
<b>PDGF</b>	<b>0.223 ± 0.03</b>	<b>0.025 ± 0.010**</b>
<b>Insulin</b>	<b>0.097 ± 0.032</b>	<b>0.025 ± 0.001**</b>

Figure 1



**Figure 2**



**Figure 3**

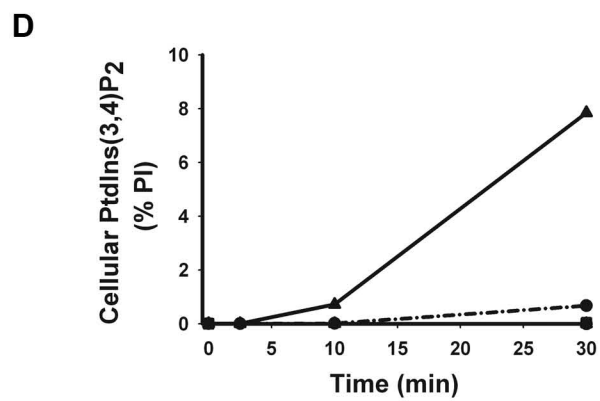
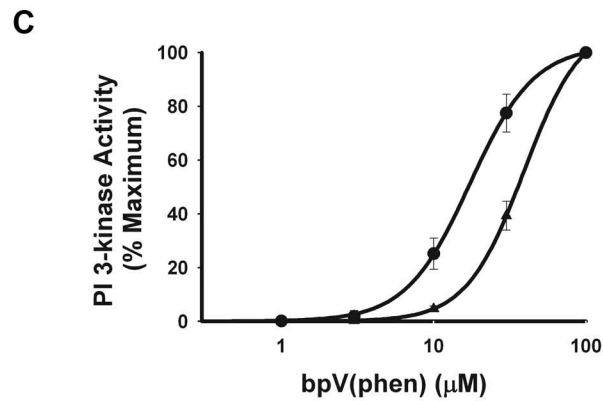
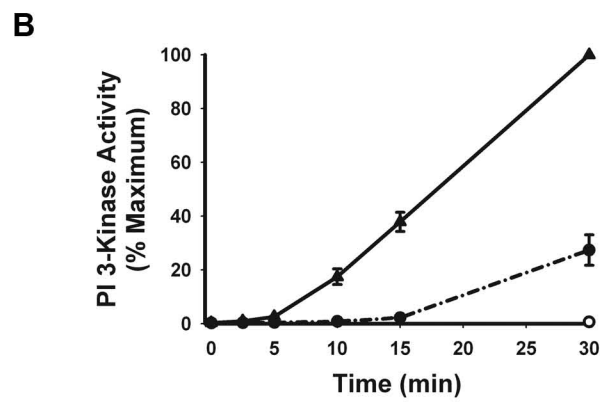
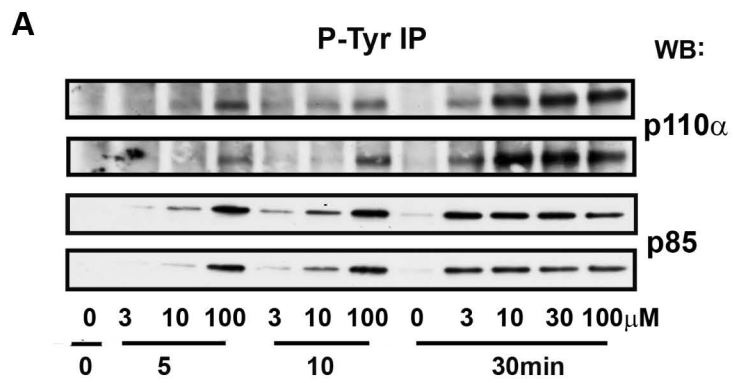
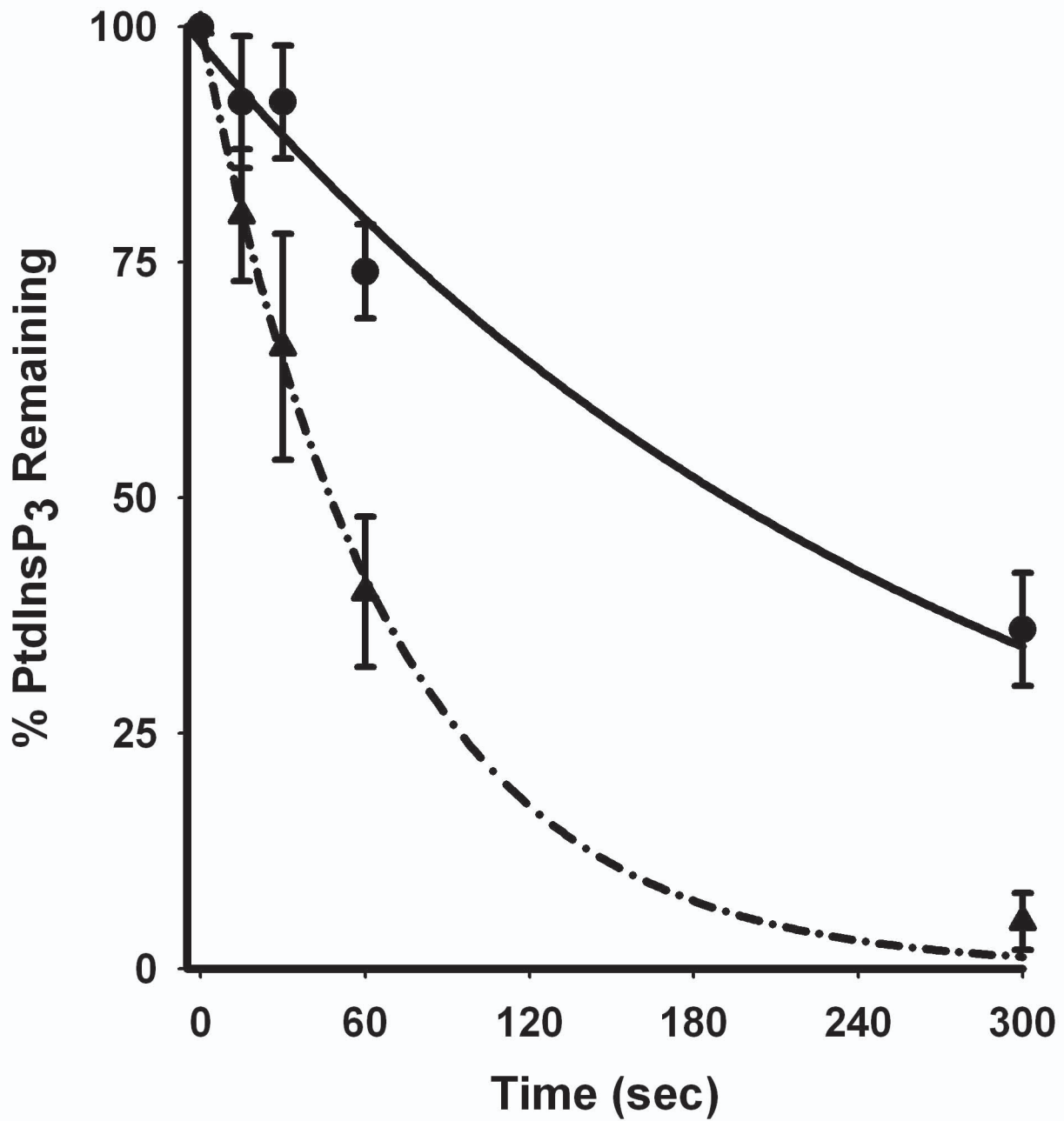
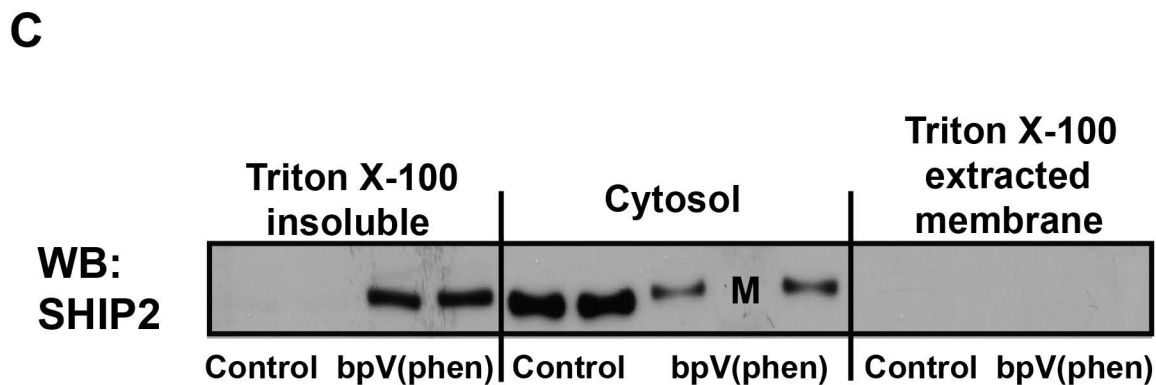
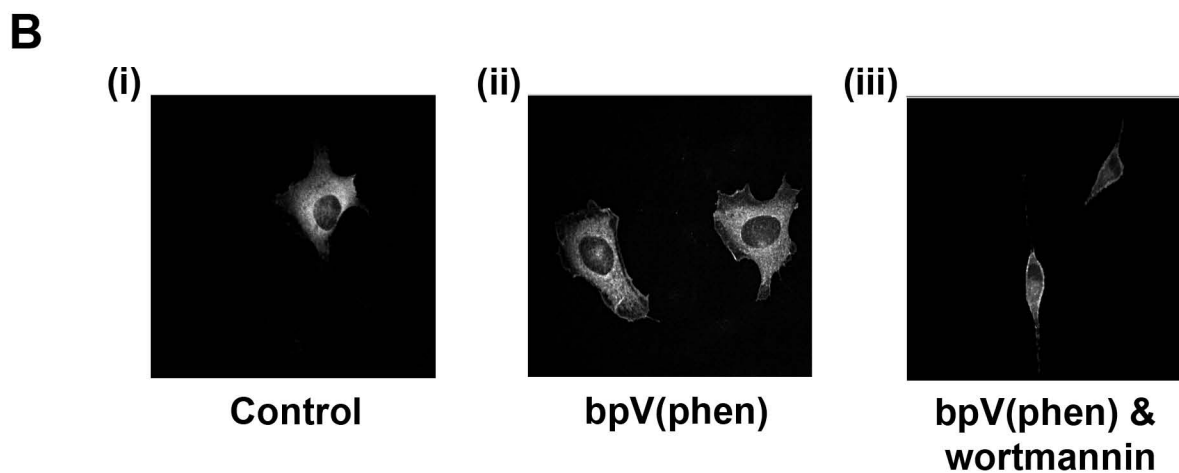
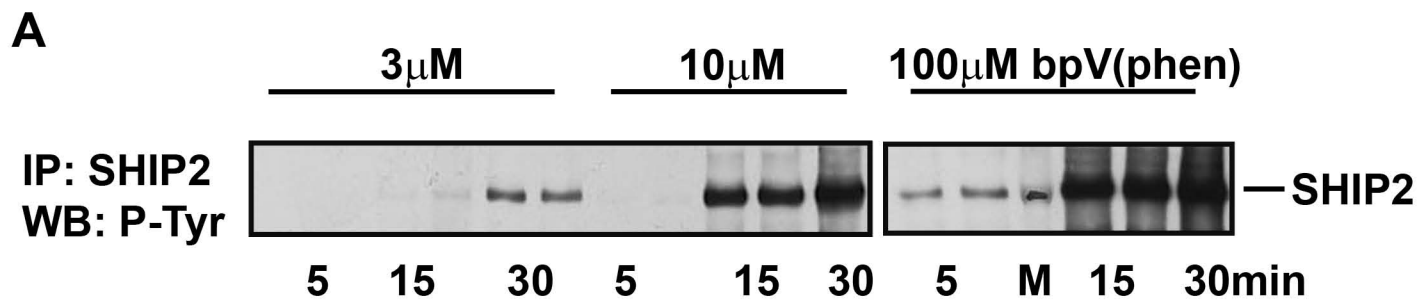




Figure 4



**Figure 5**



**Figure 6**

